DEVELOPMENT OF eDNA TECHNIQUES FOR THE DETECTION OF *TRACHEMYS SCRIPTA* IN AQUATIC SAMPLES AND DETECTION OF PREY ITEMS (*DANIO RERIO*) IN *TRACHEMYS SCRIPTA* FECAL SAMPLES

_____

A Thesis

Presented

to the Faculty of

California State University, Chico

_____

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biological Sciences

_____

by

Gin Lawson

Summer 2017

DEVELOPMENT OF eDNA TECHNIQUES FOR THE DETECTION OF *TRACHEMYS SCRIPTA* IN AQUATIC SAMPLES AND DETECTION OF PREY ITEMS (*DANIO RERIO*) IN *TRACHEMYS SCRIPTA* FECAL SAMPLES

A Thesis

by

Gin Lawson

Summer 2017

APPROVED BY THE INTERIM DEAN OF GRADUATE STUDIES:

_____

Sharon Barrios, Ph.D.

APPROVED BY THE GRADUATE ADVISORY COMMITTEE:

_____

Tag Engstrom, Ph.D., Chair

_____

David Keller, Ph.D.

_____

David Stachura, Ph.D.

PUBLICATION RIGHTS

DEDICATION

This thesis is dedicated to my husband

who stood beside me through personal, academic,

and parental trials and triumphs.

ACKNOWLEDGMENTS

I would like to acknowledge David Keller, Ph.D., and David Stachura, Ph.D. for their generosity, knowledge, and patience. I truly enjoyed sharing a laboratory with everyone in Holt 301 over these last few years.

I would also like to acknowledge Christopher Jerde, Ph.D., and Mark Renshaw, M.S., for their guidance during the initial assay development phase of my research. I always received a cheery, prompt reply to my many inquires, and their feedback was invaluable.

I would be remiss if I didn't mention Caren Goldberg, Ph.D., and her graduate student, Mallory Bedwell. I enjoyed spending the summer learning eDNA sampling techniques in the Sierra Nevada Mountains with the crew from Washington State University. The practical experience I gained helped answer many of the concerns that had arisen in the laboratory.

Finally, I would like to thank my committee chair, Tag Engstrom, Ph.D. Without his enthusiasm for herpetology, I might never have transferred to Chico State. He supplied the blood I needed for a lower division course, and the nudge I needed to consider a graduate-level molecular ecology research project on turtles.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

DEVELOPMENT OF eDNA TECHNIQUES FOR THE DETECTION OF *TRACHEMYS SCRIPTA* IN AQUATIC SAMPLES AND DETECTION OF PREY ITEMS (*DANIO RERIO*) IN *TRACHEMYS SCRIPTA* FECAL SAMPLES

by

Gin Lawson

Master of Science in Biological Sciences

California State University, Chico

Summer 2017

Early detection of invasive species and evaluation of their impact on native ecosystems is key to conservation efforts. The use of environmental DNA (eDNA) has emerged as a useful tool to complement traditional field surveys in studying both the range and the effect of invasive species. eDNA is genetic material released by an organism into the environment; eDNA extracted from environmental samples can be used to determine the location of a species and its distribution, or to identify prey items contained within fecal samples.

In this study, I developed eDNA techniques to detect an invasive turtle species, the red-eared slider (*Trachemys scripta*) in water samples collected under both laboratory and field conditions and to detect specific prey items in fecal samples collected from *Trachemys scripta* in the laboratory. Turtle detection used qPCR primers

designed to target the D-loop region of mitochondria. eDNA in water samples was concentrated onto a cellulose nitrate filter, and extracted DNA was amplified using qPCR. Water samples taken from laboratory aquaria and from a wastewater treatment facility in Chico produced two positive detections. Water samples from another site not known to house turtles, the Sacramento Zoo, produced a weak positive detection.

I also detected *Danio rerio* in fecal samples collected from *Trachemys scripta*. Zebrafish detection used qPCR primers designed to target the D-loop region of mitochondria. After adding *Danio rerio* to food pellets in *Trachemys scripta*‘s diet, I used a PCI precipitation method to extract eDNA from feces and was able to positively identify *Danio rerio* in fecal samples. For dietary analysis, eDNA provides ecologists with a powerful tool for identifying food items in feces.

Of 328 extant species of turtle, approximately 50% are listed as endangered, and many are threatened by invasive species. These techniques for the detection of invasive species and evaluation of their impact can contribute to global turtle conservation efforts.

CHAPTER I

INTRODUCTION

Environmental DNA

Whether on land, in the soil, or within aquatic environments, organisms release DNA into the environment.[1] Sources of environmental DNA (eDNA) include shed skin, hair or scales, mucous, gametes, and feces.[1,2] eDNA breaks down through exposure to acidity, heat or sunlight. The forms of eDNA detected in water can be cellular or extracellular.[2] All organisms contain DNA which exists as double-stranded strings of deoxynucleotide molecules called guanosine (G), adenosine (A), thymidine (T), and cytidine(C). Specific patterns of these deoxynucleotides allow us to distinguish between species, populations, and even individuals.[2] An advantage of eDNA is that identification occurs by capturing DNA instead of the entire organism.[3] In cases where the organism is rare, elusive, or when capture is likely to cause harm, eDNA has tremendous advantages. With eDNA, a sample is collected and DNA extracted without having to capture the target organism.[3] In the late 1990s, eDNA techniques were applied to soil samples,[5] but a more recent application includes aquatic sampling. Analysis of eDNA taken from water samples has been used to detect macro-organisms, including macroinvertebrates,[6] amphibians,[7-10] freshwater fish,[11-17] reptiles,[18-21] terrestrial,[22] and marine mammals.[23]

Ecologists understand the importance of early detection of invasive species in addressing concerns involving ecosystem management, especially in aquatic environments. Detecting an invasive species before they have a chance to establish a

population requires tools sensitive enough to detect a relatively small number of organisms.[1,3-4] Applying traditional sampling techniques can be challenging, especially in aquatic environments where low species abundance, cryptic behaviors, and tiny larval stages can make aquatic species difficult to detect. Compared to traditional methods, eDNA is cheaper, non-invasive, and less time consuming than methods such as visual encounter surveys, electrofishing, snorkeling, trapping, and netting.[2] Another advantage eDNA has as a detection tool is that no specialized taxonomy knowledge is needed prior to sampling; therefore, individuals with no previous taxonomic training can be used to collect water samples because species verification occurs in the lab using primers and genetic sequencing. Previously, taxonomic experts identified species at various developmental states, such as eggs and larvae, while conducting visual encounter surveys in the field.[24] eDNA was first applied to aquatic invasive species by French researchers in 2008. American bullfrogs (*Rana catesbiana*), which are harmful predators of native amphibians, were detected in wetland areas across France using eDNA techniques.[7] In the United States, along the Chicago Area Waterway (CAWS), eDNA was used to determine whether silver and bighead Asian carp (*Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis*) had entered the Great Lakes basin from its connecting tributary, the Mississippi River, where established populations of Asian carp exist. With only a handful of positive detections, it is unlikely the invaders have enough individuals to establish a self-sustaining population, especially considering the lack of capture by commercial fisheries in the area over the last decade.[11]

eDNA research has three primary areas of focus: detection of endangered species,[10,14-15,19-21] tracking of invasive species,[7,9,11-13,17-18] and refinement of field and

laboratory methods.[26-30] For endangered and invasive species, management relies on information about the location and distribution of organisms. eDNA enables researchers to quickly evaluate more sites for presence data, as well as provide population abundance estimates. In situations where invasive and endangered species occupy the same environment, such as giant salamanders in Japan,[10] species-specific primers help identify closely related species difficult to distinguish in the field by appearance. For non-native species, the best-case scenario involves detection while the number of individuals present is low and before self-sustaining populations can be established. In locations where rare a species is present, detection informs conservation managers to its need for protection. Facilitating the accurate detection of eDNA requires practitioners refine their field and laboratory techniques. Laboratory methods studies have examined several questions including: the number of water samples and their volume, the differences between filter types, the proper pore size for capturing DNA efficiently, the best sample preservation methods, and comparing extraction methods to commercially available kits.

Three factors affect the detection of eDNA: production, transportation, and degradation.[1,24,26] The ecology of eDNA encompasses the origin, state, transportation, and fate of shed genetic material.[1] The origin of eDNA refers to primary physiological sources of genetic material, such as sloughed skin, feces, slimy coatings, and other bodily fluids.[26] Although identifying the exact sources of specific eDNA samples is relatively unstudied, Turner's research on particle size distribution provides clues about possible eDNA origins.[26] The amount of genetic material produced depends on the size, health, and density of the organisms.[1,21,24-25] For example, in two species of carp, diet had a noticeable impact on eDNA production leading researchers to identify the gut lining as a

potential origin source.[1,24] eDNA production also increased during breeding season even in lone, captive animals.[21,24] In addition, a large amount of variation in eDNA production was observed among individuals of the same species. Under controlled laboratory conditions, bullfrog tadpoles showed a thirteen-fold difference among individuals, and the shed rate among Idaho giant salamanders showed a six-fold difference.[24,26] Although the rate of eDNA production in Idaho giant salamanders was related to their biomass, Bluegill Sunfish juveniles released eDNA at a higher rate than adults despite having smaller bodies.[1] The production of eDNA is influenced by behavioral changes, activity level, metabolic influences, as well as interspecies and intraspecies differences.

eDNA can be extracellular, cellular, or even an aggregation of cells. Genetic material's shape and length, as well as the presence of a protective membrane, influences its interactions with the environment. After being shed by an organism, eDNA is subject to degredation. While the state of eDNA within aquatic environments has not received much research attention, it is likely organisms shed genetic material as whole cells, and when those cells break down they release extracellular DNA.[1] Degradation begins almost immediately, and its form changes.

Transportation or dispersal describes the distance travelled by eDNA starting at its source and ending when the eDNA is no longer detectable within the environment. Understanding how eDNA moves through the environment makes temporal and spatial estimates about an organism's whereabouts possible.[1] eDNA helps us relate detection data to species presence as it moves through an aquatic habitat. The two basic types of freshwater environments are still, lentic bodies and rapid-moving, lotic bodies. At low flows, the distance traveled by eDNA is reduced because diffusion is limited in ponds

and lakes.[24] In streams and rivers, spatial inferences are difficult because no consistent relationship has been found between distance travelled and eDNA concentrations.[24] However, the DNA of macroorganisms has been detected up to 12 km from established populations.[1,24] Despite swift moving currents, eDNA does not appear to accumulate in downstream reaches due to settling and destructive forces.

The fate of eDNA involves environmental interactions such as absorption and biological intake. Factors affecting eDNA persistence fall into three general categories: (1) its state, such as cellular or extracellular forms; (2) the abiotic environment, such as UV light, temperature, pH, salinity, and conductivity; (3) and the biotic environment, such as the activity and composition of microbes as well as extracellular enzymes such as RNases and DNases.[1] Aquatic eDNA uses surface water as the best indicator of the recent presence of an organism. eDNA only lasts a matter of hours to days in water, whereas eDNA absorbed into sediment persists days or even years in the environment.[24] Therefore, care must be taken to avoid collecting sediment when collecting water samples to avoid the possibility of a false detection.[1,24] Abiotic conditions such as UV light, temperature and pH break down eDNA; it persists longer under cold, dark, alkaline conditions. When considered together, the origin, state, and fate helps determine how effectively eDNA can serve as a surveying tool in place of directly observing an organism.[26] Although applied more frequently in recent years, eDNA suffers from discontinuity in lab procedures, and a general lack of knowledge about macrobial eDNA's persistence in the water and whether it is composed mostly of intercellular or intracellular material.

Sampling Methods

Water sampling methods depend heavily on local conditions. Aquatic eDNA samples have been collected from oceans,[23] rivers and streams,[6,8,10,13-14,20-21] lakes and ponds,[11,15,17,26,28] as well as laboratory tanks.[7,16,18-19,22,27,30] Ocean environments have proven particularly challenging for eDNA work. Genetic material degrades in under a week,[25] the volume of water contained in the ocean dilutes and disperses DNA to a greater extent, and higher levels of false negatives occur.[23] In comparing lakes versus rivers, detection rates were higher in rivers when filtering twice the volume of water. Rivers experience higher rates of dispersion and lower levels of suspended particulate which makes higher samples volumes feasible. In tanks, PCR versus qPCR made little difference in detection success,[17] but the pH level of aquarium water noticeably affected eDNA degradation.[28]

An array of factors must be considered when choosing an aquatic eDNA sampling method. Characteristics such as pH, temperature, ultraviolet light, conductivity, and salinity contribute to DNA degradation.[28] The interactions can be complex, such as with temperature. In tanks, higher temperatures resulted in higher levels of eDNA concentration, but this was attributed to increased activity levels in the fish [16]. When the impact of seasonality on eDNA was studied, the behavioral patterns of the turtles, not temperature, mattered most in detection probability.[21] The likely form of DNA captured in the water samples also makes a difference because extracellular and cellular DNA respond differently to pH levels. In pond samples, pH didn't have a noticeable affect, but in tank experiments, the pH level mattered.[28] In lakes and ponds, higher levels of suspended particulate clog filters easily, but these particulates contain higher amounts of

cellular DNA, so filters with larger pore sizes are used to process smaller volumes of water.[17]

Before collecting the water sample, other methodological choices must be made. Samples can be processed on-site,[29] or transported to the laboratory on ice or in portable refrigeration units. The choice between filtration versus precipitation influences the volume of water collected, and the number of samples collected per site is determined at least partly by whether PCR or qPCR is being used. Filtration works well in concentrating eDNA taken from one liter or larger volumes of water, but at the risk of losing dissolved DNA that may increase detection.[24] Precipitation is a non-size selective method used to capture eDNA that is applied to much smaller 15mL volumes of water.[16] Water samples not processed on-site require either a cold-storage option, or a buffering solution added directly to the sample. When using cold-storage options, repeated freezing and thawing significantly reduces DNA detection success.[24] While PCR versus qPCR doesn't noticeably change detection in tank samples, in field samples where low quality DNA occurs in extremely small amounts, qPCR does result in better detection success. When using PCR for field samples, a higher number of samples must be taken from each site due to the increased possibility of false negatives.

In bodies of water where the genetic material is dilute, distributed heterogeneously, and present in low amounts, filtration has the advantage of processing large volumes of water, up to several liters at a time.[27] The materials used for the filter fibers vary and include – mixed cellulose ester,[16] polyethylensulfone, nitrocellulose, cellulose nitrate,[8-9,15] glass fiber,[6,10-11,14,16,19-20,27-29] polycarbonate,[17,26-27] cellulose acetate, and nylon.[22] Pore size is another consideration when selecting a filter. While eDNA

retention is greater with a smaller pore size,[16] the appropriate size largely depends on the application. For example, the size distribution of suspended particles is different between tanks, ponds, and streams.[27] Turner used sequential filter pore sizes to measure particle size distributions in aquatic eDNA field samples. In lakes and ponds, the most abundant particles sizes ranged between 1-10 um, but most eDNA fell below 0.2 um.[26] The sum of eDNA captured was greatest at the smallest pore sizes and decreased with increasing pore size.[26] However, any benefits associated with a smaller pore size must be weighed against the filter clogging and the amount of time spent filtering.[27]

After the filter type and pore size has been determined, the next decision involves whether to filter on-site[29] or transport the water sample to the laboratory. The genetic material in the water will continue to degrade unless steps are taken to slow the process. Water samples can be preserved either at room-temperature with a buffering solution,[30-31] or at cold ambient temperatures using ice or a portable freezer.[29,31] Yamanaka compared on-site filtering versus transporting water samples either cold or at room-temperature; on-site filtration resulted in higher DNA concentrations followed by cold temperature transport.[29] Another study compared water samples with Longmire's solution added versus cold-storage transport and found the buffering solution as effective as freezing at higher concentrations.[30] Yamanaka conducted another study comparing the relative effectiveness of on-site filtration, cold storage transport, and three separate buffering solutions: Bensalkonium chloride (BAC), Benzethonium chloride (BEC), and Didecyldimethylammonium chloride (DDAC).[31] On-site filtration provided the best estimate of initial DNA concentrations followed by the addition of BAC as a chemical preservative.[31]

When filtering water samples at remote sites, filters can be preserved through freezing,[29,31] adding a chemical buffer,[32] or drying with silica beads.[4] Preserving the filters makes a significant difference. If left untreated, the detectable DNA on the filter decreases by 73% in one day.[27] In addition, the interaction between filter type and the intended extraction method should be considered. Due to cost considerations, initially I decided to use a phenol-chloroform-isoamyl alcohol (PCI) extraction method. Renshaw found that CN filters combined with PCI extraction yielded more DNA than the other three treatments tested,[32] so I opted to use cellulose nitrate (CN) filters along with Longmire's buffer to chemically preserve the filters. Longmire's solution is a storage buffer used for preserving filters, especially when eDNA water samples are filtered in the field. In addition to eliminating the need for cold storage for water samples or filters, Longmire's solution also starts the process of releasing DNA strands by lysing external cellular structures. This soluble DNA accumulates over time and is protected from further degradation by the Longmire's Buffer.

Sampling and extraction methods affect detection probabilities and PCR replication. In fact, collection and extraction methods had greater effects on PCR replication than amplicon size or the marker region used for species specific primers.[15] Three main collection and extraction combinations were used: precipitation with extraction from a commercially available kit,[7] filtration with extraction from a commercially available kit,[8] and filtration with extraction using PCI method.[32] A direct comparison between precipitation and filtration methods is difficult because the sample volumes used differ greatly. Precipitation uses 15 uL sample volumes, and filtration sample volumes vary between 100 mL and 10 liters.[6] Aqueous eDNA, 0.2 um in size or

smaller, gets captured more effectively by precipitation and is preferable when conducting tank studies.[27] However, aqueous eDNA is present in very low concentrations[4] and distributed in a clumped, patchy pattern in outdoor bodies of water.[17] For field applications, filtration produces higher eDNA yields than precipitation.[15,17] Qiagen DNeasy Blood and Tissue kits worked well with cellulose nitrate filters and produced more detections than precipitation and glass fiber filters .[4] Phenol-chloroform extractions generally yield more DNA than commercially available kits such as MoBio or Qiagen.[4] Especially in field samples with an increased amount of cellular DNA in suspended particulate, Proteinase K helps boost eDNA recovery from water samples by breaking down the proteins associated with cells and organelles.[28] In addition, a silica based inhibitor removal kit, such as OneStep by Zymo Research, can be used as needed when inhibitors are present, but as much as 25% of eDNA is lost while removing any inhibitors present,[24] so this step isn't typically applied unless an initial eDNA amplification attempt has failed.

Polymerase Chain Reaction

eDNA fragments from target species are detected using polymerase chain reaction (PCR). This technique requires several ingredients including a small amount of template DNA and a thermostable DNA polymerase enzyme that can withstand the nearly boiling conditions that are needed to denature the DNA, and gene-specific primers. A PCR amplification cycle consists of three basic steps: denaturing, annealing, and extending. The initial denaturing step occurs at high temperatures. High temperatures break hydrogen bonds and allow double-stranded DNA to separate into two strands. The

next step is the annealing process; this is when primers attach to their complementary sequences on the single stranded DNA template. Lastly, the Taq polymerase extends the complementary stands. Copies of the DNA are made during this final extension step. PCR doubles the amount of template present with each new amplification cycle. However, PCR has a major drawback – quantification occurs after amplification takes place, usually by running a gel.

An extension of PCR is quantitative PCR (qPCR), which uses a fluorescent dye called SYBR Green to help make the amplification of double stranded DNA visible. After annealing takes place, dye molecules bind to double stranded DNA, resulting in an increase of light emitting molecules upon excitation. This increase in fluorescence can be viewed using a camera. Absolute quantification is achieved by using standards created with serial dilutions prepared from a control template of known concentration. The ability to quantify the DNA in experimental samples provides qPCR several advantages over PCR. It detects DNA present in much smaller amounts, it provides an index of population size, and since DNA breaks down relatively quickly in surface water, it amplifies even low quality samples.[3]

Previous Research on Freshwater Turtles

Worldwide, the survival of turtles species is threatened. Of the 328 recognized modern species, between 48% - 54% are threatened with extinction.[35] The two main cause responsible for declining turtle populations are human exploitation and habitat loss.[35] In fact, turtles and tortoises face higher risks of extinction than most other vertebrates, except primates, which are equally imperiled.[35] Most turtles require access to

a combination of both wetlands and terrestrial environments and move seasonally between these types of habitats, making them vulnerable to a wide variety of habitat changes[36] Turtles lay their eggs outside the water, and some hibernating species overwinter on land. In many areas, legislation protects riparian zones by providing a terrestrial buffer zone of 30 meters upland from streams and wetlands.[37] However, turtles have been known to overwinter up to 500 meters from shore and change their hibernation site multiple times during a single season, so this 30 meter zone may not be adequate.[37] In addition, turtles require basking sites for adequate thermoregulation, but suitable sites such as floating logs and branches are often removed for flood control.[37]

The life history pattern of turtles, which in the past produced a stable population age structure, can mask the extent of their decline. Turtles have high fecundity, and the mortality rate for eggs and hatchings is high. Those individuals who survive to adulthood must experience a low risk for mortality for population demographics to remain stable. In addition, turtles have delayed sexual maturity with 18 freshwater turtle species engaging in initial reproductive events, on average, at about 12 years of age.[37] Natural predators such as skunks, raccoons, otters, foxes, coyotes, rats, and storks feed on turtle eggs and young hatchings.[37] However, adult turtles have very few natural predators, other than humans. Breeding females are more vulnerable to removal from the population because they spend more time on land as they nest and lay eggs resulting in an increased exposure to threats, including predators, road mortality, and poachers.[37] In a healthy population, only a fraction of the population should consist of adult turtles with the largest portion composed of juveniles and subadults. Conversely

otherwise healthy appearing populations of mostly adult turtles may actually be senescent, or so-called ghost populations.[37]

At one time, the western pond turtle (*Emys marmorata*) was common throughout the Pacific Northwest from Washington to Baja California. Currently the western pond turtle is listed as endangered in Washington, threatened in Oregon, and a species of special concern in California. Western pond turtles are still relatively common in undisturbed rivers and creeks in the foothills that surround the Sacramento Valley.[37] With the largest *Emys marmorata* population, the Sacramento River basin represents one of the last remaining strongholds, and a key area of focus for conservation efforts.[38] In California, suitable turtle habitat has been lost to urban development and agricultural land use. Habitat fragmentation hurts *Emys marmorata*, and even subtle changes can affect it. For example, *Emys marmorata* produces hard-shelled eggs incapable of responding to internal pressure caused by moist incubation substrates. Both irrigated farmland and artificially groomed landscapes with automatic sprinkler systems create areas too moist for western pond turtle eggs, so the eggshells rupture.[37] An additional threat faced by this native freshwater turtle is competition from an introduced turtle species, red-eared sliders (*Trachemys scripta elegans*).

The red-eared slider (*Trachemys scripta elegans*) is the most common turtle in the pet trade,[37] the most widely invasive reptile species,[38] and the most traded reptile world-wide in the last century.[39] *Trachemys scripta elegans* exists on all continents, except Antarctica. Because its distribution consists of large populations restricted mainly to areas with high human traffic, this suggests human introduction – as opposed to the natural expansion of established populations – has caused the spread of this species.[38] In

many habitats, red-eared sliders have several advantages over other native species: larger body size, more diverse diet, higher fecundity, earlier sexual maturity, adaptation to a wider range of water temperatures, and greater tolerance to pollution and human presence. *Trachemys scripta* also threatens native turtle populations as a possible disease vector. In Washington, a population of *Emys marmarata* suffered extensive losses from a respiratory infection in 1990, and *Trachemys scripta* was found culpable for the introduction.[37]

The interaction between *Trachemys scripta* and native turtle species involves competition for basking, nesting, and feeding resources.[40] During a longitudinal study conducted at UC Davis, Spinks found that *Trachemys scripta elegans* females grew to twice the size of *Emys marmorata* and were 38% heavier.[37] Size does matter because the largest turtle successfully displaces the smaller turtle for premium basking and nesting sites, regardless of the species.[37] And Spinks confirmed that competition exists between *Trachemys scripta elegans* and *Emys marmorata* for basking sites.[37] Access to better basking sites affects the survival of native turtles due to temperature dependent sex determination. Mortality rates and incubation success contribute to population sex ratios. At UC Davis, *Emys marmorata* had male biased sex ratios compared to *Trachemys scripta elegans* that showed balanced or female biased ratios indicating potential populations declines in western pond turtles.[37]

As a means of thermoregulation, basking is a crucial activity for turtles. It activates their metabolism and helps accelerate digestive turnover.[40] When a turtle is threatened, an active escape from its basking site into the water interrupts digestion and other metabolic processes. In Spain, when competing with *Mauremys leprosa*, (a species

which is ecologically similar to *Emys marmorata)* the invasive *Trachemys scripta* was more reluctant to flee, and this behavior provided a competitive edge over the more skittish native species.[40] When competing for food resources, *Trachemys scripta* aren't particular about prey items. Although omnivores by nature, red-eared sliders are mainly carnivores as juveniles and transition to a more herbivorous diet as adults. As an exotic species, they consume a wide range of food resources and are able to adapt their diet to whatever food items are abundant.[39]

Understanding how red-eared sliders affect native turtle species is an important concern,[38] and eDNA can help with estimates of distribution and population densities as well as diet studies. When I first began exploring the possibility of applying eDNA to turtles, research using molecular methods to detect aquatic turtles had not yet been published. Starting in 2015, three separate herpetological studies were published using eDNA survey methods for detecting freshwater turtles.[19-21] At the University of Ontario, eDNA samples were collected on eight native turtle species kept in tanks. The study also included samples taken from an outdoor pond containing six red-eared sliders, *Trachemys scripta,* to confirm detectability from field samples.[19] Primers were developed for nine turtle species in the Canadian study using the mitochondrial cytochrome c oxidase subunit I (COI) gene and amplified using PCR. In Alabama, researchers use two aquatic species with overlapping distribution (i.e. sympatric) to examine seasonal effects on eDNA detection in river systems using qPCR.[21] Whereas the Black Warrior Waterdog (*Necturus alabamensis)* is active during cool months, the Flattened Musk Turtle (*Sternotherus depressus*) is more active during warmer months. Souza's findings showed eDNA detection increases when sampling occurs during active periods that align with

behavioral considerations, such as sexual reproduction.[21] In Quebec, the wood turtle (*Glyptemys insculpta*) was the target species. Samples were collected from nine rivers and seven lakes in Canada, and qPCR was used for analysis of eDNA. The results showed a positive correlation between DNA amplifications and the relative abundance of wood turtles.[20]

In my thesis, I set out to address four specific points: (1) development of novel species-specific qPCR primers for *Trachemys scripta* and *Danio rerio*; (2) detection of the presence of *Trachemys scripta* under controlled conditions in the laboratory; (3) detection of *Danio rerio* in fecal samples taken from turtles; (4) detection of *Trachemys scripta* under field conditions. Important points of departure exist between this study and previous work with *Trachemys scripta*. I developed qPCR primers using the D-loop region of the mitochondria. The D-loop contains a hypervariable region in which base pairs of nucleotides have substitutions. Changes in the hypervariable region are highly polymorphic, which is good for distinguishing between closely related organisms. Unlike the Ontario study, both laboratory and field samples were analyzed for *Trachemys scripta* using qPCR. In addition, no previous studies on turtles have used eDNA for the detection of prey items in fecal samples to our knowledge.

CHAPTER II

MATERIALS AND METHODS

Tank Setup and Husbandry

Husbandry for the turtles consisted of three individual tank setups; the equipment was identical, and the conditions controlled. The tanks measured 25.4 cm (width) x 45.7 cm (length) x 23.5 cm (height). Each tank was equipped with a heat lamp (50V ZooMed UVA basking spot lamp), a mini compact fluorescent bulb (13W ZooMed ReptiSun 5.0 UVB), an air pump (Marina 75), Internal Biological filter (CA-750 Catalina Aquarium C110/120V), basking platform (Walmart), and smooth stones with an average length of 2.54 cm. In addition, metal mesh and wood-framed screens were constructed for each tank.

During the sampling period, tank water was drained every morning at 8:00 am ensuring all the turtles basked for the same amount of time each day, and different thermoregulation among turtles could be managed. At 10:00 am, water balloons filled with feces were collected, and the tanks were refilled with water. The tanks were filled with 5.7 liters of water, and the tank conditions were kept at 7.5 - 8.5 pH levels and a temperature range of 24˚C - 29˚C. At 11:00 am, every turtle was fed a single zebrafish (*Danio rerio*) along with enough aquatic turtle food (ZooMed) to ensure that the turtles fed as much as desired. Turtles swam for six hours until 5:00 pm when the fecal apparatus was checked a second time. However, juvenile turtles did not produce enough feces for a second collection. The zebrafish diet was initiated on October 1, 2016 and continued through November 30, 2016.

Tank Water Sampling Procedure

In the laboratory, experimental tanks were bleached using a 10% bleach solution and rinsed thoroughly prior to use in order to remove all potential DNA contamination. One turtles was placed in each of three sterilized tanks with 5.7 liters of water for three days prior to sampling. At the end of three days, 14% of the total volume of the each tank (800 mL) was sampled using Whirl-Paks. Water samples were collected along the surface of the water without any attempt to resuspend settled particulate. Three biological replicates were obtained, one from each of three individual tanks. In addition, contamination negatives were created for each individual tank using distilled water poured into Whirl-Paks and processed alongside the experimental samples. The filter funnel, vacuum flask, silicone tubing, and rubber stopper were sterilized using a 10% bleach solution and autoclaved before each use. Negative samples were filtered first, followed by the experimental sample for each tank. Because of the volume of water processed (800 mL) per sample, and the use of a filtration method for concentrating the DNA as opposed to a precipitation method, three biological replicates of eDNA sampling should be sufficient to produce a positive detection.

Filtration of Field Samples

When samples were collected in the field, one falcon tube filled with 50% bleach, and another filled with distilled water were used to clean hand tools such as tweezers; nitrile gloves were worn to prevent contamination. Samples were taken from sites spaced 100 meters apart and collected in Whirl-Paks labeled with the site number. Identical filter replicates were created by filtering 150 uL of water from every Whirl-Pak

through each filter. The flask, tubing, vacuum pump, rubber stopper, and filter funnel were assembled before processing samples (Figure 1a and b). The environmental water

(a)

(b)

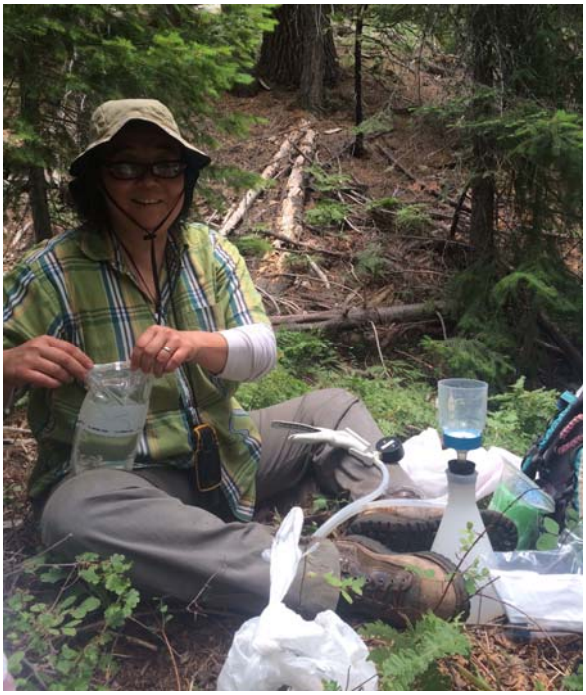

Figure 1 Water samples filtered onsite eliminated the need for cold storage. (a) 1-filter funnel; 2 -rubber stopper; 3 -vacuum flask; 4-silicone tubing; 5 -hand-operated vacuum pump. **(b)** Water samples collected in Whirl-Pak bags were poured into a collection cup with a cellulose nitrate filter. Samples were filtered onsite with a hand pump and Masterflex tubing which created suction, pulling the water down through the filter housing and into the Nalgene vacuum flask situated below.

samples were pumped through the equipment until the funnel was empty, and then the filter was removed and the tubing was detached to release pressure. Folded filters were put into coin envelopes and placed inside Ziplock bags filled with silica beads.

Longmire Lysis Buffer Filter Preservation

Longmire's solution consists of 100 mM Tris (Trizma Base, pH 8.0), 100 mM EDTA (Ethylenedinitrilo tetraacetic acid disodium salt, pH 8.0), 10mM NaCl (sodium chloride), 0.5% SDS (sodium dodecyl sulfate), and 0.2% sodium azide (prevents growth by biological contaminates). When filters were preserved using Longmire's buffer (700uL), buffer was added to a 2mL microcentrifuge tube. A folded filter was placed inside the microcentrifuge tube and completely submerged. In the lab, a pipette tip was used to mechanically break the filter into smaller pieces before the tube was placed into a rotating spinner for at least 24 hours. Then the DNA was extracted from the filter.

Dry Method of Filter Preservation

As a result of work done with a research team from Washington State University, experimental methods changed from using PCI extraction to using a commercially available kit, the DNeasy Blood and Tissue Kit (Qiagen, Germany), along with a dry filter preservation method. For remote collection sites, drying filters with silica beads is convenient because it does not require carrying cold-storage equipment or chemical preservatives. At remote sampling sites, silica beads have several advantages. Ziplock bags filled with silica beads occupy less space in a backpack than the equivalent number of falcon tubes filled with buffering solution. In addition, the buffering solution can leak and weighs slightly more than silica. Although not ideal, multiple filters can be

placed in the same silica bag because separate coin envelopes house each filter. Finally, dry filters crumble when placed into microcentrifuge tubes for DNA extraction. This provides more exposed surface area during the DNA extraction process.

In the field, contamination presented a challenge, so precautions were taken to minimize its impact on any samples collected. Powder-free, nitrile gloves were worn, tweezers were washed in a 50% bleach solution and then rinsed thoroughly after each use, autoclaved coin envelopes were kept in Ziplock bags, and pre-proportioned silica was also stored in Ziplock bags.

After the water sample was filtered, tweezers were used to remove the filter from the funnel housing. The filter was folded in half before placing it inside a coin envelope. Once labeled, the envelope was placed in a snack bag containing silica beads. The beads dried the filters and kept the DNA from degrading further until the DNA could be extracted.

Apparatus for Fecal Sample Collection

All procedures involving animals were conducted with permission from the Animal Care and Use Committee at California State University, Chico. In Figure 2, the apparatus design for fecal collection in aquatic turtles was taken from a dissertation from the University of Florida in 2004.[41] As shown in Figure 3, the fecal collection device consisted of a small Nalgene tubing connector with a water balloon attached to one end. Since collecting feces from larger turtles would require buying expensive condoms, juveniles were used instead. The study turtles measured 4-5 inches in length from tip to tail across the carapace. A wire was needed to hold the tubing securely in place, so a

Figure 2 Apparatus for collecting fecal samples in *Trachemys scripta*. Two small holes were made in the posterior marginal scutes of the carapace for threading through wire. A metal wire held tubing in place, and water balloons were attached for catching feces. Fecal samples were collected daily.

brass ring was affixed to the plastron and two tiny holes were created in the posterior marginal scutes of the carapace. A wire was coiled around the tubing before each end was threaded through a hole in the carapace and twisted into place. A second wire wrapped up and over the tubing before being looped through the brass ring on the plastron. The tubing connector sat flush against the turtle, and silicon caulk was inserted into any remaining gaps between the connector and skin. The feces flowed through the connector into the balloon, and the balloon was easily removed. Twist ties were used to prevent the water balloons from slipping off.

PCI Extraction Method

DNA extractions using Phenol-chloroform-isoamyl alcohol were used in three different applications – DNA extractions from tissue, filters, and feces. Depending on the

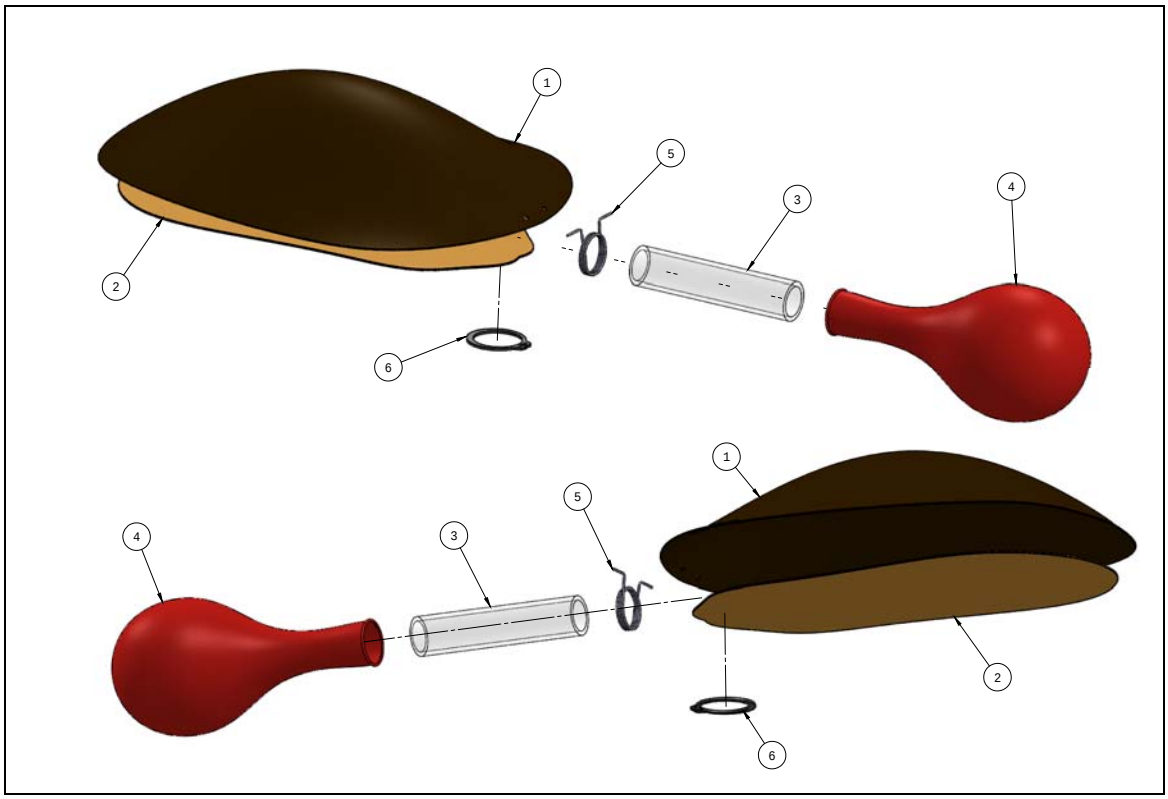

Figure 3 Technical drawing of fecal apparatus with a carapace and plastron exploded view. 1) Carapace with two small holes created in the rear marginal scutes. 2) Plastron 3) Vinyl faucet spray hose 4) Water balloon 5) Galvanized steel wire 6) Custom flat washer.

application, either embryo lysis buffer [10 mM Tris-HCl, 50mM KCl, 0.3% Tween 20 (w/v), 0.3% NP40 (w/v)] or Longmire's solution [0.1 M Tris, 0.1 M EDTA, 10 mM NaCl, 0.5% (w/v) SDS, 100 mM sodium azide] were used as initial steps.

DNA tissue extractions were performed using an embryo lysis buffer along with an initial 98˚C incubation step for 10 minutes that helped initiate cell lysing. The solution was cooled on ice before Proteinase K (10mg/ml) was added. Then the solution was placed in a water bath at 55˚C overnight. Once the solution removed, a second 98˚C incubation step destroyed any remaining Proteinase K. Finally, the digested genetic

material was centrifuged at 3,000 rpm for 10 minutes, and the supernatant transferred to a clean microcentrifuge tube (2mL).

Phenol-chloroform-isoamyl alcohol (PCI) provided additional purification by extracting proteins away from the DNA. The DNA was diluted before adding the PCI, and the solution was spun in a centrifuge [Eppendorf Centrifuge 5471, Germany] for three minutes. After transferring the supernatant to a fresh tube, acetate (1/10 volume) and ice-cold 100% ethanol (2.5x volume) were added to precipitate the DNA. Microcentrifuge tubes were taken to the cold room and spun [Beckman Coulter Microfuge 18 Centrifuge, Germany] for 15 minutes. The supernatant was then removed before washing the resulting pellet with 70% ethanol. The pellet was then dried before resuspending the DNA in 50 uL of Tris (50 mM, pH 7).

Cellulose nitrate filters were used to process up to 1 L samples of water in the field. With a preservation buffer, the solution is typically added while at the field site, and therefore must work at room temperature. 700 uL Longmire's solution was added to microcentrifuge tubes (2 mL) containing cellulose nitrate filters for an initial room-temperature incubation period which helped release DNA trapped inside cellular structures. This initial incubation period can be extended for up to two weeks without a significant loss in DNA recovery. Next, the microcentrifuge tubes were transferred to a 65˚C water bath. The subsequent water bath incubation period took only 10 minutes to an hour, due to increased cell lysis efficiency acquired over the previous incubation period. 700 uL Chloroform-Isoamyl alcohol was added to each tube and then vortexed [Scientific Industries Vortex-T Genie 2, USA]. The mixture was centrifuged at 14,000 rpm for 5 minutes and 500 uL of the aqueous layer was transferred into a new 2 mL tube. Ice-cold

isopropanol (500 uL) and room temperature 5M NaCl (250 uL) were added to the supernatant and allowed to precipitate at -20˚C for at least 1 hour. The precipitate was pelleted by centrifugation at 14,000 rpm for 10 minutes before decanting the liquid. The pellet was rinsed with 70% ethanol (150 uL), centrifuged at 14,000 rpm for 5 minutes, and then rinsed again with ethanol before decanting the remaining liquid. A final centrifugation helped in removing any remaining ethanol before the tubes were placed upside down on a paper towel to dry. The resulting pellet was resuspended in 50 uL of Tris (50 mM, pH 7).

Phenol Chloroform Isoamyl alcohol (PCI) extractions were also performed on fecal samples. A preserved filter was placed in a microcentrifuge tube with 500 uL embryo lysis buffer [10 mM Tris-HCl, 50mM KCl, 0.3% Tween 20 (w/v), 0.3% NP40 (w/v)] and incubated at 98˚C on a [Fisher Scientific Isotemp 205, USA] for 10 minutes. Then the tube was quenched on ice for 5 minutes before 20 uL Proteinase K along with 20 uL DTT were added. The filter was incubated in a 55˚C water bath for at least 18 hours. The filter and lysis buffer mixture was incubated a second time at 98˚C for 10 minutes to destroy any remaining Proteinase K. The mixture was spun in the centrifuge [Eppendorf Centrifuge 5417C, Germany] at 3,000 rpm for 10 minutes, and the resulting supernatant drawn off into a clean tube.

At this point, further purification of the DNA was required, so a PCI extraction method was performed to remove any excess proteins still present in the DNA mixture. Before further purification began, the DNA was diluted (3:1) in order to minimize the percent of DNA loss when pipetting the supernatant. Then, an equal volume of phenol chloroform isoamyl alcohol was added to the DNA mixture and vortexed for 10

seconds before being spun at 14,000 rpm for 3 minutes. Once the phases separated, 500 uL supernatant was transferred to a new microcentrifuge tube (2 mL) and sodium acetate (50 uL) and ice cold 100% ethanol (1,250 uL) were added. The mixture was centrifuged using a in the cold room for 15 minutes and then the supernatant was removed. The resulting pellet was rinsed with 70% ethanol (1 mL), centrifuged for 1 minute, and the ethanol removed before a final dry spin was performed. The remaining ethanol was carefully removed and the microcentrifuge tubes sat on the bench for five minutes or until completely dry. The pellets were resuspended in 50 uL Tris (50mM, pH 7.0).

Qiagen DNeasy Extraction Method

Qiagen uses a spin column in its DNeasy kits. This consists of a filter and column nestled inside a tube. DNA is negatively charged, so an acid solution is used to ensure the filter inside the column is positively charged. This helps the DNA adhere to the filter. When it is time to release the DNA, a basic eluting solution is added to the spin column, and the DNA flows through the filter into the tube below. During the project, the DNeasy kit was used in a few different ways: extracting DNA from tissue, cellulose nitrate filters, and fecal matter. Each application required a slight modification of the manufacturers recommended protocol.

All procedures involving animals were conducted with permission from the Animal Care and Use Committee at California State University, Chico. Before extracting DNA from tissue, a small tail clipping (~ 20mg) was taken from laboratory turtles. The tissue sample was mechanically disrupted and placed in a fresh microcentrifuge tube (2 mL). DNA was extracted using Qiagen DNeasy Blood and Tissue Kit. 300 uL Buffer

ATL, 20 uL Proteinase K (20mg/mL), and 20 uL DTT (1 M) were added to the tissue sample, and then incubated at 56˚C overnight or until completely lysed. After being incubated, the digested material was spun in a centrifuge at 14,000 rpm for two minutes before the supernatant was drawn off into a clean tube. Next, equal amounts of Buffer AL and 100% ethanol (300 uL per sample) were added simultaneously before vortexing [Scientific Industries Vortex-T Genie 2, USA] for 15 seconds. The remaining part of the assay was performed according to the manufacturer's instructions.

Regardless of whether ethanol, Longmire's solution, or a dry method was used in preserving the filters, Qiagen DNeasy Blood and Tissue kits could be used for extracting DNA from cellulose nitrate filters. I preferred using the unpublished, dry method of filter preservation developed by Dr. Caren Goldbgerg, which uses coin envelopes and desiccating silica beads. Once dried, filters can be stored for several months until DNA extractions can be performed.

In preparing for extraction, dried cellulose nitrate (CN) filters were removed from labeled envelopes and put into a microcentrifuge tube (2 mL). 500 uL ATL Buffer was added to the tube along with 20 uL Proteinase K and 20 uL of Dithiothreitol (DTT). The critical next step involved squishing down the CN filter with the pipette tip. This helped break the filter into smaller pieces and ensured the tube's contents were completely submerged. In addition, the mechanical disruption of the filter increased the surface area exposed to the lysis buffer. The microcentrifuge tube was placed in a rotating spinner for at least 24 hours. Next, the lysis buffer and filter were placed in a water bath at 56˚C for 1 hour, and vortexed occasionally during the incubation period to disperse the sample. After removing from the water bath, equal amounts of Buffer AL

and 100% ethanol (300 uL per sample) were added simultaneously before vortexing [Scientific Industries Vortex-T Genie 2, USA] for 15 seconds. The remaining part of the assay was performed according to the manufacturer's instructions.

A modified protocol for DNA extraction from fecal matter started with 25 mg fecal matter being added to a 2 uL microcentrifuge tube along with 800 uL Lysis Buffer [500 mM NaCl, 50 mM Tris HCl (pH 8.0), 50 mM EDTA, and 4% sodium dodecyl sulfate(SDS)]. Any large chunks were mechanically disrupted with a pipette tip and then vortexed. The mixture was incubated on a heat block [Fisher Scientific Dry Bath Incubator, USA] at 70˚C for 20 minutes with periodic vortexing. Samples were centrifuged at 7,000 rpm for 5 minutes and the supernatant transferred to a fresh Eppendorf tube (1.5 uL). 200 uL of ammonium acetate (10 mM) was added, and then vortexed before the lysates were incubated on ice for 5 minutes. Next, the tubes were spun at max speed (14,000 rpm) for 2 minutes, and then centrifuged at 7,000 rpm for an additional 3 minutes. 800 uL supernatant were transferred to a new Eppendorf tube (1.5 mL) and 800 uL of chilled isopropanol added. The mixture was centrifuged [Beckman Coulter Microfuge 18 Microcentrifuge, Germany] in the cold room for 15 minutes at 14,000 rpm, and the resulting pellet washed with 70% ethanol (1 mL). After decanting the ethanol, the pellet was resuspended in 150 uL Tris (50 mM, pH 7.0).

At this point, further purification of the fecal DNA was required, so the DNeasy Blood and Tissue kit was used. 200 uL of AL Buffer and 15 uL of Proteinase K were added to the resuspended pellet. The samples were incubated at 70˚C for 10 minutes and then 200 uL ethanol (100%) was added to the tube. The remaining part of the assay was performed according to the manufacturer's instructions.

Primer Design for *Trachemys Scripta*

Primers were identified for *Danio rerio* using a partial sequence of mitochondrial DNA from the D-loop region on GenBank (accession# AY940118). Primers were identified for *Trachemys scripta* using a partial sequence of mitochondrial DNA from the D-loop region on GenBank (accession# FJ392294). Primers were designed using Primer3 software (Primer3, http://www.biotools.umassmed.edu/bioapps/primer3/) using the parameters listed below (see Table 1).

Table 1 Species specific primers developed for *Trachemys scripta* (Ts), *Danio rerio* (Dr), and (GFP plasmid)

Species	Forward primer (5' -3')	Reversd primer (3' -5')	bp
Ts	CCTGCCGACTTGATGAAACTGGA	TGCAAGACCAACCAACTTGAACGA	50
GFP	GCAGAAGAACGGCATCAAGGTGA	GGGTGCTCAGGTAGTGGTTGTCG	143
Dr	CCCTTACGTCCAGCGATTCCTGT	TCTCGGGCCTTCCTTGGTTTAGG	61

A product was selected with a minimum size of 50, an optimum of 100, and a maximum of 150. The number of returns selected was 20, which is the number of results the program provides. The primer size selected was a minimum of 20, an optimum of 23, and a maximum of 27. The primer temperature selected was a minimum of 60, an optimum of 68, and a maximum of 70. The max self-complementary selected was 5, and the max 3' self-complementary was 3. Self-complementary refers to the likelihood or tendency of the PCT primer to bind to itself; this is not desirable. The primer should bind

to our target DNA sequence. The max poly-x selected was 3. Max poly refers to how many times in a row a base pair repeats itself.

PCR/Quantitative PCR

Conventional PCR was conducted on a [BioRad iCycler Thermal Cycler, USA] using the species specific primers developed for the species of interest, *Trachemys scripta* and *Danio rerio*. A PCR negative control and positive control were included with each plate, and three PCR technical replicates were typically performed for each experimental sample. The PCR was carried out in 20 uL reactions containing 11.2 uL DEPC H2O, 1 x PCR Buffer containing1.5 mM MgCl2, 0.2 mM dNTP mix, 5uM primer, 0.5ng/uL extracted DNA (5ng/uL), and 1 unit Taq Polymerase (5' prime). The thermal cycling treatment was 95˚C for 2 minutes, followed by 30 cycles of 95˚C for 15 seconds, 63˚C for 30 seconds, and 68˚C for 30 seconds with a final holding step of 4˚C for ∞.

Quantitative PCR was conducted on a [Eppendorf Realplex2, Germany] using the species specific primers developed for the species of interest, *Trachemys scripta* and *Danio rerio*. A PCR negative control and positive control were included with each plate, and three PCR technical replicates were typically performed for each experimental sample. The qPCR was carried out in 15 uL reactions containing 7.5 ul 2X Mix (Luminaris), 0.5 uM of each primer, 3.0 uL DEPC H2O and 1 ng/uL extracted DNA. The thermal cycling treatment was 95˚C for 2 minutes, followed by 40 cycles of 95˚C for 15 seconds, 68˚C for 45 seconds as well as a meltcurve step-up to 95˚C.

Gel Electrophoresis

Conventional PCR products were visualized on a [BioRad PowerPac HC, Singapore & Thermo Scientific OWL Easycast B2, USA] using gel electrophoresis with a 2% agarose gel and stained with ethidium bromide (1 mg/ml). Next 1-2 uL of 10X loading dye were spotted separately and individually mixed with each 10 uL cPCR product on parafilm. A full range DNA ladder was loaded into the far left well, followed by the rest of the samples. The gel was run at 100 Volts and 1.0 AMP for 30-45 minutes. The resulting bands were visualized on the Kodak gel-document imaging system [Kodak Gel Logic 100 Imaging System High Performance Ultraviolet Transilluminator, USA], and the image analyzed.

Field Sampling Sites

Water Pollution Control Plant (Chico, CA)

Field sampling sites for *Trachemys scripta* were selected based on the likely presence or absence of the target species. The positive control site selected was the Water Pollution Control Plant in Chico, CA. Just southwest of Chico, the plant is located at 4827 Chico River Road and is 1.7 miles from the Sacramento River. The waste treatment plant uses a gravity flow system consisting of collection, conveyance, treatment, and disposal facilities. At its current capacity, the plant can move 12 million gallons of water per day. Several rectangular holding ponds contain wastewater undergoing bioremediation. This is the final step before treated wastewater, or effluent, is quality tested and released into the Sacramento River. These holding ponds host several species of birds, plants, and also our target species, *Trachemys scripta*.

Permission to access the plant was obtained prior to sampling. Sampling occurred on September 7, 2016. The weather was sunny with no clouds, and the temperature was in the high 90s. The water was murky and had small green floating vegetation and abundant macro invertebrates. Readings were taken with a PCSTestr 35 WP [Oakton Instruments, Singapore] before collecting water samples The water temperature was 28.6˚C, conductivity was 720 uS/cm, and the pH was 7.07. The length of the pond was 104 meters in length and 90 meters in width. Eight samples were taken around the perimeter of the holding pond, one at each corner and also at the midline of all four sides (Figure 4). Water samples were collected in Whirl-Paks labeled with the site number. All labeled bags were kept cold in ice chests sterilized with a 50% bleach solution before use and transported to the lab for filtering.

Filter replicates were created for the holding pond by combining samples taken from sites 1-3, sites 5-6, and sites 7-8. Unfortunately, the Whirl-Pak from site 4 leaked, and the sample was lost. For the first filter, a hand-pump was used to process unstrained pond water, but the filtering took two hours. In an attempt to shorten the time spent filtering through the second filter, the sample was strained through a kitchen strainer washed in a 50% bleach solution and through an autoclaved cheesecloth before filtering with a hand-powered vacuum pump. The rest of the filters were processed by pre-straining samples and using an electric vacuum pump in the laboratory. In addition, the volume of sample processed through each filter was reduced (see Table 2). A dry method of preservation was used for storing cellulose nitrate sample filters.

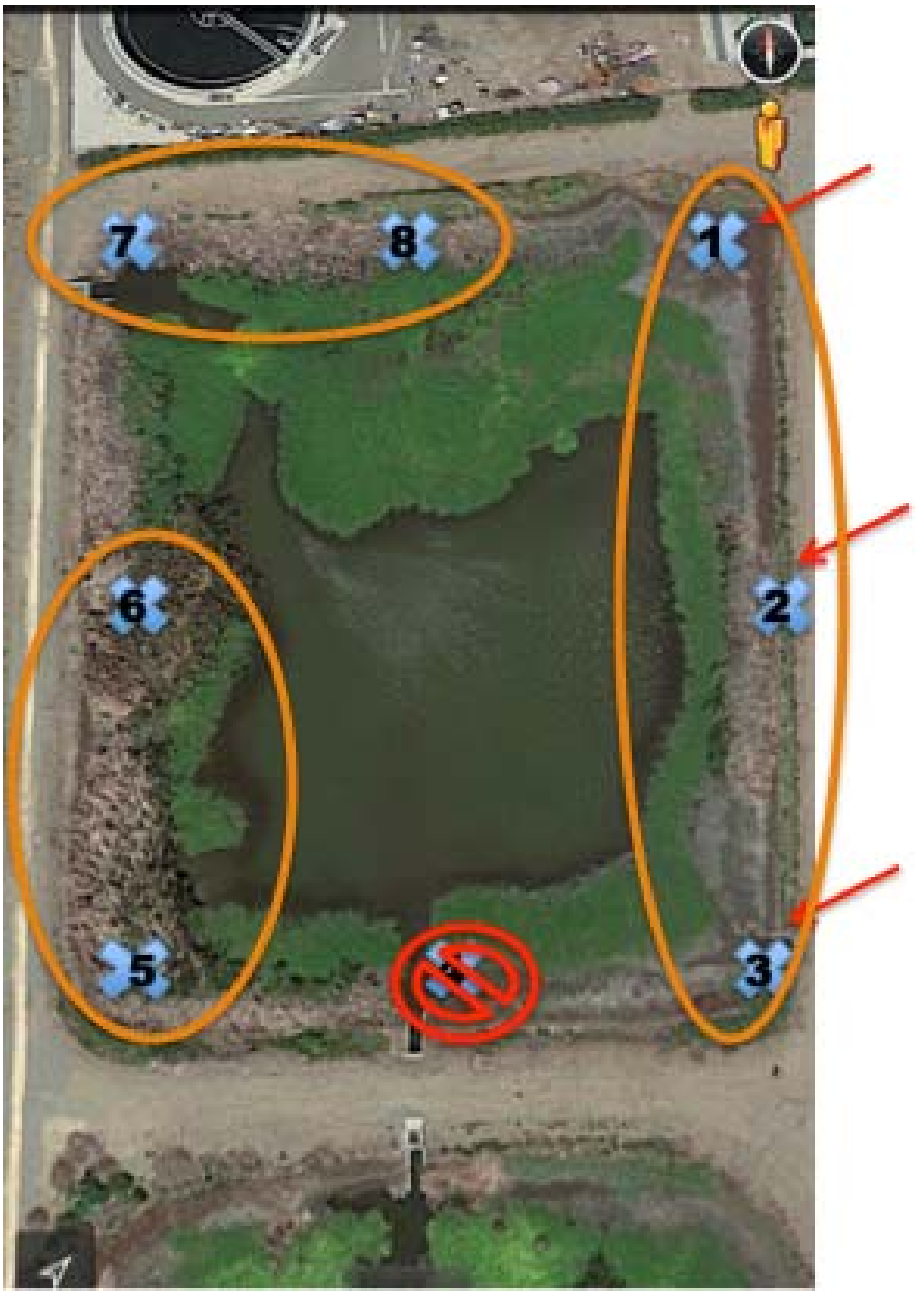

Figure 4 eDNA water sampling sites at the waste water treatment plant in Chico. A single (1L) sample bag was collected from each of eight sites along the perimeter of the pond; the bag from site four leaked. Samples 1-3, samples 5-6, and samples 7-8 were pooled together before creating filter replicates. The red arrows indicate the sampling sites where detection occurred.

Table 2 eDNA filtering data on water samples collected at the Water Pollution Control Plant in Chico. Water was collected from eight sites along the perimeter of a single holding pond. Site samples were combined to create filter replicates. An electric pump and straining were employed after the first two samples because filters kept clogging. Due to leaking, the sample from site #4 was lost.

Filter#	Site#	Strained	Pump	Amount Filtered
#1	1-3	No	No	400 mL
#2	1-3	Yes	No	400 mL
#3	1-3	Yes	Yes	450 mL
#4	1-3	Yes	Yes	450 mL
#5	5-6	Yes	Yes	300 mL
#6	5-6	Yes	Yes	300 mL
#7	5-6	Yes	Yes	300 mL
#8	7-8	Yes	Yes	300 mL
#9	7-8	Yes	Yes	300 mL
#10	7-8	Yes	Yes	300 mL

Sacramento Zoo

Located in William Land Park, the Sacramento Zoo occupies 5.8 hectares of land. The zoo first opened its doors on June 2, 1927, with 40 animals on exhibit. Currently the zoo has just over 500 animals on site but does not have any red-eared sliders in any of its current exhibits. The zoo has undergone multiple renovations over the years including an expansion in the early 1960s and construction of a giraffe habitat in 2009 (https://www.saczoo.org/about-us/history/).

The Sacramento Zoo contains two connected ponds, which are enclosed with buried wire mesh and wood fencing. The zoo staff communicated that no *Trachemys*

scripta lived in the ponds. However, the ponds outside the zoo in Williams Land Park contain *Trachemys scripta*, and city water pumped to the zoo might be routed through the park before entering the zoo. In addition, during the previous week, a staff member spotted a *Trachemys scripta* inside the zoo wandering along the walkways. This animal, unable to enter any of the exhibits, was removed from the zoo by staff.

Sampling occurred on May 14, 2017. The weather was clear with temperatures in the mid 70s. The ponds were murky with quite a few bird feathers scattered throughout the enclosure. Readings were taken separately at each pond before collecting water samples with the smaller site sampled mid-morning, and the larger pond sampled in the early afternoon. At the smaller pond, the water temperature was 17.8˚C, conductivity was 107.2 uS/cm, and the pH was 8.14. For the larger pond, the water temperature was 21.1˚C, conductivity was 108.9 uS/cm, and the pH was 8.33. The distance around the small pond was ~67 meters and ~125 meters around the larger pond. Five samples were taken around the perimeter of the smaller pond with 15 meters between sample sites, and seven samples taken around the larger pond; one sample site was deducted from the larger pond in order to accommodate the flamingo breeding area (Figure 5). Water samples were collected in Whirl-Paks labeled with the site number. All labeled bags were kept cold in ice chests sterilized with a 50% bleach solution before use and filtered on-site. After water collection was completed at the small pond, the five samples were pooled, and two replicates were filtered on-site. A fence divides the flamingo and waterfowl habitats. A second, sterilized filter funnel and housing are used before sampling the big pond. After water collection was completed at the big pond, the seven samples were pooled, and two replicates were filtered on-site (See Table 3).

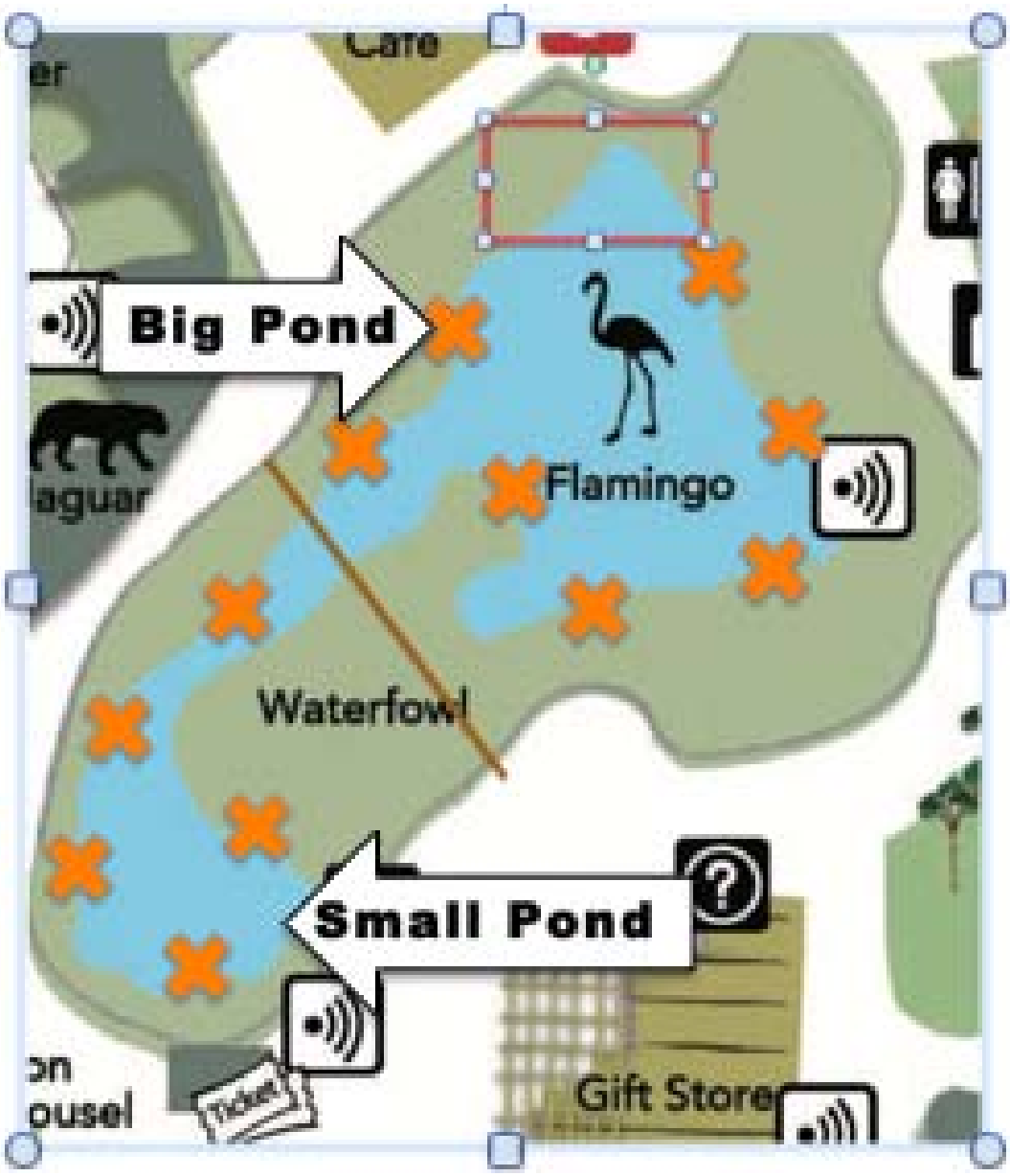

Figure 5: Sacramento Zoo Map showing field site where negative control eDNA samples taken. A single (1L) sample bag was collected from each of seven sites along the perimeter of the flamingo (big pond). The red box indicates the location of a breeding site where no sampling was permitted. A single (1L) sample bag was collected from each of five sites along the perimeter of the waterfowl (small pond). Samples from the big pond were pooled together before creating two filter replicates. All samples from the small pond were also pooled, and two filter replicated were made.

Table 3 eDNA filtering data on water samples collected at the Sacramento Zoo. Water was collected from seven sites along the big pond and five sites along the small pond. Site samples were combined to create two filter replicates, and distilled water was used as a blank. All filtering was performed on-site using a manual vacuum pump.

Filter#	Location	Pump	Amount filtered
1	Big Pond	Manual	300 mL
2	Big Pond	Manual	300 mL
3	Bottle	Manual	Blank
4	Small Pond	Manual	300 mL
5	Small Pond	Manual	300 mL
6	Bottle	Manual	Blank

CHAPTER III

FINDINGS

Species specific primers targeting the D-Loop region of mitochondrial DNA were developed for *Trachemys scripta elegans* using Primer 3. To verify the efficacy of the specific primer pair created, we ran a PCR using DNA extracted from tail clippings as a template. In Figure 6, bands appear at 50 base pairs for each of the three technical replicates performed (lanes 2-4). Primer only and template only negative controls were run, and all negative controls were negative (lanes 5-6).

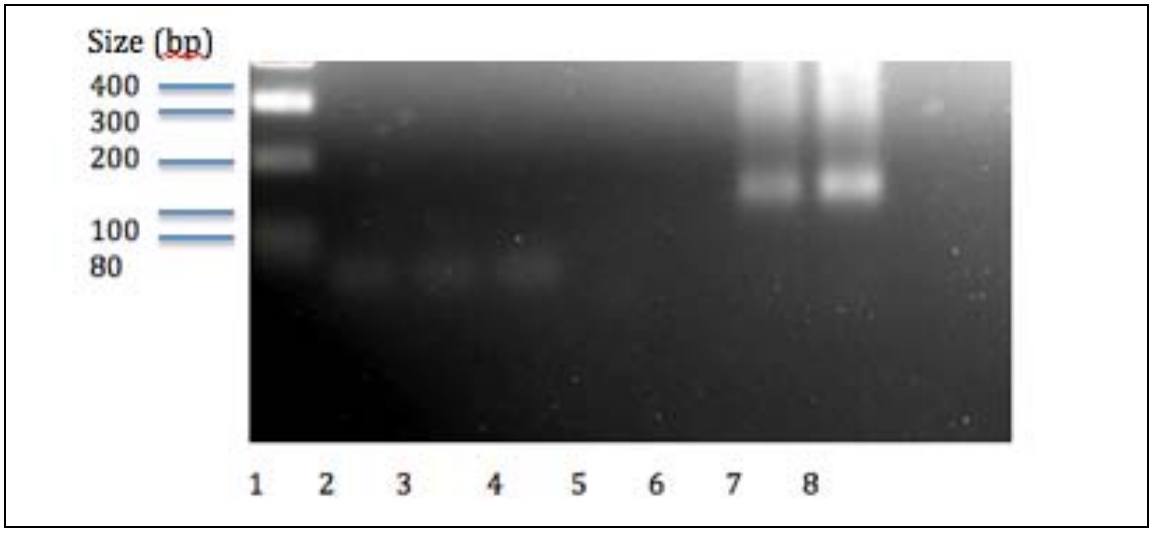

Figure 6 In vitro primer pair specificity shown for *Trachemys scripta* using tissue derived DNA. PCR products were separated by electrophoresis on a 2% agarose gel. The sizes of the molecular weight standards (lane 1) are shown on the left. Gene specific primer pair TSDLOOP50F/R was used with Trachemys template DNA (lanes 2-4). Negative controls used were primer only (lane 5) and template only (lane 6). A PCR product using a GFP plasmid with GFPRT_143F/R primers was used as a positive control (lanes 7-8).

To verify the specificity of the primer pair developed for *Trachemys scripta*, we performed qPCR to get a standard curve (Figure 7). The fluorescence detected from a five-step, serial dilution (1/5) on purified tail tissue DNA indicated a difference of approximately two threshold cycles (Ct) between dilution steps, except at the lowest concentration, and threshold cycles ranged between 16 and 30 (Figure 7a-b). In Figure 7c, the melt curve showed five products with slight shifts in peaks, and the negative control appeared as background noise. The standard curve showed 71% efficiency with a slope of -4.279 and R^2 value of 0.973.

Real-time PCR primers targeting the D-Loop region of mitochondrial DNA were also developed for *Danio rerio* using Primer 3. Using DNA templates taken from fin clips, we ran a PCR to verify the efficacy of the specific primer pair we created (Figure 8). In Figure 8, bands appear at 61 base pairs for each of the three technical replicates performed (lanes 2-4). Primer only and template only negative controls were run, however the template only negative control showed a smear due to the presence of genomic DNA template (lane 6). A positive control was run using a GFP plasmid with GFPRT_143F/R primers and showed an amplified product (lane 7-8).

The specificity of the primer pair developed for *Danio rerio* was verified using qPCR to get a standard curve (Figure 9a-b). The fluorescence detected from four serial dilutions (1/5) on purified DNA from fin clips indicated a difference of approximately four threshold cycles (Ct) between dilution steps, and threshold cycles ranged between 20 and 35. In Figure 9c, the melt curve showed four products neatly nested one atop another; however, no negative control is shown. The standard curve showed 71% efficiency with a slope of -4.305 and R^2 value of 0.996.

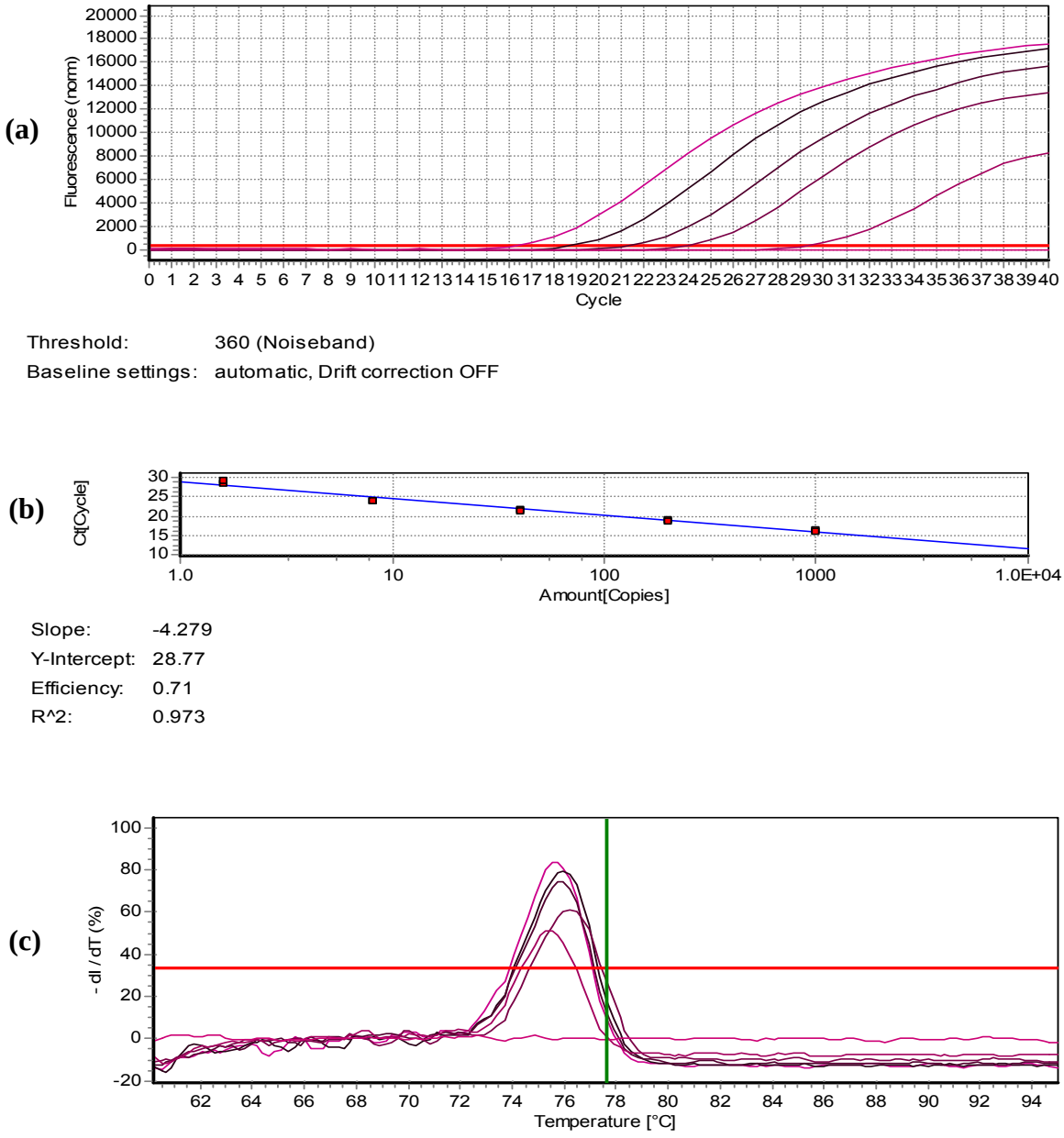

Figure 7 Verification of primer pair verified using qPCR standard curve for Trachemys *scripta*. After a five step serial dilution (1/10), DNA purified from tissue (turtle) was amplified using PCR primers specific for *Trachemys scripta*. (a) The amplification plot shows a consistent spread in C_t values overall, except in the lowest concentration. (b) Standard curve shows R^2 value of 0.973 with an efficiency of 71%. (c) The melt curve shows five products with slight shifts in Tm. The negative control appears as noise.

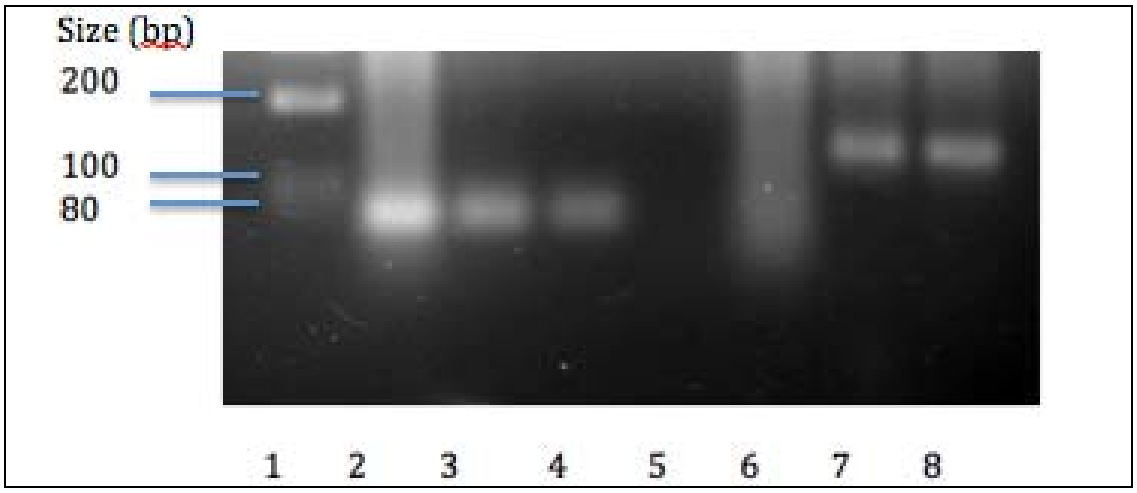

Figure 8 In vitro primer pair specificity shown for *Danio rerio* (zebrafish) using tissue derived DNA. PCR products were separated by electrophoresis on a 2% agarose gel. The sizes of the molecular weight standards (lane 1) are shown on the left. Gene specific primer pair DANIO_DLOOP61F/R were used with zebrafish template DNA (lanes 2-4). Negative controls used were primer only (lane 5) and template only (lane 6). A PCR product using a GFP plasmid with GFPRT_143F/R primers was used as a positive control (lanes 7-8). A single band does appear at the expected 61 bp.

Different serial dilutions of purified *Trachemys scripta* DNA were used to discover the concentration limitations for consistent qPCR amplification. Starting with an initial concentration of 2100 ng, a four-step serial dilution (1/10) was performed. A known amount of DNA (3 uL) was added to 250 mL water samples. The water was filtered using cellulose nitrate filters (0.7 um pore size), the DNA removed using PCI extraction, and the target DNA amplified using qPCR. In Table 4, the results showed amplification in sample concentrations as low as 0.21 ng of DNA, but detection rates were not consistent with DNA concentrations lower than 2 ng.

Preliminary eDNA assays were tested using water samples collected from experimental tanks containing *Trachemys scripta*. Three biological replicates were obtained using three separate individuals housed in their own tanks (Turtle A, Turtle B, and Turtle C). The funnel, flask, and filter paper used for sampling were changed in

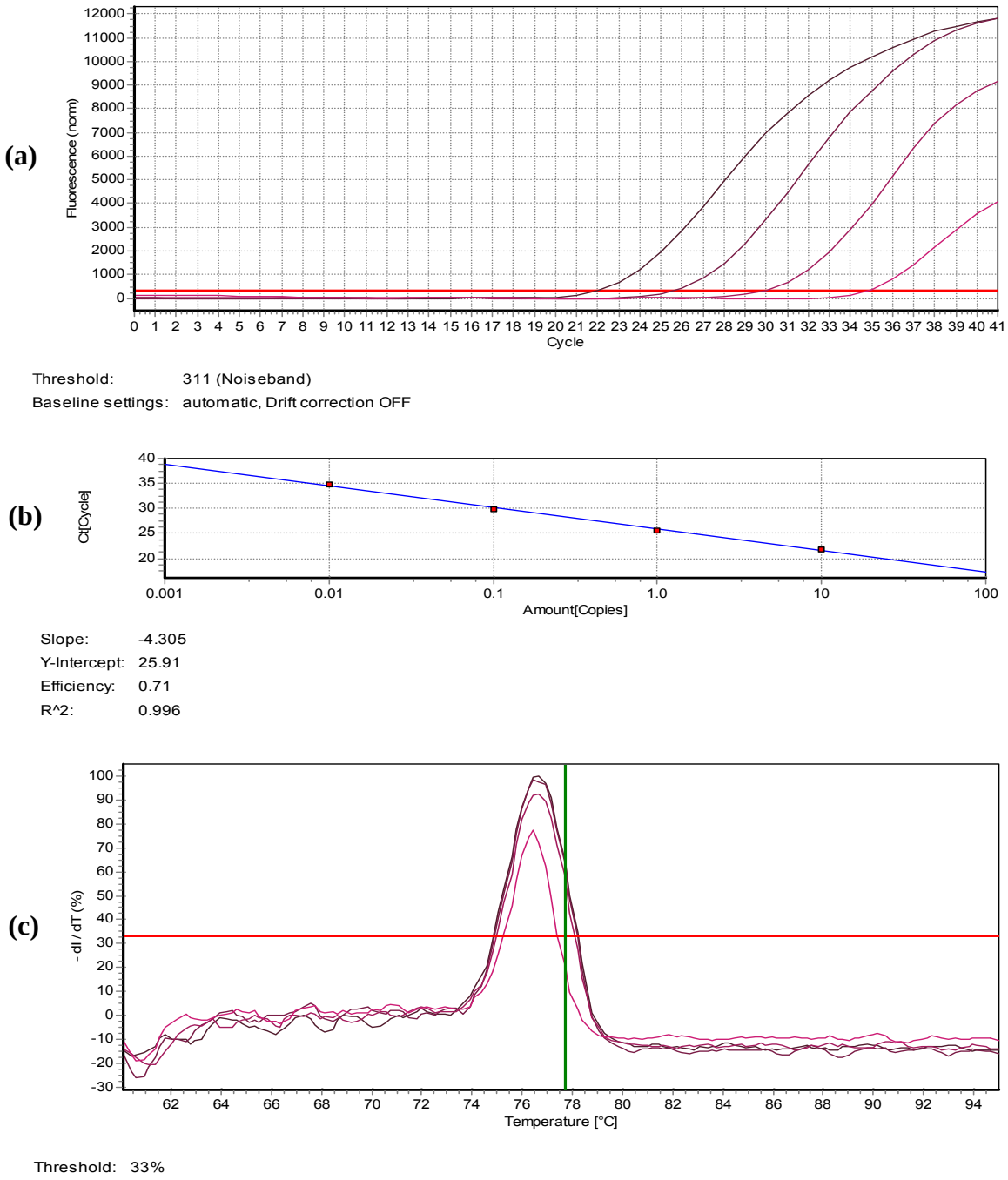

Figure 9 Verification of primer pair using qPCR standard curve for *Danio rerio*. After an eight step serial dilution (1/10), DNA purified from tissue (zebrafish) was amplified using PCR primers specific for *Danio rerio*. (a) The amplification plot shows a consistent spread in C_t values. The two lowest and highest concentrations are absent. (b) Standard curve shows an R_2value of 0.996 with an efficiency of 71%. (c) The melt curve shows four products with similar Tm.

Table 4 The ability to consistently detect *Trachemys scripta* DNA from aquatic samples decreases as the quantity of available genetic material decreases. In this experiment, a known amount of purified turtle DNA (2100 ng) was artificially added to 250mL of water. DNA was extracted from filtered water samples and then amplified using qPCR. The table lists sample mass and the number of positive PCR amplifications for each. All the experimental samples showed consistent amplification, except the lowest concentration (0.21 ng).

Mass of DNA (in ng)	Total number of positive amplifications
Blank	0/3
.21 ng	1/3
2.1 ng	3/3
21 ng	3/3
210 ng	3/3

between tanks. In addition, three technical replicates were run for each tank water sample, but only one negative was performed per tank. After filtering the water samples, Qiagen DNeasy kits were used to extract DNA from the filters before running qPCR.

In Figure 10, the graph indicates eDNA was recovered in varying amounts from water samples taken from all three tanks. Turtle C had the highest levels of amplification relative to the standard. Turtle A had the lowest levels of amplified qPCR product whereas Turtle B produced mid-level eDNA amplification when compared to the other two turtles. As shown, the error bars indicate similar values for amplification levels within each turtle's sample set. No amplification was detected in the negative control samples.

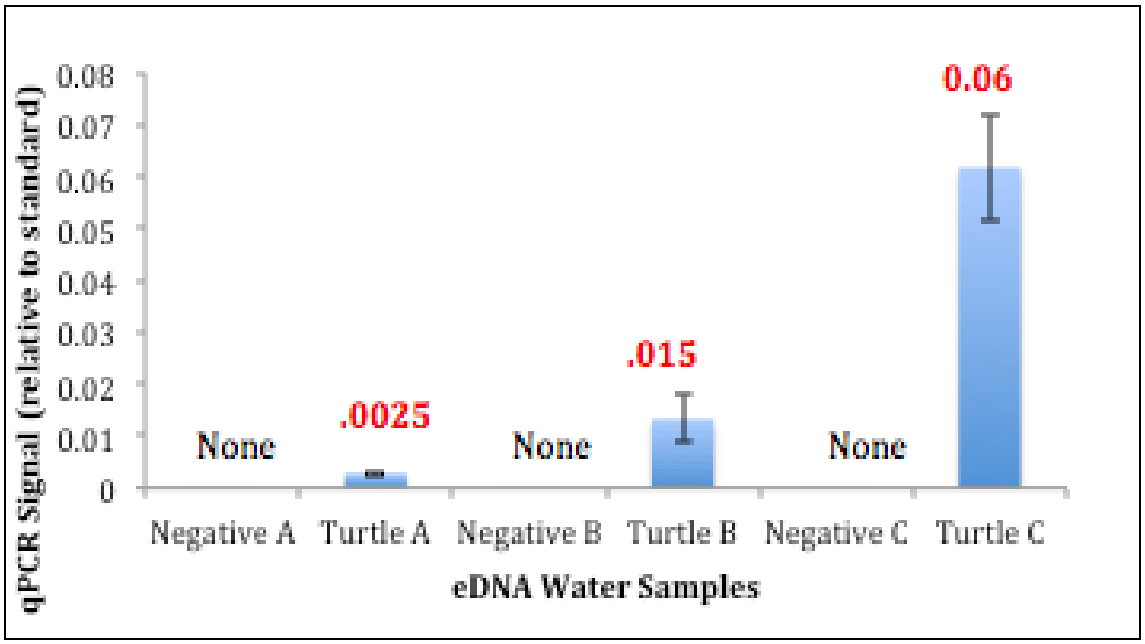

Figure 10 The presence of *Trachemys scripta* DNA in tank water was detected using quantitative PCR. Turtles were housed for 3 days in sterilized tanks containing 5.7 liters of water before sampling. Three biological replicates were obtained using separate tanks with each water sample (800 uL) representing 14% of the total volume of each tank. Aquatic eDNA samples were filtered with cellulose nitrate filters (0.7 um pore size), purified with the Qiagen DNeasy kit, and amplified using qPCR. Negative controls were created for each tank using distilled water poured into Whirl-Paks and processed alongside experimental samples. A gene specific primer pair TSDLOOP50F/R was used for qPCR, and experimental samples compared against standards using DNA from purified turtle tissue. The quantification of eDNA in experimental samples is determined through direct comparison to a standard created from tissue derived DNA in red-eared slider turtles. Comparisons between experimental samples shows variation in shedding rates between individuals. Turtle B had 6X the value of turtle A, and Turtle C had 4X the value of Turtle B as well as 24X the value of Turtle C.

Preliminary eDNA assays were tested using *Trachemys scripta* fecal samples collected from an fecal apparatus. Three biological replicates were obtained using three separate individuals each with their own apparatuses (Turtle A, Turtle B, and Turtle C). In addition, three technical replicates were run for each fecal sample, but only one negative was performed per turtle. After detaching the feces filled balloons, a phenol-

chloroform-isoamyl alcohol purification method was used to extract DNA from the feces before running qPCR.

In Figure 11, the graph shows stronger DNA detection in samples taken from Turtle B and Turtle C compared to Turtle A; DNA detection from Turtle A was much

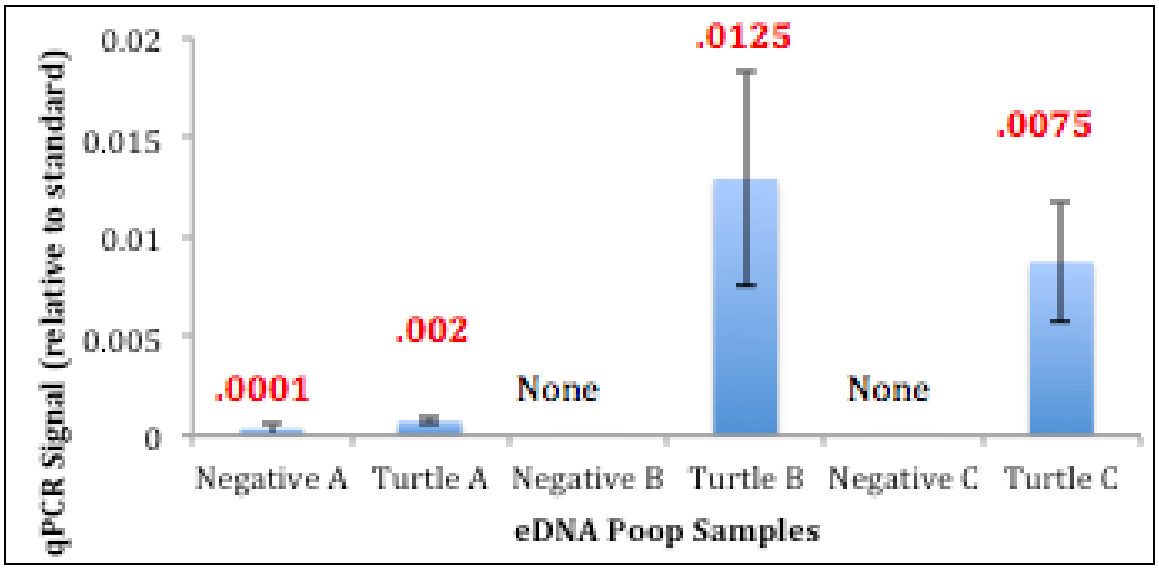

Figure 11 Turtle eDNA taken from *Trachemys scripta* feces detected using species specific primers. Fecal samples were collected in water balloons shortly after the turtles arrive in the lab. Three technical replicates were performed from a single DNA extraction, using the same water balloon fecal sample, which was taken from an individual turtle. Fecal samples were purified with the Qiagen DNeasy kit and amplified using qPCR. Negative controls were created by adding DEPC water instead of purified DNA onto the well plate. A gene specific primer pair TSDLOOP50F/R was used for qPCR, and experimental samples were compared against standards using DNA from purified turtle tissue.

weaker. The error bars indicate similar values for amplification levels taken on individual turtles. Background noise was detected in the negative control for Turtle A, but differences in detection level and error bar size suggest the presence of qPCR product in the sample but not the negative control.

Danio rerio DNA found in *Trachemys scripta* feces can be detected using qPCR. Feces collected prior to adding zebrafish to the turtles' diet provided a baseline for eDNA detection (Figure 12, 'No Fish'). qPCR was run on DNA extracted from commercially available food pellets (ZooMed) to verify the absence of *Danio rerio* DNA; zebrafish DNA was not detected (data not shown). eDNA was also extracted from turtle feces after adding zebrafish to the diet for seven weeks (Figure 12, 'Fish'). For both 'No Fish' and 'Fish' treatments, two separate *Trachemys scripta* individuals were used with three technical replicates processed from each turtle. The 'No Fish' treatment used *Trachemys scripta* primers as a positive control (data not shown), and the 'Fish' treatment was run using *Danio rerio* primers.

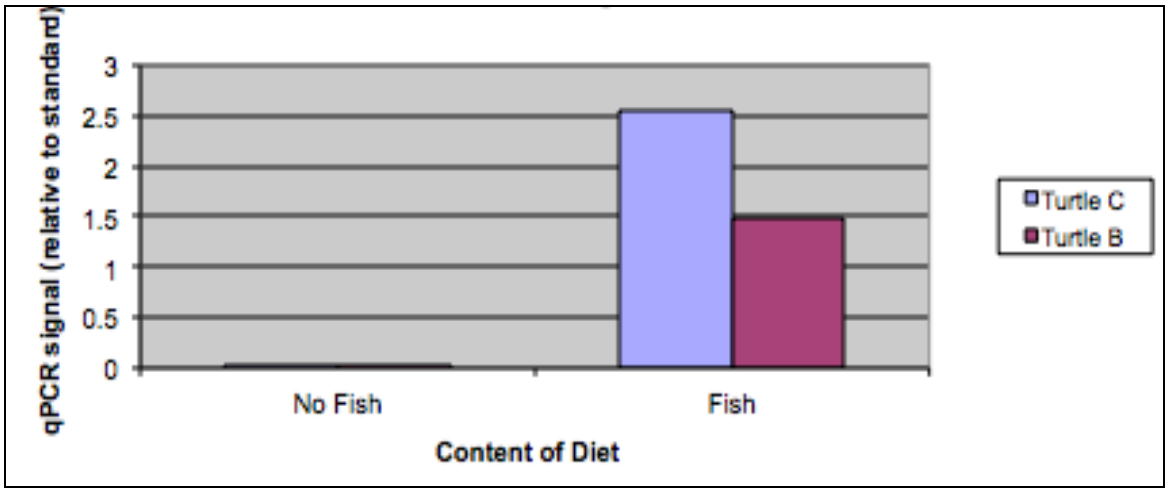

Figure12 *Danio rerio* (zebrafish) detected in turtle feces after added to *Trachemys scripta* diet. Fecal samples were collected in water balloons. An extraction was performed using PCI, and eDNA amplified by qPCR with DANIO_DLOOP61F/R primer pair for *Danio rerio*. Three technical replicates were performed from a single DNA extraction, using the same water balloon fecal sample, which was taken from an individual turtle. The quantification of eDNA in experimental samples was determined through direct comparison to a standard created from tissue derived DNA in zebrafish. In week one, the diet consisted completely of aquatic turtle food (ZooMed); whereas by week eight, zebrafish had been fed to the turtles daily almost two months.

Although samples run using *Danio rerio* primers should not have amplified, at least one produced a signal in Figure 12. But when comparing the relative amounts of zebrafish DNA found in samples collected with 'no fish' versus the relative amounts seen in samples with fish in the diet, the difference is clear. The relatively low signals from the 'no fish' samples are most likely background noise.

After developing assays in the laboratory, the next step involved collecting aquatic eDNA samples to detect *Trachemys scripta* in the field. In Figure 13, PCRs were analyzed by melt curve after extracting eDNA purified from water samples collected at the waste water treatment plant (experimental site) and Sacramento Zoo (negative control). At the wastewater treatment plant, two out of ten experimental samples showed a positive detection at or above the 10% detection threshold (Figure 13a, Table 5). A 10% threshold was selected because it showed positive detections without including any background noise. At the Sacramento Zoo, a sample extracted from filter 1 collected from the flamingo (big) pond showed a product slightly below the 10% detection threshold, but filter 2 (replicate) showed no detection (Figure 13a, Table 5).

After analyzing the meltcurves by qPCR, products approaching or exceeding the 10% detection threshold were separated by electrophoresis on a 2% agarose gel. In Figure 14, a band appears at 50 base pairs for the wastewater sample with strongest PCR signal in lane 4, but the weaker signal appearing in the meltcurve at the 10% threshold detection was not visualized on the gel in lane 3. A band appears at 50 base pairs for the negative control field site at the Sacramento Zoo in lane 6. A product is not visualized for the other filter replicated taken from the big pond at the zoo. A positive control using

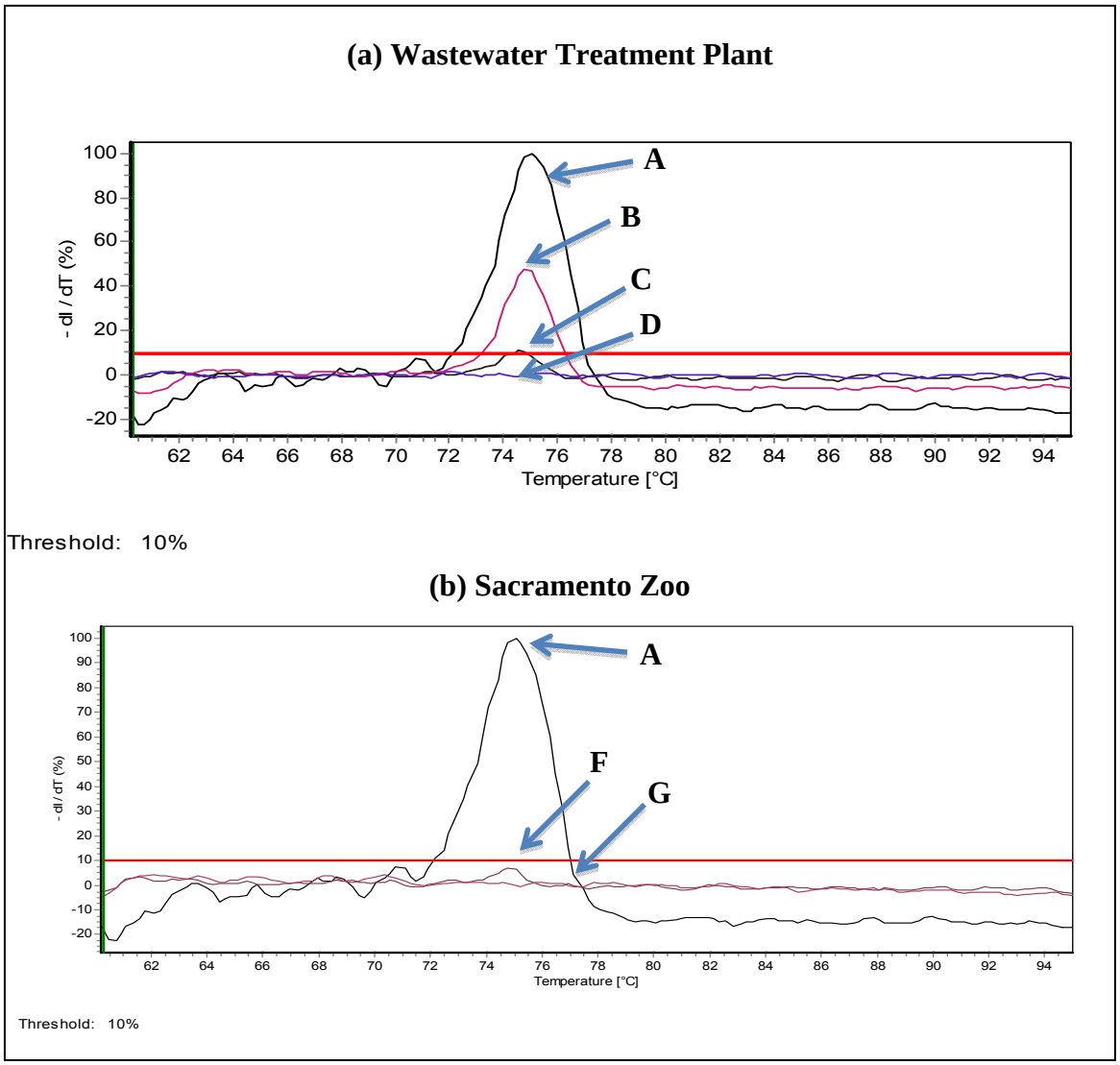

Figure 13 Detection of *Trachemys scripta* eDNA from field samples. PCRs were analyzed by melt curve after extracting eDNA purified from water samples collected at the waste water treatment plant (experimental site) and Sacramento Zoo (negative control). (a) At the waste water treatment facility: (A) shows amplification from tissue derived DNA, (B) product appears at ~50% tissue derived sample, (C) product appears slightly above the 10% threshold; (D) the field blank appears as a negative detection. (b) At the Sacramento Zoo: (A) shows amplification from tissue derived DNA, (F) product appears slightly below the 10% threshold, (G) the field blank appears as a negative detection.

DNA extracted from turtle tail tissue does show a band in lane 7, and the distilled water process in the field alongside pond samples does not show a band in lane 8.

Table 5 Detection of *Trachemys scripts* using water samples taken at the Chico waste water treatment plant and the Sacramento Zoo. At the waste water treatment plant, four filter replicates were created from water samples collected from sites 1-3, and two positive detections were obtained. Three filter replicates each were created from sites 5-6 and 7-8 with no positive detections. At the zoo, two filter replicates were created for both the small and big pond. No positive detections were observed from the small pond. The asterisk indicates a slight product was observed from a single sample taken at the big pond, but the melt curve fell below the 10% threshold for detection

Waste Water Treatment Plant		Sacramento Zoo	
Sites	Positives	Sites	Positives
1-3	2/4	Small Pond	0/2
5-6	0/3	Big Pond	0*/2
7-8	0/3		

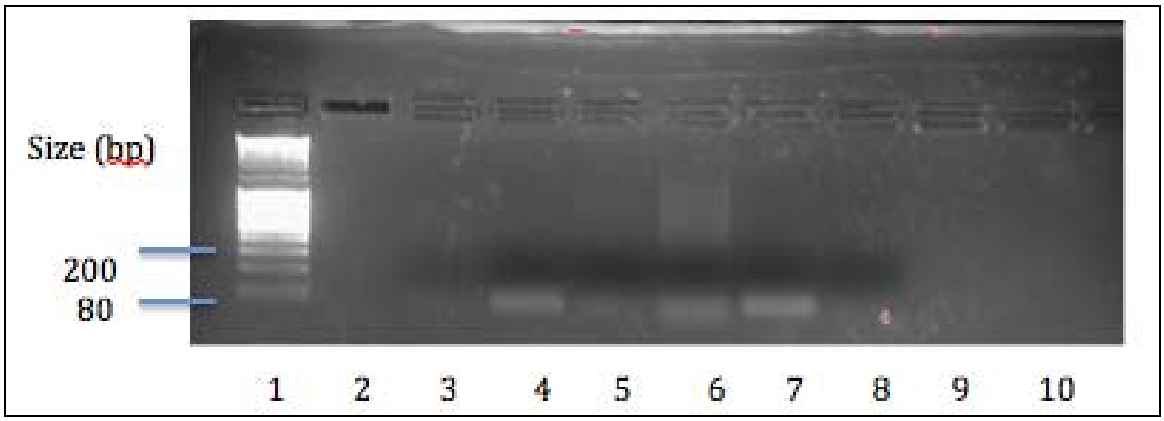

Figure 14 Detection of *Trachemys scripta* eDNA from field sample. PCR products were separated by electrophoresis on a 2% agarose gel. The sizes of the molecular weight standards (lane 1) are shown on the left. Gene specific primer pair TSDLOOP50F/R was used with eDNA taken from the waste water treatment facility (lanes 3-4). Gene specific primer pair TSDLOOP50F/R was used with eDNA taken from the Sacramento Zoo (lanes 5-6) A tissue derived DNA template for *Trachemys scripta* was used as a positive control (lane 7). A negative control used distilled water filtered at the field site(lane 8). A single band does appear at the expected 50 bp in lanes 4 and 7. However, an unexpected product was visualized in lane 6, the sample collected at a negative control site – the Sacramento Zoo.

Quantitative PCR primers, developed for *Trachemys scripta,* were tested under field conditions at the wastewater treatment plant in Chico (experimental) and the Sacramento Zoo (negative control). At the wastewater treatment plant, samples (1L) were collected from eight sites along the perimeter of the pond. Four filter replicates were collected from sites 1-3, and two positive detections were obtained. Three filter replicates each were created from sites 5-6 and 7-8 with no positive detections. Positive detection results in samples taken from sites 1-3 indicate the turtles were located along the southern perimeter of the pond at the time of sampling.

At the zoo, samples (1L) were collected from seven sites at the big pond and five sites at the small pond. Two filter replicates were created for both the small and big pond. No positive detections were observed from the small pond. However, a weak signal, indicated by an asterisk (Table 5) was observed from a single sample taken at the big pond, but the slight product shown on a melt curve fell below the 10% threshold for detection. If a positive detection occurred in the absence of *Trachemys scripta* within the zoo ponds, then this result represents a false positive.

CHAPTER IV

CONCLUSIONS AND RECOMMENDATIONS

Water Sampling Findings/Limitations

In my study, I developed qPCR primers for *Trachemys scripta elegans* and obtained positive detections for the target species under field conditions. Water samples were collected at the waste water treatment plant in Chico (experimental site) and the Sacramento Zoo (negative control). Previously, a Canadian study tested species-specific PCR primers on a man-made pond known to contain six red-eared sliders (*Trachemys scripta*), but no negative field site was used.[19] In Alabama, Souza sampled for two imperiled native species in the Black Warrior River basin using species-specific qPCR primers. Few studies have evaluated the efficacy and sensitivity of eDNA sampling methods on turtle species in natural aquatic environments.[20]

On 7 September 2016, eight (1L) samples were collected at the experimental site and transported back to the lab in a cooler filled with ice. Samples from sites 1-3, sites 5-6, and sites 7-8 were combined to create homogeneous sample replicates. Initially I filtered the sample without any modifications, but filtering debris-filled samples with a manual vacuum pump took two hours. As a result, I tried straining filter #2 through a kitchen strainer lined with cheesecloth before filtering with a manual vacuum pump. Filters #3-10 were strained after which I used an electric vacuum pump. The filters were preserved with a dry method using silica beads, and extracted later using the DNeasy extraction kit by Qiagen.

I encountered similar difficulties with clogging while filtering on-site at the zoo on 14 May 2017. Due to concerns over potential sample loss due to bags leaking and the two-hour commute back to Chico, all water samples were processed immediately after collection with a manual vacuum pump. Seven samples (1 L) from big pond, and five samples (1L) from the small pond were collected. The samples taken at each pond were combined to create homogenous samples, and two filter replicates were made for each pond by filtering 300 mL samples. The filters were preserved with a dry method using silica beads, and extracted later using the DNeasy extraction kit (Qiagen, Germany).

Different environmental sampling conditions require an adjustment in filter pore size. Laboratory tanks contain a higher proportion of extracellular DNA and fewer suspended particles compared to pond water.[28] Filter pore size requires balancing the ability to process larger volumes with suspended particles quickly (and possibly losing extracellular sources of eDNA) versus prolonged periods spent filtering smaller volumes of water. For both aquarium and pond water samples, I used cellulose nitrate filters with 0.7um pore size. I experienced no clogged filters with tank samples where extracellular DNA sizes averaged 0.2 um.[26] However, in pond samples where suspended particulate matter range between 1-10um, the filters clogged frequently.[17]

After extracting eDNA purified from water samples collected at the waste water treatment plant and the Sacramento Zoo, PCRs were analyzed by melt curve and products separated on a gel. With the wastewater treatment samples, two out of ten filter extractions showed a positive detection at or above the 10% detection threshold. At the Sacramento Zoo, one sample extracted from the first filter collected from the big pond

showed a product slightly below the 10% detection threshold, but its filter replicate showed no detection signal. The two positive samples from the wastewater treatment plant and both filter replicates from the big pond at the Sacramento Zoo were separated by electrophoresis on a 2% agarose gel. The wastewater sample with strongest signal produced a visible band, but the weaker signal was not visualized on the gel. For the zoo samples, a band appeared for the first filter replicate that showed a slight PCR detection signal, but a band was not visualized for its filter replicate, which didn't produce a PCR detection signal.

The positive result from the negative field site presents a problem. Field samples weren't in the linear range of the standard curve, so quantification was not possible.The question arises whether the detection signal represents *Trachemys scripta* physically present inside the zoo ponds (positive detection), or whether DNA entered from outside the system through vectors or other sources of contamination (false positive). For example, a *Trachemys scripta* was observed wandering around inside the zoo a week before eDNA samples were collected. However, the entire pond area is enclosed by a wooden fence and reinforced with wire mesh which extended below the ground down to half a meter. Therefore it's unlikely this unsolicited visitor gained access to the zoo's ponds. Fecal contamination by pelicans at the zoo is another possibility. Pelicans eat turtles, and the zoo's ponds are accessible by air. William Land Park, a recreational area located adjacent to the Sacramento Zoo, contains a population of *Trachemys scripta*.

Another possibility involves the route city water takes into the zoo ponds, as well as life history considerations for *Trachemys scripta*. If the water is piped directly

into the zoo, then city water isn't an issue. However, if the water is first sent to the ponds located in William Land Park and then piped into the zoo, the water supply itself may represent the source of DNA contamination. Seasonal differences may also have contributed to a possible false positive. *Trachemys scripta* engage in mating behavior between March and July. Increased activity levels in the turtles along with an increase in gametes present in the water make positive eDNA detection more likely.[21] Despite the distance traversed by pipe, if pond water filled with gametes from *Trachemys scripta* mating in William Land Park enters the aquatic zoo habitat, it's likely eDNA sampling would produce a positive or near positive detection without *Trachemys scripta* inhabiting the zoo's pond.

Sample contamination in the laboratory is a third possible explanation to explain the presence of a false positive detection. Strict laboratory protocols are necessary to protect the integrity of experimental samples and results. Due to the low quality of eDNA samples versus the high quality of PCR products, Goldberg recommends using separate rooms for PCR assembly and amplification. In addition, all equipment should remain in their respective rooms, and technicians should undergo a decontamination procedure consisting of a shower and clean clothes before switching between rooms.[4] DNA and PCR products present on surfaces and equipment within the laboratory can be removed in multiple ways. Autoclaving works well in destroying nucleic acids. Bleach is also effective at removing DNA and PCR products; 10% bleach solution is standard, but 50% bleach solution is necessary to eliminate extraneous contaminants on equipment and surfaces that cannot be autoclaved or exposed to UV sterilization. Workstations with UV sterilization and HEPA filters are another option for

addressing contamination issues. Other clean laboratory techniques include using filtered pipette tips and changing gloves whenever contact with a potential contaminant occurs.

For my project, I did not use separate rooms for PCR assembly and amplification. However, I did use 50% bleach solutions, an autoclave, clean gloves, exhaust hoods, and filtered pipette tips. Despite the lack of separation between PCR assembly and amplification, I rarely experienced amplification in negative controls. In my wastewater treatment and Sacramento Zoo samples, none of the negative field samples (distilled water poured over the equipment and filtered in the field) produced positive detection results, so laboratory contamination seems less likely.

In examining the positive detections at the waste water treatment facility, two of ten filter samples produced positive detection results. Although no criteria have been established regarding the proportion of positive eDNA detections necessary to infer the presence of a species,[4] published proportions range between 1/4 to 1/12. The samples taken from the waste water treatment pond resulted in a positive detection ratio of 1/5. Even though I did not visually spot the presence of *Trachemys scripta* in the waste water pond at the time of sampling, I have observed them during previous visits. When in doubt, eDNA results can always be paired with traditional sampling methods to verify species presence. When using traditional capture methods, there is no such thing as a false positive.[34]

Fecal Sampling Findings Limitations

I also developed an assay for the detection of prey items in turtle feces. In order to provide consistency in metabolic and thermal regulation between individuals, a

strict basking and feeding regimen was followed, and an apparatus was used to collect fecal samples from laboratory turtles. During an initial one-week period before introducing fish into the diet, two *Trachemys scripta* individuals were fed commercial food pellets (ZooMed), and fecal samples were collected daily. After the end of the first week, *Danio rerio* was added to the diet, and each turtle was fed one *Danio rerio* per day for seven weeks.

After completing the fecal sampling, extractions were performed using PCI, and eDNA amplified by qPCR with DANIO_DLOOP61F/R primer pair for *Danio rerio*. The quantification of eDNA in experimental samples was determined through direct comparison to a standard created from tissue-derived DNA in zebrafish. In week one, the feeding pellets produced a weak signal for two of the nine samples. By week eight, the zebrafish diet produced a signal 50,000 times larger than the feeding pellet signal, on average. These lower Ct values indicate higher amounts of *Danio rerio* DNA was found in the feces. When comparing the relative amounts of zebrafish DNA found in samples collected with 'no fish' versus the relative amounts seen in samples with fish in the diet, the difference is clear. The relatively low signals from the 'no fish' samples are most likely background noise.

No previous work has been conducted by using eDNA diet analysis on fecal matter from turtles. In Spain, a study was conducted manually examining the stomach contents of both *Emys orbicularis* and *Trachemys scripta*.[39] In exotic turtles, individuals were euthanized before examining the stomach contents; fecal samples from native turtles were collected from tanks.[39] eDNA analysis of fecal samples from both native and exotic species would allow for direct comparison of results. In addition, Deagle et al.

found DNA analysis of feces produced more consistent results over time compared to traditional hardpart analysis.[42]

Unlike aquatic eDNA, fecal sample collections require capturing the study animal. In this process, the apparatus is secured in place with a metal wire threaded through tiny holes created in the carapace of the turtle. This method of attachment limits its application to turtle species with hard shells. The apparatus is removed easily once the study has been completed; however, the turtle keeps the piercings it received. A non-invasive alternative involves keeping turtles of interest in a tank. Once the turtle defecates, the tank water is drained, and the recovered feces mixed into a slurry with 70% ethanol in a 1:3 ratio.[42] The disadvantage of collecting fecal samples directly from an aquarium is degradation and dilution of DNA. While omitting water from the tank eliminates problems with dilution, it makes recovery more difficult due to the distribution of thin layers of fecal matter along the glass surfaces and mostly likely exacerbates degradation due to exposure to oxygen and UV lamps.

Future Directions

eDNA detection from aquatic and fecal samples in turtle species is important considering half of all modern turtle species are endangered.[35] eDNA detection in aquatic environments makes sampling for target species faster and cheaper.[2] The sensitivity of the technique enables detection despite the presence of only a few individuals. This is important because either the species are endangered or they are the leading edge of an invasive species introduction.[43] eDNA also makes possible the identification of diet content from DNA contained in feces. Competition with other invasive species

contributes to population declines in endangered turtle species. For example, *Trachemys scripta* and *Emys marmorata* compete for food, basking, and nesting resources.[37] With eDNA verification of food resources, competition between native and invasive species can be made.

Sometimes predation by an invasive species threatens native species. In the state of Hawaii, a complex conservation situation exists for two endangered species, wattle-necked turtles (*Palea steindachneri*) and Goby fishes (*Lentipes concolor, Sicyopterus stimpsoni, Awaous guamensis, Stenogobius hawaiiensis,* and *Eleotris sandwicensis*). The wattle-necked, soft-shelled turtle might be eating Goby fishes, and in situations where the invasive and native species are both protected, eDNA provides a viable mean of determining prey items from feces. With its soft-shelled exterior, *Palea steindachneri* would require a capture and contain method for collecting feces. However, *Palea steindachneri* is a cryptic species, and trapping can prove difficult. In such cases, aquatic eDNA may provide an efficient means of locating the turtle before trapping begins, and once fecal samples are collected, species specific primers can be used for presence detection of Goby fishes in the extracted DNA.

Conclusion

In my study, I successfully developed qPCR primers for *Trachemys scripta* and *Danio rerio* using the D-loop region of mitochondrial DNA, tested the efficacy of those primers under laboratory conditions, devised a means of collecting fecal samples from hard-shelled aquatic turtles, and detected the presence of *Trachemys scripta* under real-world conditions. This is only the second time qPCR primers for turtles have been

used successfully in outdoor environments,[21] and it is the first time qPCR primers have been developed for *Trachemys scripta*.

Developing qPCR primers (as opposed to PCR primers) is important because field samples require larger volumes of water be processed in order to capture enough of the low quality DNA available in miniscule amounts to enable detection of a target species. Quantitative PCR decreases the likelihood of a false detection[33] where the presence of an animal likely would be missed by another sampling method. From a management perspective, a failure to detect a species does not equal absence.[25]

Devising a method for collecting feces in aquatic, hard-shelled turtles is important because diet studies provide insight into food consumption among native and invasive species. Prey item DNA can be extracted from fecal samples and detected using qPCR; moreover, the results are more consistent over time than traditional stomach content methods, and sample collection does not require sacrificing an animal. eDNA can be applied to fecal samples taken from soft-shelled turtles; this involves capturing and temporarily containing the animal.

Detecting eDNA in the field using qPCR primers is important because difficulties such as false positives and false negatives aren't necessarily confronted unless tested in an uncontrolled natural environment. Including a negative control field sampling site demonstrates the complexities of sampling outdoors and the relevance of protocols in reducing the contamination of samples under conditions both in the laboratory and the field.

REFERENCES

REFERENCES

1. Barnes MA, Turner CR. The ecology of environmental DNA and implications for conservation genetics. Conserv Genet. 2016;(17):1-17.

2. Pilliod DS, Goldberg CS, Laramie MB, Waits LP. Application of environmental DNA for inventory and monitoring of aquatic species [Internet]. Corvallis (OR): US Geological Survey; 2013. USGS Fact Sheet 2012–3146 [modified 2016 Nov 29; cited 27 May 2017]. Available from: https://pubs.usgs.gov/fs/2012/3146/

3. Lodge DM, Turner CR, Jerde CL, Barnes MA, Chadderton L, Egan SP, Feder JL, Mahon AR, Pfrender ME. Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. Molecular Ecology. 2012;(21):2555-2558.

4. Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS, et al. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol Evol. 2016;(7):1299–1307.

5. Paget E, Michel L, Georges F, Pascal S. The fate of recombinant plant DNA in soil. European Journal of Soil Biology. 1998;34(2):81-88.

6. Mächler E, Deiner K, Spahn F, Altermatt F. Fishing in the water: effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. Environmental Science & Technology. 2016;50(1):305-312.

7. Ficetola, GF, Miaud C, Pompanon F, and Taberlet P. Species detection using environmental DNA from water samples. Biology Letters. 2008;4(4):423-25.

8. Goldberg CS, Pilliod DS, Arkle RS, Waits LP. Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. PLoS ONE. 2011;6(7):1-5.

9. Smart AS, Tingley R, Weeks AR, Van Rooyen AR, Mccarthy MA. Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. Ecological Applications. 2015;25(7):1944-1952.

10. Fukumoto S, Ushimaru A, Minamoto T. A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: a case study of giant salamanders in Japan. Journal of Applied Ecology. 2015;52(2):358-365.

11. Jerde CL, Chadderton WL, Mahon AR, Renshaw MA, Corush J, Budny ML, Mysorekar S, Lodge DM. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. Canadian Journal of Fisheries and Aquatic Sciences. 2013;(70):522-526.

12. Mahon AR., Jerde CL, Galaska M, Bergner JL, Chadderton WL, Lodge DM, Hunter ME, Nico LG. Validation of eDNA surveillance sensitivity for detection of Asian carps in controlled and field experiments. PLoS ONE. 2013;8(3):1-6.

13. Farrington HL, Edwards CE, Guan X, Carr MR, Baerwaldt K, Lance RF. Mitochondrial genome sequencing and development of genetic markers for the detection of DNA of invasive bighead and silver carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in environmental water samples from the United States. Plos One. 2015;10(2):1-17

14. Janosik AM, Johnston CE. Environmental DNA as an effective tool for detection of imperiled fishes. Environmental Biology of Fishes. 2015;98(8):1889-1893.

15. Piggott MP. Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. Ecology and Evolution. 2016;6(9):2739-2750.

16. Lacoursière-Roussel A, Rosabal M, Bernatchez L. Estimating fish abundance and biomass from eDNA concentrations: variability among capture methods and environmental conditions. Molecular Ecology Resources. 2016;16(6):1401-414.

17. Davison PI, Créach V, Liang WJ, D. Andreou D, Britton JR, Copp GH. Laboratory and field validation of a simple method for detecting four species of non-native freshwater fish using eDNA. Journal of Fish Biology. 2016;89(3):1782-1793.

18. Piaggio, AJ, Engeman RM, Hopken MW, Humphrey JS, Keacher KL, Bruce WE, Avery ML. Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. Molecular Ecology Resources. 2013;14(2):374-380.

19. Davy CM, Kidd AG, Wilson CC. Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. PLoS ONE. 2015;10(7):1-9.

20. Lacoursière-Roussel, Anaïs, Yohann Dubois, Eric Normandeau, Louis Bernatchez, and Sarah Adamowicz. Improving herpetological surveys in eastern North America using the environmental DNA method. Genome. 2014;59(11):991-1007.

21. De Souza LS, Godwin JC, Renshaw RA, Larson E. Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. Plos One. 2016;11(10):1-15.

22. Rodgers, TW, Mock KE. Drinking water as a source of environmental DNA for the detection of terrestrial wildlife species. Conservation Genetics Resources. 2015;7(3):693-96.

23. Foote, AD, Thomsen PF, Sveegaard S, Wahlberg M, Kielgast J, Kyhn LA, Salling AB, Galatius A, Orlando L, Gilbert T. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. PLoS One. 2012;7(8):1-6.

24. Goldberg CS, Strickler KM, Pilliod DS. Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. Biological Conservation. 2015;(183):1-3.

25. Díaz-Ferguson EE, Moyer GR. History, applications, methodological issues and perspectives for the use environmental DNA (eDNA) in marine and freshwater environments. Revista de Biología Tropical. 2014;62(4):1273-1284.

26. Turner CR, Barnes MA, Xu CC, Jones SE, Jerde CL, Lodge DM. Particle size distribution and optimal capture of aqueous macrobial eDNA. Methods Ecol Evol. 2014;(5):676-684.

27. Minamoto T, Naka T, Moji K, Maruyama A. Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction. Limnology. 2016;(17):23-32.

28. Tsuji S, Yamanaka H, Minamoto T. Effects of water pH and proteinase K treatment on the yield of environmental DNA from water samples. Limnology. 2016;18(1):1-7.

29. Yamanaka H, Motozawa H, Tsuji S, Miyazawa RC, Takahara T, Minamoto T. On-site filtration of water samples for environmental DNA analysis to avoid DNA degradation during transportation. Ecological Research. 2016;31(6):963-67.

30. Williams KE, Huyvaert KP, Piaggio AJ. No filters, no fridges: a method for preservation of water samples for eDNA analysis. BMC Research Notes. 2016;9(298):1-5.

31. Yamanaka H, Minamoto T, Matsuura J, Sakurai S, Tsuji S, Motozawa H, Hongo M, Sogo Y, Kakimi N, Teramura I, Sugita M, Baba M, Kondo A. A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. Limnology. 2016;18(2):233-41.

32. Renshaw MA, Olds BP, Jerde CL, Mcveigh MM, Lodge DM. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. Molecular Ecology Resources. 2014;15(1):168-76.

33. Kelly RP, Port JA, Yamahara KM, Martone RG, Lowell N, Thomsen PF, Mach ME, Bennet M, Prahler E, Caldwell MR, Crowder LB. Harnessing DNA to improve environmental management. Science. 2014;27(6):1455-1456.

34. Roussel J-M, Paillisson J-M, Tréguier A, Petit E. The downside of eDNA as a survey tool in water bodies. Journal of Applied Ecology. 2015;52(4):823-826.

35. Turtle Conservation Coalition. Turtles in trouble: the world's 25+ most endangered tortoises and freshwater turtles—2011. Rhodin AGJ, Walde AD, Horne BD, van Dijk PP, Blanck T, Hudson, R, editors. Lunenburg (MA): IUCN/SSC Tortoise and Freshwater Turtle Specialist Group, Turtle Conservation Fund, Turtle Survival Alliance, Turtle Conservancy, Chelonian Research Foundation, Conservation International, Wildlife Conservation Society, and San Diego Zoo Global; 2011.

36. Browne CL, Hecnar SJ. Species loss and shifting population structure of freshwater turtles despite habitat protection. Biological Conservation. 2007;(38):421-429.

37. Spinks PQ, Pauly GB, Crayon JJ, Shaffer HB. Survival of the western pond turtle (*Emys marmorata*) in an urban California environment. Biological Conservation. 2003;113(2):257-267.

38. Thomson RC, Spinks PQ, Shaffer HB. Distribution and abundance of invasive red-eared sliders (*Trachemys scripta elegans*) in California's Sacramento River basin and possible impacts on native western pond turtles (*Emys marmorata*). Chelonian Conservation and Biology. 2010;9(2):297-302.

39. Pérez-Santigosa N, Hidalgo-Vila J, Florencio M, Díaz-Paniagua C. Does the exotic invader turtle, *Trachemys scripta elegans*, compete for food with coexisting native turtles? Amphibia-Reptilia. 2011;32(2):167-175.

40. Polo-Cavia N, López P, Martín J. Interspecific differences in responses to predation risk may confer competitive advantages to invasive freshwater turtle species. Ethology. 2008;114(2):115-123.

41. Bouchard SS. Diet selection in the yellow-bellied slider turtle, *Trachemys scripta*: ontogenetic diet shifts and associative effects between animal and plant diet items. [dissertation]. [Gainesville (FL]: University of Florida; 2004.

42. Deagle BE, Gales NJ, Hindell MA. Detecting prey from DNA in predator scats: A comparison with morphological analysis, using *Arctocephalus* seals fed a known diet. Journal of Experimental Marine Biology and Ecology. 2007;347(1):144-154.

43. Jerde CL, Mahon AR, Chadderton WL, Lodge DM. Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters. 2011;4(2):150-157.