SEASONAL COMPARISON OF ENVIRONMENTAL DNA AND TRADITIONAL SAMPLING TECHNIQUES FOR DETECTING COASTAL TAILED FROGS (*ASCAPHUS TRUEI*) IN NORTHERN CALIFORNIA

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ABSTRACT

SEASONAL COMPARISON OF ENVIRONMENTAL DNA AND TRADITIONAL SAMPLING TECHNIQUES FOR DETECTING COASTAL TAILED FROGS (*ASCAPHUS TRUEI*) IN NORTHERN CALIFORNIA

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While environmental DNA (eDNA) sampling has been shown to provide higher detection rates for aquatic amphibians compared to traditional sampling, the effect of season and stream characteristics on the efficacy of eDNA sampling remains unclear. The pH, turbidity, water temperature, and flow rate of streams may affect eDNA concentrations, and consequently influence detection rate. The purpose of this research was to (1) compare the detection rates of eDNA and traditional sampling techniques during different seasons, (2) observe the effects of stream characteristics on eDNA concentrations, and (3) review the relationship between animal abundance and eDNA concentrations at specific sampling sites. I used eDNA and traditional sampling techniques to detect coastal tailed frogs (*Ascaphus truei*) in cold, fast-moving streams. At three streams in northern California, we performed a "rubble rousing" technique and collected eDNA water samples every 100m during summer, fall, and winter. Water temperature, pH, flow rate, and turbidity data were collected from each stream. Detection rates for eDNA sampling (≥94%) were higher than those for traditional sampling (≤91%) when stream data was combined during the summer and fall. During winter, the detection rate for traditional sampling was higher (91%) than that for eDNA sampling (58%). With

season and water temperature excluded, flow rate had a significant, negative effect on eDNA concentrations, while higher eDNA concentrations were observed when eDNA sampling was performed in our largest stream. During summer and fall, a positive correlation between animal abundance and mean eDNA concentration was found for each stream, but not at specific sampling sites. Our findings indicate that mean eDNA concentrations found in streams can be used to monitor fluctuations in population size. Our results show that eDNA sampling is effective for monitoring tailed frogs during the spring and summer, but is not recommended for use during winter months due to increased flow rates.

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INTRODUCTION

Amphibians make excellent indicators for monitoring ecological changes such as pollution, habitat loss, and climate change, because of their sensitivity to environmental conditions. Larval amphibians, which are restricted to water before metamorphosis, can be greatly impacted by human disturbances, such as logging, that result in increased suspended sediment, bank erosion, and water temperatures (Noble and Putnam 1931, Semlitsch et al. 2009). Amphibian populations need to be regularly monitored across their range to prevent population declines due to changes in water quality following canopy removal and soil erosion. Traditional sampling techniques used to monitor aquatic amphibians include dip-netting, kick-netting, visual observations, and auditory observations. These techniques can be plagued by observer bias and limited detection rates due to low density or cryptic populations; they may also contribute to the deterioration of suitable habitat from continuous survey efforts (Herrick 2015, Gingera et al. 2016). As an alternative, environmental DNA (eDNA) techniques have rapidly become a reliable method for monitoring aquatic species across a range of habitat types. Environmental DNA techniques involve the collection and analysis of water samples for the presence of genetic material that is shed or released by an organism into its environment, most commonly in the form of blood, urine, feces, and intact skin cells (Dejean et al. 2012, Ficetola et al. 2008, Goldberg et al. 2011, Jerde et al. 2011, Pilliod et al. 2013, Pilliod et al. 2014, Takahara et al. 2012, Thomsen et al. 2012a).

The application of eDNA techniques promises several advantages over traditional sampling techniques. Specifically, eDNA techniques are more cost effective, reduce stress on the animals, require less time and labor in the field, and have higher detection rates (Dejean et al. 2012, Ficetola et al. 2008, Jerde et al., 2011, Goldberg et al. 2013, Pilliod et al. 2013). Given the same budget, the reduced costs associated with eDNA sampling allow for more sites to be sampled relative to the use of traditional sampling (Goldberg et al. 2011). In addition, eDNA sampling involves only the collection of water and therefore requires limited contact with the species of interest. Finally, the high detection rates provided by eDNA techniques can overcome under-representations of population size estimated using traditional techniques when animal population densities are low and/or individuals are extremely cryptic (Herrick 2015).

Environmental DNA techniques have been employed to characterize the distribution of threatened species, assess the biodiversity of an area ("metabarcoding"), and detect invasive species and monitor their spread (Deiner et al. 2015, Laramie et al. 2015, Goldberg et al. 2013, Thomsen et al. 2012a, Shaw et al. 2016, Spear et al. 2015). Over the past several years, eDNA techniques have become a popular sampling method for monitoring aquatic species, including fish and amphibians. Like amphibians, fish can be susceptible to increases in suspended sediments and water temperatures. The increased implementation of eDNA sampling to detect several fish species during breeding or spawning events is most likely due to the imperiled status and commercial value of some species (Janosik and Johnston 2015, Laramie et al. 2015, Sigsgaard et al. 2015, Takahara et al. 2012). In addition, several studies have used eDNA sampling to detect highly

elusive amphibians such as hellbenders (*Cryptobranchus alleganiensis*), Japanese giant salamanders (*Andrias davidianus*), and blind cave salamanders (*Proteus anguinus*) (Fukumoto et al. 2015, Spear et al. 2015, Gorički et al. 2017). In addition, the Rocky Mountain tailed frog (*Ascaphus montanus*) in central Idaho was detected using eDNA sampling (Goldberg et al. 2013, Nielson et al. 2001). These frogs are secretive, reside in high-gradient streams that are difficult to survey, and typically remain at low densities. Unfortunately, there is no "one size fits all" method to using eDNA techniques because different species and environments require somewhat different approaches.

Environmental DNA techniques are known to provide high detection rates in various aquatic environments (Biggs et al. 2015, Deiner et al. 2015, Dejean et al. 2012, Ficetola et al. 2008, Foote et al. 2012, Golberg et al. 2011, Goldberg et al. 2013, Janosik and Johnston 2015, Jerde et al. 2011, Pilliod et al. 2013, Takahara et al. 2013, Thomsen et al. 2012b) but very few of these studies have explicitly compared eDNA detection rates to the detection rates of traditional sampling techniques. It remains unclear how seasonal changes and variation in stream characteristics (i.e., pH, temperature, turbidity and flow rate) will affect the detection rates of eDNA and traditional techniques. One recent study assessed the effect of species behavior and activity during cool and warm seasons on the detection probability of eDNA sampling (deSouza et al. 2016). They found that season had a strong effect on eDNA detection probabilities because species that were more active during one season had higher detection probabilities during that time (deSouza et al. 2016). It has also been shown that colder temperatures and alkaline conditions are optimal for DNA persistence (Strickler et al. 2015). While many studies

collected turbidity measurements from their study sites, there has been limited research explicitly examining the effects of turbidity on the persistence of DNA. Studies have shown that increased flow rate can decrease the availability of eDNA in streams due to a "dilution effect" (Jane et al. 2015, Roussel et al. 2015). Most of the research assessing DNA persistence and degradation was conducted using mesocosms, or included the addition of animals into areas where they do not naturally occur (Barnes et al. 2014, Dejean et al. 2012, Klymus et al. 2015, Pilliod et al. 2013, Strickler et al. 2015, Takahara et al. 2012, Thomsen et al. 2012a). The majority of these studies saw no significant effect of temperature or pH on eDNA degradation. When eDNA sampling is used to detect species that are not added in streams but naturally occur in them, the animals can continuously contribute DNA and increase the likelihood of collecting eDNA for this species. However, in a natural setting, the pH, temperature, turbidity, and flow rate will not be controlled and may have a significant, compounding effect on the ability to detect eDNA. Studies comparing both techniques during different seasons needs to be conducted to address how changes in season and stream characteristics will alter the ability to detect animals.

Previous studies have found a positive correlation between species abundance/biomass and eDNA concentrations (Baldigo et al. 2017, Erickson et al. 2016, Jane et al. 2015, Klymus et al. 2015, Pilliod et al. 2013, Mahon et al. 2013, Spear et al. 2015, Takahara et al. 2012, Thomsen et al. 2012b). For example, a study conducted in central Idaho found a positive correlation between mean eDNA concentrations and density, biomass, and occupancy of Rocky Mountain tailed frogs (Goldberg et al. 2013).

These authors suggested that the eDNA concentrations collected at any point within a stream will represent the animal abundance upstream. However, studies have not successfully linked eDNA concentrations to species abundance at specific locations within a water source, so additional research is warranted.

My research assessed the efficacy of eDNA sampling relative to a traditional sampling technique for detecting coastal tailed frogs (*Ascaphus truei*) during three seasons: summer, fall, and winter. Like the Rocky Mountain tailed frog, coastal tailed frogs are extremely cryptic due to their small size, coloration, and lack of vocal calls, making adults difficult to survey (Stebbins 2003). Coastal tailed frogs typically remain at relatively low densities in cold, fast moving streams. Failure to detect these frogs using traditional sampling methods may provide misinformation about the status of known populations. Their range extends from northern California to the border of British Columbia and Alaska. In northern California, logging is prevalent in areas near tailed frog populations, making these frogs important indicators of stream health. The removal of nearby timber can increase stream temperatures, making tailed frogs susceptible to population decline due to their narrow range of temperature tolerance (Noble and Putnam 1931). During each season, I performed extensive eDNA and traditional surveys concurrently in three streams and recorded the number of animals, pH, temperature, turbidity and flow rate for each stream. The purpose of this research was to (1) compare the detection rates of eDNA and traditional sampling techniques, (2) evaluate the effect of pH, temperature, turbidity, and flow rate on eDNA concentrations, and (3) investigate if there is a positive correlation between animal abundance and eDNA concentrations for

each stream and for specific sampling sites within each stream. My goal was to determine an appropriate time frame for performing eDNA sampling in order to provide accurate detection rates for tailed frogs in streams located in northern California.

METHODS AND MATERIALS

Biology of Study Species

Coastal tailed frogs warrant attention due to their sensitivity to the stream conditions in which they reside (Brown 1975). In some areas, like British Columbia, little is known about tailed frog abundance, habitat requirements, and range, but several studies in the United States have deemed these frogs to be at risk after observing population declines following timber harvesting (Dupuis and Steventon 1999). Their restriction to streams during growth and breeding makes them susceptible to population decline in logged areas, due to increased water temperatures and sedimentation (Bury and Corn 1988, Diller and Wallace 1999, Welsh Jr. and Lind 1991). Metamorphosis occurs in one to four years, and tadpoles spend at least one winter in the stream. Tadpoles are found more often in high gradient riffles than pools or runs (Diller and Wallace 1999). Males become sexually mature two years after metamorphosis, and females most likely become mature three years after metamorphosis (Burkholder and Diller 2007). Adults have been found higher upstream compared to younger life stages; they move downstream to breed (Hayes et al. 2006). Breeding typically occurs during early fall and fertilization is internal (Stebbins 2003). Females usually breed every year, but coastal populations are known to breed every other year (Burkholder and Diller 2007, Sever et al. 2001). Eggs are laid the following spring and summer, and tadpoles hatch three to six weeks later (Brown 1975). Newly metamorphosed frogs disperse from the streams in the fall (Stebbins 2003).

Field Sampling of Streams in Northwestern California

eDNA collection

Three streams in Humboldt County, CA were extensively surveyed for the presence of *A. truei*: Mule Creek, North Fork (NF) Maple Creek, and South Fork (SF) Ah Pah Creek (Figure 1). These creeks were known to harbor *Ascaphus truei* in relatively low, medium and high densities, respectively, based on prior surveys. Mule Creek is located near Korbel, CA and is a tributary to the North Fork of the Mad River. It was the smallest of the three streams, with a watershed area of 1,413 km^2. The initial 75% of Mule Creek is classified as a first order stream and the remaining 25% is a second order stream. North Fork Maple Creek is a primary inflow to Big Lagoon and has a watershed area of 2,024 km^2. The initial 50% of the sampling reach for NF Maple Creek is considered a second order stream and the remaining 50% is a third order stream. South Fork Ah Pah Creek is a tributary to the lower Klamath River, and was the largest stream, with a watershed area of 5,367 km^2. This stream is classified as a fourth order stream. We surveyed a reach of approximately 1200 meters for Mule Creek, and reaches of approximately 2000 meters each for NF Maple Creek and SF Ah Pah Creek. The sampling reach for Mule Creek was smaller than the others because the habitat outside the 1200 meter sampling reach was unsearchable and unsuitable for amphibians. This stream was still chosen for study because I wanted to include a stream with a relatively low density of tailed frogs.

Before each stream was surveyed for animals, two Green Diamond Resource Company field technicians and I collected eDNA samples every 100m along the sampling reach of each stream. Multiple people took water samples throughout the watershed in order to collect all water samples during the shortest time interval possible. For each stream, we collected water between 09:00 and 15:00 hours, completing sampling within a single day. Each sample consisted of one liter of water that was collected in a sterile bottle. The bottle was rinsed three times with stream water to remove residual sterilizing agents before collecting the sample. Three replicate one-liter samples were taken from 25% of randomly chosen sampling sites to investigate whether there was any significant variation between eDNA concentrations collected from specific sites. After samples were collected, bottles were temporarily placed back into the stream (for up to 30 minutes) until they could be stored on ice. The water was filtered through a 0.45μm pore-size cellulose nitrate filter (Sterlitech Co., Kent, WA) using a vacuum pump and captured in a flask on a sterile workbench. Each filter was cut in half, and each half (labeled "A" and "B") was placed into a separate vial containing 95% ethanol. Half of all filters ("A" sides) were taken to a sterile lab on the Humboldt State University campus, and remaining samples ("B" sides) were stored in a cold room at Green Diamond's office in Korbel, CA. For a negative control, water was collected and filtered from an enclosed, outdoor tank near the Fisheries field crew office in Korbel, CA. The tank was visually surveyed to ensure that no vertebrates (and *A. truei* in particular) were present and one 1-L water sample was collected from this site during each season.

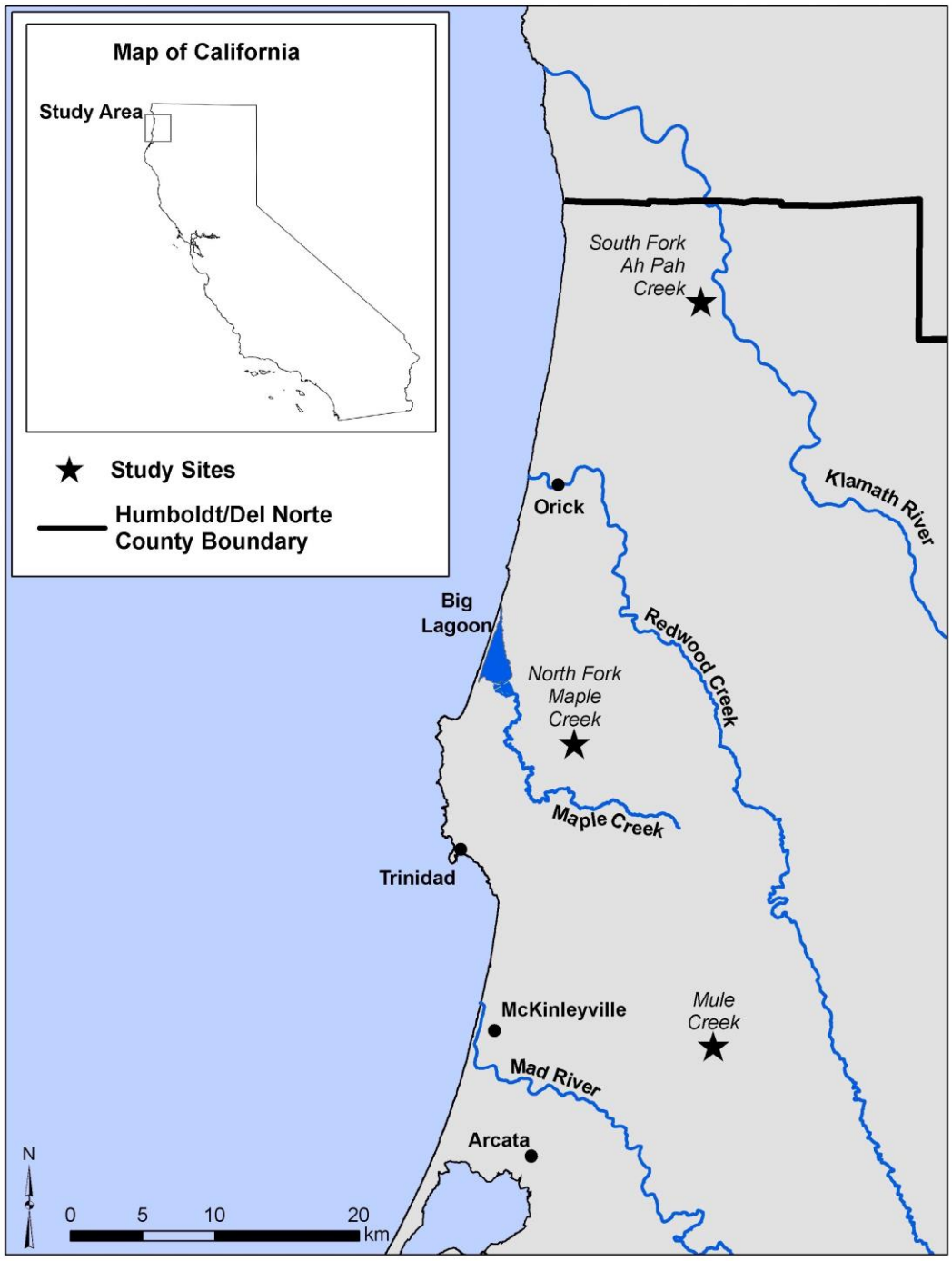

Figure 1. Map of northern California representing the three sampling sites: Mule Creek, NF Maple Creek, and SF Ah Pah Creek. Stars represent the exact locations of our sampling sites and their proximity to the bodies of water into which they flow.

To observe seasonal differences in eDNA concentrations, we sampled during three different intervals. The summer sampling period took place during July 2014, before the tailed frog tadpoles metamorphosed. The fall sampling period ran from late September to early October 2014, before newly hatched tailed frog tadpoles appeared in the streams. The winter sampling period took place between February and early April 2015 when newly metamorphosed tailed frogs had dispersed from streams.

During each field season, three environmental variables were measured at each sampling site in each stream to assess their effects on the ability to detect eDNA: water temperature, pH, and turbidity. Water temperature and pH were measured at each sampling site for each stream during each field season. Temperature measurements were accurate to a tenth of a degree (°C). Measurements for pH were made to the one-hundredth value on the pH scale. A 300mL water sample was collected from each sampling site to determine the turbidity during eDNA collection. Each turbidity sample was shaken vigorously and partitioned into three, clean 30 mL lab turbidity sample cells (Hach, Loveland, CO), with excess available if additional subsamples were needed. Each sample cell was inserted into a 2100N Laboratory Turbidimeter (Hach, Loveland, CO) that provides accurate Nephelometric Turbidity Unit (NTU) readings. Each sample cell provided one NTU reading for a total of three NTU readings for each turbidity sample. A fourth reading was taken from an additional sample cell if the first three values were not within ±10% of each other. (Hach Method 8195 Determination of Turbidity by Nephelometry). The NTU measurements were averaged to obtain the turbidity for each sampling site.

Several flow rate measurements were collected from each stream during each field season. I calculated the flow rate of several cross-sections of each stream by (1) multiplying the distance a floating object traveled downstream, stream width, and average depth, and then (2) dividing the product by the average time it took for the object to travel along the specific length of stream. First, we measured the cross-sectional width with a tape measure at a minimum of three different locations along the stream. Locations were chosen near the beginning, middle, and end of the sampling reach. The number of locations where cross sectional measurements were taken increased to at least four for the two larger streams, NF Maple Creek and SF Ah Pah Creek. This number increased to at least five locations per stream when water was more available during our winter field season. At each location, we measured the flow rate of an arbitrarily chosen length of stream. The length of stream that was chosen at each location was contingent upon water availability. A floating object was released and as it traveled down the stream, the amount of time it took to travel along each length of stream was recorded using a stopwatch. This was repeated a total of three times; a fourth time measurement was taken if the first three times were not within ±10% of each other. Depth measurements were taken at 25% of the wetted width, middle channel, and 75% of the wetted width at each cross section using a yardstick. The average depth of each cross-section was determined by averaging the three depth measurements. The flow rate of each cross-section was then adjusted to account for the fact that our measurement took place at the stream surface instead of in the middle of the water column (Arizona Board of Regents 2007). Average flow rate was calculated for each stream by averaging the flow rates of all cross-sections of that stream.

Animal abundance surveys

A “rubble rousing” technique was applied to each stream the day after water samples were collected (O’Donnell et al. 2007, Quinn and Hayes 2007). Assisted by Green Diamond technicians, I used clear-bottomed view buckets and nets to search the lotic habitat (e.g., riffles, eddies, thalwegs) and cover objects (e.g., un-embedded substrate) for tailed frogs (Edelman et al. 2015). Using a hip chain distance measurer (Forestry Suppliers, Inc.), we started our meter count at the beginning of each water sampling site and measured the location and number of observed animals as we moved up the sampling reach (~100 m). Tadpoles were our main focus since they reside entirely within streams, but we also included the location and number of adults observed.

These data were used to calculate detection rate, species abundance, and density of tadpoles and adults (Goldberg et al. 2013). The detection rate for traditional sampling was calculated for each stream by dividing the number of sampling sites where tailed frogs were detected by the total number of sampling sites in each stream. The species abundance in each stream was estimated from the total number of tadpoles and adults found during our animal surveys. Tailed frog density was calculated for the entire stream and for each individual sampling site by dividing the total number of tadpoles and adults observed by the total area searched.

Genetic and Molecular Analysis

Generation and validation of an *A. truei* eDNA qPCR assay

To quantify eDNA concentrations from water samples, a sensitive and accurate quantitative PCR assay is required. Although previous studies have developed such assays for *A. montanus*, these assays have not been used on *A. truei*. I therefore developed such an assay for *A. truei* based on those available for *A. montanus*. First, I investigated whether there are sequence differences in the commonly used cytochrome b (cyt b) gene between *A.truei* and *A. montanus* by sequencing a region of this gene from local *A. truei*. The cytochrome b (cyt b) region was chosen because mitochondrial DNA is more stable and readily available in the environment compared to nuclear DNA (Nielson et al. 2001). Also, existing sequences and primers for *A. truei* using the cyt b region are available in GenBank.

Tissue samples were taken from the toes of a male and female *A. truei* collected on 21 August 2012 (permit# SC-3295) from Jiggs Creek, a stream near Korbel, Humboldt County, California (123.9304W, 40.8854N, GPS Datum WGS84). Purified DNA was extracted from 20 mg of tissue using a Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, MA) and the Mammalian Tissue and Rodent Tail Genomic DNA Purification protocol (Thermo Scientific GeneJET Genomic DNA Purification kit, Genomic DNA Purification Protocols, pp. 4-5). Each extracted sample was labeled and stored at -20°C until it could be amplified using Polymerase Chain Reaction (PCR). The sequences of two sets of standard primers were

obtained from a previous study on the phylogeography of tailed frogs (Nielson et al. 2001. The cyt b gene was amplified from DNA extracted from the tissue samples using the L14115 and H14963 primers designed by Sullivan and Swofford (1997) (obtained from Integrated DNA Technologies, Coralville, Iowa). An additional *Ascaphus*-specific primer--SUV (Nielson et al. 2001--was used in combination with the L14115 primer. A final PCR product of approximately 730 base pairs was obtained using both primer sets (L14115-H14963 and L14115-SUV). PCR conditions for both primer combinations (L14115-H14963 or L14115-SUV) were as follows: EconoTaq PLUS 2X MasterMix (Lucigen Corporation, Middleton, WI; 50mM Tris-HCl; 50mM NaCl; 5mM $MgCl_2$; 200µM each of dATP, dGTP, dCTP, dTTP; 10µg activated calf thymus DNA; and 0.1mg/ml BSA in a final volume of 50µl) and sterile water were mixed by vortexing to make a PCR reaction solution. I added 1µL of extracted DNA sample to 24µL of the PCR reaction solution. Samples were centrifuged and placed in a thermocycler for 40 cycles, with a one-minute dwell time for the three steps within each cycle. Each cycle included a denaturation, annealing, and primer extension step. Samples mixed with primer combinations L14115-H14963 and L14115-SUV were run with an annealing temperature of 54.8°C. Standard agarose gel electrophoresis was performed using a 1.5% agarose gel and Promega blue/orange loading dye, 6X (0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0)) to confirm amplification and fragment size.

After confirming amplification, the PCR products and primers were sent to Sequetech Corporation (Mountain View, CA) for PCR clean-up and sequencing. I used

CodonCode Aligner (CodonCode Corporation, Centerville, MA) to compare my *A. truei* sequences to those obtained from GenBank (NCBI; http://www.ncbi.nlm.nih.gov/genbank/) for *A. truei, A. montanus*, and amphibian species that may co-occur with *A. truei* (specifically, *Rhyacotriton variegatus*, *Rana boylii*, and *Dicamptodon tenebrosus*). After aligning the sequences and recording any sequence differences, new primers were designed (hereafter referred to as ASTR F and ASTR R) by modifying the *Ascaphus montanus*-specific primer set (ASMO F and ASMO R) created by Goldberg et al. (2011). The alignment revealed a one base difference between my *A. truei* sequences and those from GenBank. As a result, I also created a degenerate forward primer (hereafter referred to as ASMO_TR F) that would amplify sequences of *A. montanus* and northern California *A. truei* (Table 1). Both primer sets (ASTR F/ASTR R and ASMO_TR F/ASTR R) produced PCR products of 85 base pairs.

Table 1. Primer sequences used to amplify species-specific regions of cyt B.

Species	**Primer name**	**Primer sequence (5′→3′)**
Coastal tailed frog (*Ascaphus truei*)	ASTR F	CGTCAACTATGGCTGACTAA
Coastal tailed frog (*Ascaphus truei*)	ASTR R	TCGGCCAATGTGAAGATAAA
Ascaphus truei and *Ascaphus montanus*	ASMO_TR F	CGTCAACTATGGCTG**R**CTAA
Ascaphus truei and *Ascaphus montanus*	ASTR R/ASMO_TR R	TCGGCCAATGTGAAGATAAA
Rocky Mountain tailed frog (*Ascaphus montanus*)	ASMO F	CGTCAACTATGGCTGGCTAA
Rocky Mountain tailed frog (*Ascaphus montanus*)	ASMO R	TCGGCCAATGTGAAGATAAA

To ensure that the modified primers would reproducibly amplify DNA of *A. truei*, they were tested using eDNA extracted from water collected from an aquarium housing *A. truei*. While wearing gloves, I fully submerged a sterile container to collect 100mL of the water. Following collection, the water was centrifuged at maximum speed (20,000 x g) for 10 minutes to pellet tissue before it was extracted using the same purification protocol as described previously for tissue extraction. Additionally, the primers were entered into a Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to test the specificity of the modified primer and demonstrate that there was no amplification of DNA from amphibian species co-occurring with *A. truei.* Skin swabs were taken from a captive adult coastal giant salamander (*Dicamptodon tenebrosus)* and DNA was extracted using the same purification protocol. The extracted DNA from the swabs was run with the ASTR F/ASTR R and ASMO_TR F/ASTR R primers during PCR, and it was concluded from gel electrophoresis that no amplification of *Dicamptodon tenebrosus* eDNA occurred. Finally, I collected water in a sterile, one liter container from Jacoby Creek in Bayside, Humboldt County, California, where coastal tailed frogs are known to be present. An adult tailed frog was found along the bank of the stream at the time that the water was collected. The eDNA was extracted (using the same procedure as described previously) and DNA was amplified from the Jacoby Creek water sample using the *Ascaphus*-specific primers. All purified PCR products, derived from DNA extracted from water collected in the lab and in the field, and the primers used to amplify them, were sent to

Sequetech Corporation for sequencing. The modified primers consistently amplified DNA from *A. truei* and failed to amplify DNA from co-occurring amphibian species found in Humboldt County, California.

DNA extractions and qPCR

Extractions were performed in a sterile lab where no pre- or post- PCR had been conducted. The filters and ethanol were removed from all "A" sample vials and placed in new 1.5 mL microcentrifuge tubes labeled with their original Sample ID. These tubes were placed with the lids open in a sterilized, eDNA-specific laboratory hood to air dry for 24 hours. The following day, I removed any remaining ethanol from the filter with a pipette. I extracted *A. truei* DNA from the air-dried filters with the GeneJET Genomic DNA Purification Kit using the Mammalian Tissue and Rodent Tail Genomic DNA Purification protocol. To begin the extraction procedure, 180μL of Digestion solution (water >50%) was added to the filter and the solution was mixed with the contents of the filter by crushing the filter with the end of a pipette tip. Next, 20μL of Proteinase K (enzyme; serine protease, *Tritirachium album* 1-2.5%) was added and the sample was vortexed for 1 minute. Samples were then placed in an incubator overnight at 56°C. In the morning, they were removed and vortexed for 30 seconds before being placed back into the incubator for ten minutes. The samples were removed from the incubator and the liquid was pulled off of the filter and placed in a new, labeled microcentrifuge tube. I added 20μL of RNase A Solution (glycerol >50%; water 25-50%) to each sample and mixed by vortexing. The samples were incubated at room temperature for 10 minutes before adding 200μL of Lysis solution (guanidinium chloride 25-50%), then were mixed

by vortexing for 15 seconds. At this point, the purification protocol was modified by adding a QIAshredder homogenizer that consisted of a biopolymer-shredding system in the form of a microcentrifuge spin-column (Qiagen Biotechnology Business, Venlo, Netherlands) to remove any DNA inhibitors, such as residual polypeptides and polysaccharides, from the eDNA sample. The QIAshredder spin-column was placed in a collection tube, loaded with the sample and centrifuged for two minutes at a relative centrifugal force (RCF) of 20,000 x g. The flow-through liquid in the collection tube was removed carefully off of the pellet and placed in a new, labeled microcentrifuge tube. The remaining steps were completed as specified by the Mammalian Tissue and Rodent Tail Genomic DNA Purification protocol.

Quantatitive PCR (qPCR) was performed with the purified DNA samples and the designed ASTR F/ASTR R and ASMO_TR F/ASTR R primers to amplify an 85 base pair fragment. All samples were run in triplicate, which means that three wells contained the same qPCR reaction. Each run included 96-well reactions run in twelve, MicroAmp Optical 8-tube strips (0.2mL) (Applied Biosystems, Waltham, MA). Each well contained 2µL of sample, 12.5µL of GoTaq qPCR Master Mix (5x1ml GoTaq qPCR Master Mix 2x; 100µL carboxy-X-rhodamine reference dye; 2x13ml nuclease-free water), 1µL of forward and 1µL of reverse primer, and 8.5µL of nuclease-free water for a reaction total of 25µL. Master Mix contains SYBR Green dye, which is a fluorescent DNA-binding dye. Along with our extracted eDNA samples, all qPCR runs included a negative extraction control, negative PCR control, and a set of ten-fold serial dilution standards of *A. truei* DNA. The negative extraction control was sterile, DNA-free water that

underwent the extraction procedure. The negative PCR control included no DNA but contained all qPCR reagents such as water, primers, and Master Mix. These negative controls were used to ensure that no contamination had occurred during the extraction process or qPCR set up. Standards contained all qPCR reagents and included ten-fold serial dilutions of tailed frog DNA.

To use qPCR to measure relative quantities of eDNA, I needed to create a standard curve to compare my samples to. The standard curve was created by performing a ten-fold serial dilution of purified DNA extracted from *A. truei* tissue (1/10 to 1/100,000). I used a NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Scientific Inc., Waltham, MA) to determine the exact concentration of DNA in each diluted sample (Ellison et al. 2006). Several aliquots of the serial dilution were frozen and stored for future use. Quantitative PCR was performed three times with the standards and our species-specific primer sets to create an amplification plot. This standard curve amplification plot represented increasing concentrations of DNA in each sample that were accompanied by decreasing threshold cycle (C_t) values (Denman and McSweeney 2005). When real-time PCR was conducted with eDNA samples, the serial dilutions of tissue were run simultaneously in order to compare the standardized C_t values of the tissue samples to the C_t values of the eDNA samples. All negative controls were run simultaneously with all eDNA samples. The qPCR reaction was run for 50 cycles with a 30 second dwell time for the two steps within each cycle. Each cycle consisted of a denaturation and annealing step, and the amount of DNA was measured after each cycle. At the completion of 50 cycles, a dissociation step was added to each qPCR run.

Dissociation curves are necessary for determining specific amplification of the target species when using a non-specific DNA-binding dye like SYBR green (Denman and McSweeney 2005). Dissociation curves of my standards were compared to the curves of my eDNA samples to ensure that no non-specific amplification occurred during reactions.

Gel electrophoresis of qPCR products

To ensure any amplification that occurred was specific to *A.truei*, sixteen eDNA samples were extracted, used in qPCR with the ASTR primer set, and were run on an agarose gel. One positive and one negative sample were randomly chosen from each stream for all three field seasons. This is true for all streams except those that did not have any negative samples (Summer NF Maple Creek and Fall Mule Creek samples), so only one positive sample was randomly chosen for those streams. Samples were placed in a thermocycler for 40 cycles, with a one-minute dwell time for the three steps within each cycle. Samples were run with an annealing temperature of 53°C. All samples were run on a 1.5% agarose gel for an hour at 84V, stained with ethidium bromide, then rinsed. The gel was viewed using an ultraviolent transilluminator to ensure that the bands present were the appropriate size (85 base pairs) for positive samples, and that no band was present for negative samples. The gel yielded bands for positive eDNA samples that corresponded with the final qPCR results.

qPCR data validation and eDNA quantification

After completion of all qPCR runs, the amplification plots, threshold cycle (C_t) values, and dissociation curves were evaluated for positive amplification of *A. truei* DNA. Environmental DNA samples were deemed negative if none of the three wells

containing the same eDNA sample showed amplification of tailed frog DNA. Some negatives underwent qPCR two more times if amplification appeared to be inhibited during the first run. Evidence for inhibition included excessive background noise caused by well contamination, poor amplification curves resulting in high C_t values, or multiple dissociation peaks. Any eDNA samples that exhibited fewer than three wells with positive amplification were considered ambiguous samples and were re-run. If the re-run showed at least one well with positive amplification, the ambiguous samples were determined to be positive for tailed frog DNA. Some ambiguous samples were later determined to be negative if all three wells containing the sample showed no amplification after two re-runs (Goldberg et al. 2013, Hall et al. 2015). To ensure that false positive amplification of eDNA samples caused by contamination did not occur, our negative controls needed to show a combination of the following: no amplification curve, no C_t value, no dissociation curve, or no dissociation curve around 77°C.

Next, I examined the amplification plots, C_t values, and dissociation curves of my standards containing the ten-fold serial dilution of DNA extracted from *A. truei* tissue for signs of inhibition or non-specific amplification. Standards that showed the expected relationship between C_t values and eDNA concentrations were determined to be free of inhibition. Non-specific amplification was ruled out when the dissociation curves of the standards included a peak around 77°C. Re-runs of eDNA samples occurred when negative controls exhibited signs of contamination and/or standards showed signs of inhibition during qPCR.

Determining the concentration of *A. truei* DNA present in my eDNA samples required calculation of an average C_t value for each sample. For samples in which all wells showed positive amplification during the initial run, I averaged the C_t values for all three wells. For ambiguous samples that were re-run and were later determined to be positive, I averaged the C_t values for the wells showing positive amplification during the initial run and re-run. Negative samples were considered to have no C_t value, indicating a DNA concentration of zero. I created a standard curve amplification plot in Microsoft Excel (2016, version 15.24) for each run. The slope of the logarithmic trendline and the R-squared value of this plot were used to calculate the concentrations of my eDNA samples and represent the linear relationship of my standards (Denman and McSweeney 2005, Weksberg et al. 2005). The average C_t values for positive samples were plugged into the y-variable of the equation and solved to determine the concentrations of DNA (ng/μL) present in these samples. The eDNA concentrations were multiplied by 200, our initial DNA elution volume of our 1L sample, to convert our concentrations to ng/L. For ambiguous samples, the DNA concentrations calculated for each run were averaged to obtain a final DNA concentration.

During each field season, I quantified the eDNA concentration collected from each sampling site and the average eDNA concentration of each stream. For sampling sites where triplicate eDNA samples were collected, I averaged the calculated DNA concentration of each sample. To get the average DNA concentration for each stream, the DNA concentrations of all eDNA samples, positive and negative, taken from the stream

were averaged. This was done for each stream during each field season to observe seasonal fluctuations in DNA concentrations.

To calculate the detection rate for eDNA sampling in each stream, I divided the number of eDNA samples that showed positive amplification during qPCR by the total number of eDNA samples collected from the stream. The detection rates for the streams helped evaluate the efficacy of eDNA sampling during each field season.

Statistical Analyses

Investigating the relationship between eDNA concentrations and environmental variables

I created a general linear model (GLM) to assess the effect of water temperature, pH, predicted flow rate, season, stream, and turbidity on eDNA concentrations. The lack of individual flow rate measurements for every sampling site required us to calculate predicted flow rates for each site in RStudio (RStudio, Inc., Boston, MA; version 0.99.491) using the 'predict' function and the original flow rate and distance measurements of each cross-section. This allowed us to include flow rate measurements for each sampling site in our analysis of the effect of flow on eDNA concentrations at specific locations. The environmental variables were used to construct three global generalized mixed models. In these global models, the environmental variables were input as predictors to test their significance in affecting the response variable: log transformed eDNA concentrations. I fit each global model and carried out model selection using the 'dredge' function within the RStudio package, MuMIn (RStudio, Inc.,

Boston, MA; version 0.99.491; Martin et al. 2014). Several model permutations were created for each global model. The models were ranked and the top model was determined using the lowest, second-order Akaike information criterion (AIC_c) value and highest weight. The lowest AIC_c value indicates a model that minimizes the divergence of the model from reality (Kullback-Leibler distance; Burnham and Anderson 2003). A summary output of the statistics of this top model showed the environmental variables that had the most significant effect on eDNA concentrations based on their p-values. The first global model included all environmental variables, the second global model excluded season, and the third excluded season and water temperature. The purpose of this multiple global model approach was to reveal the significance of environmental variables that were originally confounded.

Investigating the relationship between eDNA concentrations and animal abundance

Several graphical representations, some including overlaid generalized additive models (GAMs), were used to evaluate the effect of animal abundance on eDNA concentrations. Specific stream plots were created in Rstudio, using the package ggplot2 and the GAM link function (Guisan et al. 2002, Wickham 2009). First, I created a GAM for each field season that included each stream's average eDNA concentration (ng/L) and total estimated density (individuals/m^2) determined from traditional sampling. Each GAM was plotted to reveal the effect of season on the correlation between animal abundance and eDNA concentration. Then, I plotted the number of animals observed and eDNA concentration detected at each sampling site for each field season. This was done

to review the relationship between animal abundance and eDNA concentrations at specific locations. Additionally, I explored the effect of animal density on eDNA concentrations by plotting each eDNA sample as an individual point to reveal any general trends between observed eDNA concentrations and number of animals. There was no separation of eDNA samples by stream or season in this plot. To account for any masking of the relationship between density and eDNA concentrations, I created separate GAMs for each stream and field season that included animal density and log transformed eDNA concentrations. I created a plot for each GAM with the log transformed eDNA concentrations as individual points.

Next, for each sampling site, which included 100 m of stream, I determined the distance between locations where individual animals were observed and the beginning of the sampling reach/initial eDNA water sampling location. I used these distances to review their correlation with the eDNA concentrations found at each eDNA sampling location. To do this, I created a plot that included animal detections for each stream as individual points; these points were coded by color to indicate which season the animal was observed. Each animal detection, represented as an individual point, was aligned on the y-axis according to the eDNA concentration that was collected at that sampling site. Lastly, I plotted eDNA sample concentrations as individual points according to the number of animals observed during the first 10 meters of each water sampling site to further understand the effect of distance and abundance on eDNA concentrations. There was no separation of eDNA samples by stream or season in this plot.

RESULTS

Comparison of Detection Rates for eDNA and Traditional Sampling

The eDNA detection rates, when using my species-specific primer set (ASTR), were higher than the detection rates estimated for our traditional sampling technique during the summer and fall field seasons (Table 2). During the winter field season, the detection rate for eDNA sampling dropped below that for traditional sampling. The summer and fall detection rates for NF Maple Creek and SF Ah Pah Creek were similar for both sampling techniques (Table 3). It is interesting to note that in our low density stream, Mule Creek, the detection rates for traditional sampling were fairly low (23-69%) during all three seasons. By contrast, during the summer and fall seasons, eDNA sampling at Mule Creek yielded high detection rates (95-100%) despite its relatively low frog density (Table 3). In the winter, however, the detection rate for Mule Creek was lower for eDNA sampling relative to traditional sampling (Table 3).

The detection rates for my non-specific, degenerate primer set (ASMO_TR) followed a seasonal pattern similar to that seen for the species-specific primer set (ASTR). Specifically, the eDNA detection rates with the ASMO_TR primer set were highest during the summer and fall field seasons, but dropped dramatically during the winter. However, the detection rate from this primer set was lower than the detection rate for traditional sampling during the summer and winter field seasons (Table 2). The non-specific primer yielded a slightly higher detection rate than traditional sampling for the fall field season only. As was seen with the ASTR primer set, eDNA sampling with the

degenerate primer set (ASMO_TR) at Mule Creek yielded higher detection rates during the summer and fall field seasons relative to traditional sampling (Table 3), whereas in the winter the detection rate for traditional sampling was higher than for eDNA sampling. Unlike results seen for the ASTR primer set, the detection rates for NF Maple Creek and SF Ah Pah were consistently higher for traditional sampling than for eDNA sampling for all three seasons.

Table 2. The detection rates for each field season using eDNA sampling (with the ASTR and ASMO_TR primer sets) and traditional sampling (using a rubble rousing method).

Field season	Month(s)	ASTR detection rate	ASMO_TR detection rate	Rubble rousing detection rate
Summer	July	96%	83%	91%
Fall	Late Sept. – early Oct.	94%	82%	79%
Winter	Late Feb. – early April	58%	42%	91%

Table 3. Detection rates for each season broken down by stream using eDNA sampling (with the ASTR and ASMO_TR primer sets) and traditional sampling (using a rubble rousing method).

Field Season	Stream Name	ASTR eDNA detection rate	ASMO_TR detection rate	Rubble rousing detection rate
Summer	Mule Creek	95%	95%	69%
Summer	NF Maple Creek	100%	73%	95%
Summer	SF Ah Pah Creek	93%	83%	100%
Fall	Mule Creek	100%	100%	23%
Fall	NF Maple Creek	90%	80%	95%
Fall	SF Ah Pah Creek	93%	77%	100%
Winter	Mule Creek	48%	48%	62%
Winter	NF Maple Creek	67%	43%	100%
Winter	SF Ah Pah Creek	57%	37%	100%

Influence of Season and Stream on eDNA Concentrations

Average eDNA concentrations, extracted from eDNA samples that were run with the ASTR primer set, varied between streams and field seasons. The average eDNA concentrations were highest during the summer and lowest during the winter field season (Table 4). In the fall, eDNA concentrations were lower than summer concentrations but remained relatively high. During the summer and fall field seasons, eDNA concentrations varied according to stream size: the smallest creek (Mule Creek) had the lowest average eDNA concentrations, and the largest creek (SF Ah Pah Creek) had the highest average eDNA concentrations (Table 4). During the winter field season, fluctuations in eDNA concentrations were not correlated with differences in stream size. With the ASMO_TR primer set, concentrations of eDNA were usually lower than those obtained with the

ASTR primer set for each field season (Table 4). Nonetheless, the eDNA concentrations for each stream derived from the ASMO_TR primer set followed similar patterns as those described for the ASTR primer set with regard to season and stream size -- concentrations were the highest during the summer and lowest during the winter field season, and during each field season, the largest stream had the highest eDNA concentrations while the smallest stream had the lowest.

Table 4. Total watershed area and average eDNA concentrations for each stream during every field season using the ASTR and ASMO_TR primer sets.

Field season	**Stream name**	**AVG ASTR eDNA conc. (ng/L)**	**AVG ASMO_TR eDNA conc. (ng/L)**
Summer	Mule Creek (small)	0.0748	0.0281
Summer	NF Maple Creek (medium)	0.0833	0.0305
Summer	SF Ah Pah Creek (large)	0.1068	0.0311
Fall	Mule Creek	0.0322	0.0267
Fall	NF Maple Creek	0.0553	0.0289
Fall	SF Ah Pah Creek	0.0718	0.0326
Winter	Mule Creek	0.0047	0.0001
Winter	NF Maple Creek	0.0179	0.0006
Winter	SF Ah Pah Creek	0.0087	0.0106

Influence of Environmental Variables on eDNA Sampling

The water temperature and pH did not vary significantly between streams during each field season. The average temperature of each stream did not go above 14°C during any field season. The mean temperature for streams was the highest during the summer and fall field seasons (Table 5). During the winter field season the average temperature dropped approximately 4°C. NF Maple Creek saw the most dramatic drop in temperature during the winter (-5.2°C). The pH of each stream remained neutral for each field season (Table 6).

By design, sampling of streams only occurred when the turbidity measured 5 NTUs (Nephelometric turbidity unit) or less. This was done to avoid low visibility during animal surveys and avoid an increase in the amount of filters used. High turbidity causes filters to clog, which would increase the cost per sample to conduct eDNA sampling. Turbidity values for each stream during each field season ranged from 1.00 to 4.69 NTUs (Table 7). During the summer, turbidity values were relatively high for each stream, compared to those from the fall and winter field seasons. Turbidity values were low during the winter field season except for at Mule Creek, which had the highest turbidity values during this time.

Flow rate was the only environmental variable that had a noticeable change between streams and field seasons. During the summer and fall field seasons, Mule Creek had the lowest stream discharge and SF Ah Pah Creek had the highest stream discharge

(cfs). During the winter field season, each stream experienced a substantial increase in stream discharge (Table 8).

Table 5. Average temperature values for each stream during each field season.

Field season	Stream name	Average temp. (°C)	Average temp. for season (°C)
Summer	Mule Creek	13.9	13.7
Summer	NF Maple Creek	13.5	13.7
Summer	SF Ah Pah Creek	13.7	13.7
Fall	Mule Creek	12.6	13.0
Fall	NF Maple Creek	13.5	13.0
Fall	SF Ah Pah Creek	12.9	13.0
Winter	Mule Creek	10.6	9.6
Winter	NF Maple Creek	8.3	9.6
Winter	SF Ah Pah Creek	9.9	9.6

Table 6. Average pH values for each stream during each field season.

Field season	Stream name	Average pH	Average pH for season
Summer	Mule Creek	7.34	7.24
Summer	NF Maple Creek	7.02	7.24
Summer	SF Ah Pah Creek	7.36	7.24
Fall	Mule Creek	7.33	7.20
Fall	NF Maple Creek	6.83	7.20
Fall	SF Ah Pah Creek	7.45	7.20
Winter	Mule Creek	7.37	7.07
Winter	NF Maple Creek	6.95	7.07
Winter	SF Ah Pah Creek	6.89	7.07

Table 7. Average turbidity values for each stream during each field season.

Field season	Stream name	Turbidity (NTUs)
Summer	Mule Creek	3.07
Summer	NF Maple Creek	4.61
Summer	SF Ah Pah Creek	3.16
Fall	Mule Creek	1.73
Fall	NF Maple Creek	2.33
Fall	SF Ah Pah Creek	1.00
Winter	Mule Creek	4.69
Winter	NF Maple Creek	2.01
Winter	SF Ah Pah Creek	1.56

Table 8. Flow rate of each stream, represented by stream discharge (cfs), was calculated using cross-sectional flow rate measurements for each season.

Field season	Stream name	Discharge of stream (cfs)
Summer	Mule Creek	0.08
Summer	NF Maple Creek	0.46
Summer	SF Ah Pah Creek	1.38
Fall	Mule Creek	0.01
Fall	NF Maple Creek	0.24
Fall	SF Ah Pah Creek	1.68
Winter	Mule Creek	2.76
Winter	NF Maple Creek	1.60
Winter	SF Ah Pah Creek	10.61

Statistical Analysis of Environmental Variables and ASTR eDNA Concentrations

The following environmental variables were included in three global generalized mixed models (hereafter referred as global models) as predictors for eDNA concentrations: pH, turbidity, temperature, predicted flow rate, season, and stream. The first global model (global.model1) included all environmental variables. The intercept column represents the positive or negative effect on eDNA concentrations if the values for each environmental variable included in the model permutation are zero. The top model generated from model permutations of global.model1 accounted for 0.12 of the weight and included turbidity and the winter field season as variables that affect eDNA concentrations (Table 9). A summary of this top model showed that the winter field season had a significant negative effect (estimate = -2.16, p-value = 6.07×10^{-9}) on eDNA concentrations. Turbidity also had a negative effect (estimate = -0.16 on eDNA concentrations but this effect was not significant (p-value = 0.09). Based on the F-statistic ($F = 14.78$, p-value = 3.39×10^{-8}) I can conclude that this top model is performing better than a model containing random predictors.

Table 9. Series of models generated using the dredge function from global.model1 containing the predictors: pH, turbidity, temperature, predicted flow rate, season, and stream. The first row of the top ten listed models that is highlighted in yellow represents the top model permutation based on the lowest AIC_c and highest weight values. The boldface values in the top model represent the environmental variables that are considered to have a positive or negative effect on eDNA concentrations. NA values are placed in the environmental columns when they were not included in that particular model permutation. The positive or negative symbols (+ or -) used in the Season and Stream columns signify a positive or negative effect on eDNA concentrations.

Intercept	Turb.	pH	Pred. Flow	Season	Stream	Water Temp	df	log Lik	AICc	delta	wgt
-2.83	**-0.16**	NA	NA	**+**	NA	NA	5	-209.14	428.81	0.00	**0.12**
-3.75	NA	NA	NA	+	+	NA	6	-208.13	429.02	0.20	0.11
-3.34	NA	NA	NA	+	NA	NA	4	-210.61	429.57	0.75	0.08
-3.38	NA	NA	0.06	+	NA	NA	5	-210.01	430.56	1.74	0.05
-3.43	-0.08	NA	NA	+	+	NA	7	-207.80	430.63	1.81	0.05
-2.91	-0.14	NA	0.03	+	NA	NA	6	-208.98	430.72	1.90	0.05
-3.55	-0.16	NA	NA	+	NA	0.05	6	-209.08	430.91	2.10	0.04
-2.95	-0.16	0.016	NA	+	NA	NA	6	-209.14	431.03	2.22	0.04
-3.77	NA	NA	-0.02	+	+	NA	7	-208.06	431.15	2.33	0.04
-4.19	NA	NA	NA	+	+	0.03	7	-208.11	431.24	2.43	0.04

The second global model (global.model2) excluded season but included all other environmental variables. The top model generated from model permutations of global.model2 accounted for 0.19 of the weight and included predicted flow, stream, and water temperature as variables that affect eDNA concentrations (Table 10). A summary of this top model showed that SF Ah Pah Creek is the only stream that has a significant positive effect (estimate = 1.16, p-value = 0.005) on eDNA concentrations. Water temperature also had a significant, positive effect (estimate = 0.30, p-value = 0.001) on eDNA concentrations. The predicted flow had a slight, negative effect on eDNA concentrations (estimate = -0.09) but this effect was not significant. Based on the F-

statistic (F= 10.45, p-value = 3.09×10^{-7}) I can conclude that this top model is performing better than a model containing random predictors.

Table 10. Second series of models generated using the dredge function from a global.model2 containing the predictors: pH, turbidity, temperature, predicted flow rate, and stream. The first row of the top ten listed models that is highlighted in yellow represents the top model permutation based on the lowest AIC_c and highest weight values. The boldface values in the top model represent the environmental variables that are considered to have a positive or negative effect on eDNA concentrations. NA values are placed in the environmental columns when they were not included in that particular model permutation. The positive or negative symbols (+ or -) used in the Season and Stream columns signify a positive or negative effect on eDNA concentrations.

Intercept	Turb.	pH	Pred. Flow	Stream	Water Temp	df	log Lik	AICc	delta	wgt
-8.22	NA	NA	**-0.09**	**+**	**0.30**	6	-209.96	432.68	0.00	0.19
-9.50	NA	NA	NA	+	0.40	5	-211.27	433.07	0.39	0.15
-8.74	-0.12	NA	NA	NA	0.41	4	-212.95	434.26	1.58	0.09
-8.84	NA	NA	NA	NA	0.39	3	-214.05	434.31	1.63	0.08
-8.17	-0.07	NA	-0.09	+	0.32	7	-209.71	434.43	1.75	0.08
-9.41	-0.07	NA	NA	+	0.41	6	-210.95	434.66	1.98	0.07
-10.79	NA	0.17	NA	+	0.40	6	-210.99	434.74	2.06	0.07
-8.95	NA	0.09	-0.09	+	0.31	7	-209.89	434.80	2.12	0.07
-8.29	-0.14	NA	-0.03	NA	0.38	5	-212.74	436.02	3.34	0.04
-9.72	NA	0.12	NA	NA	0.39	4	-213.91	436.17	3.49	0.03

When water temperature and season are removed from the global model and an additional set of model permutations was created from global.model3, we see that the significant effect of predicted flow was possibly confounded by the removed variables (Table 11). With season and water temperature removed, predicted flow rate has a significant, negative effect on eDNA concentrations (estimate = -0.22, p-value = 2.99×10^{-6}). From the F-statistic (F= 10.45, p-value = 1.26×10^{-5}) I can conclude that this top

model is performing better than a model containing random predictors. The weight of the top model that includes predicted flow rate and stream also accounted for a much greater portion of the weight (=0.56) relative to the models that included season and water temperature.

Table 11. Additional series of models generated using the dredge function from a global.model3 containing the predictors: pH, turbidity, predicted flow rate, and stream. The first row of the top four listed models that is highlighted in yellow represents the top model permutation based on the lowest AIC_c and highest weight values. The boldface values in the top model represent the environmental variables that are considered to have a positive or negative effect on eDNA concentrations. NA values are placed in the environmental columns when they were not included in that particular model permutation. The positive or negative symbols (+ or -) used in the Season and Stream columns signify a positive or negative effect on eDNA concentrations. These models reveal the significance of predicted flow rate, which was initially masked by season and water temperature.

	Intercept	**Turb.**	**pH**	**Pred. Flow**	**Stream**	**df**	**log Lik**	**AICc**	**delta**	**weight**
13	-4.34	NA	NA	**-0.22**	**+**	5	-215.43	441.40	0.00	0.56
15	-4.14	NA	-0.03	-0.22	+	6	-424.00	443.60	2.21	0.19
14	-4.32	-0.01	NA	-0.22	+	6	-215.43	443.61	2.22	0.19
16	-4.12	-0.01	-0.03	-0.22	+	7	-215.42	445.86	4.47	0.06

To review the effect of predicted flow rate on eDNA concentrations further, I created a plot comparing log transformed eDNA concentrations (ng/L) to the predicted flow rate (cfs) for each sampling site (Figure 2). During the summer and fall field seasons, increased predicted flow rates had a positive effect on eDNA concentrations, but this effect was not significant because flow rate measurements during these seasons remained relatively low (< 5 cfs; Table 8). During the winter field season, the predicted

flow rates were much higher and had a negative effect on eDNA concentrations, but this effect was not significant. With all seasons combined, increasing predicted flow rates had a significant, negative effect on eDNA concentrations in each stream (estimate = -2.54, p-value = 4.54×10^{-5}).

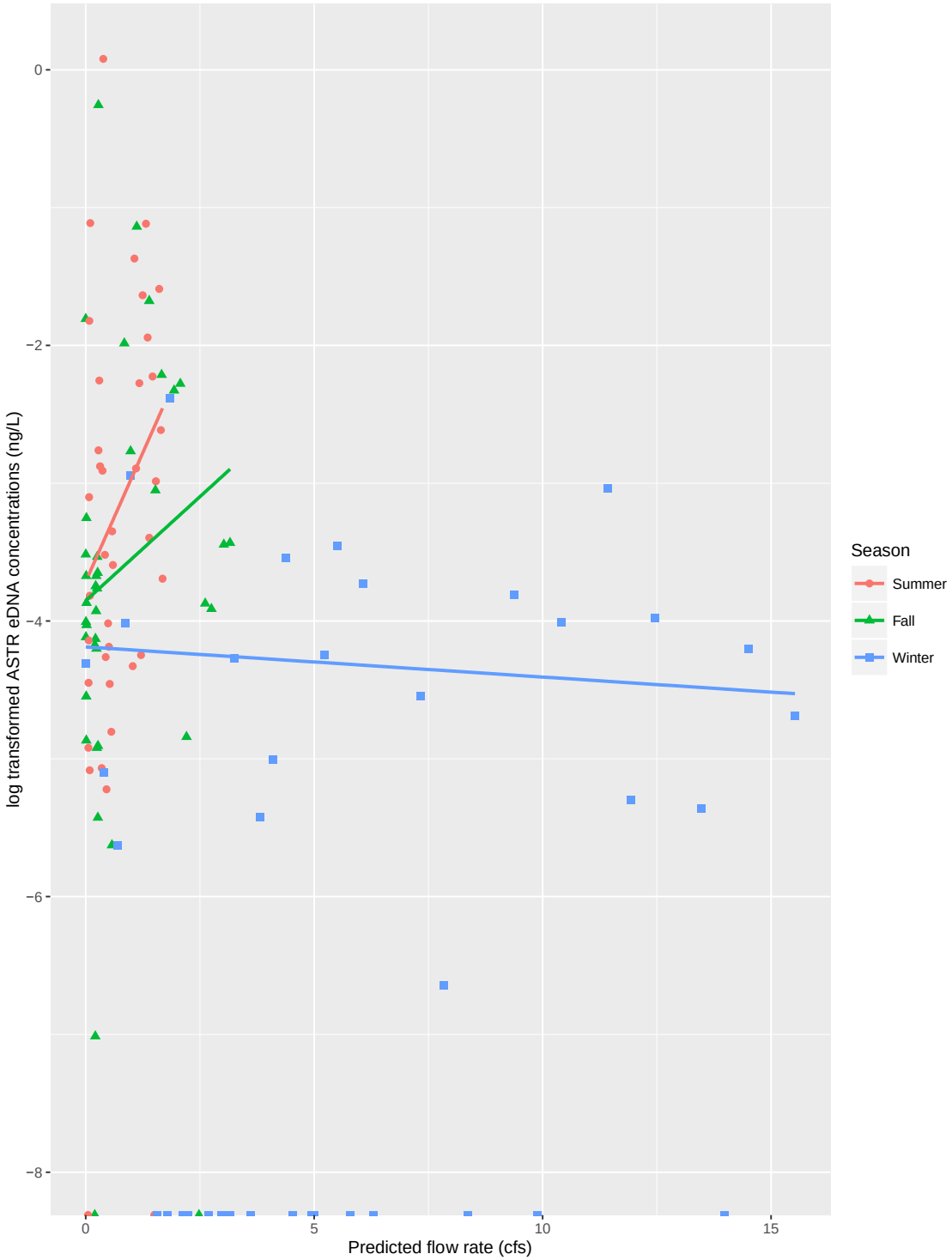

Figure 2. Linear regression plot showing the relationship between predicted flow rate (cfs) and log transformed eDNA concentrations (ng/L) for each field season.

Animal Abundance Estimates Related to eDNA Concentrations

As expected based on earlier surveys, Mule Creek had the lowest number of animals and SF Ah Pah Creek had the highest number of animals detected during all three seasons (Table 12). The number of animals detected was highest during the summer field season and lowest in the fall. Animal detections increased between fall and winter. More specifically, animal observations in Mule Creek and NF Maple Creek increased substantially during the winter field season. It is interesting to note that more animals were detected in NF Maple Creek during the summer and fall compared to Mule Creek, which had the lowest number of animals observed, but both had similar animal densities.

Table 12. Relative animal abundances and estimated density for each stream during three field seasons determined using a rubble-rousing technique.

Field season	Stream name	# of animals observed	Estimated density (individuals/m^2)
Summer	Mule Creek	53	0.05
Summer	NF Maple Creek	159	0.08
Summer	SF Ah Pah Creek	1079	0.54
Fall	Mule Creek	3	0.00
Fall	NF Maple Creek	58	0.03
Fall	SF Ah Pah Creek	634	0.32
Winter	Mule Creek	26	0.02
Winter	NF Maple Creek	375	0.19
Winter	SF Ah Pah Creek	832	0.42

There was a positive relationship between the total estimated density (individuals/m^2) and average eDNA concentrations (ng/L) of each stream during each field season (Figure 3); however, this positive correlation was not significant during any field season (p-values > 0.05, Figure 3). During the winter field season, eDNA concentrations at NF Maple Creek surpassed those at SF Ah Pah Creek, despite its lower estimated animal density (Figure 3).

From Figure 4, I investigated if there was any correlation between the number of animals and eDNA concentrations found at specific sampling sites (WaterSampleID). More peaks of eDNA and animal observations were seen during the summer field season, meaning that more animals and eDNA were detected during this season, contributing to the high detection rate for both sampling techniques. This is true for each stream except for NF Maple Creek, which had an increase in the number of animal observations during the winter. The eDNA concentrations were low for each stream during the winter despite the high number of animal observations. The peaks of large eDNA concentrations and high numbers of animal observations observed in each stream did not usually correspond with one another during each field season.

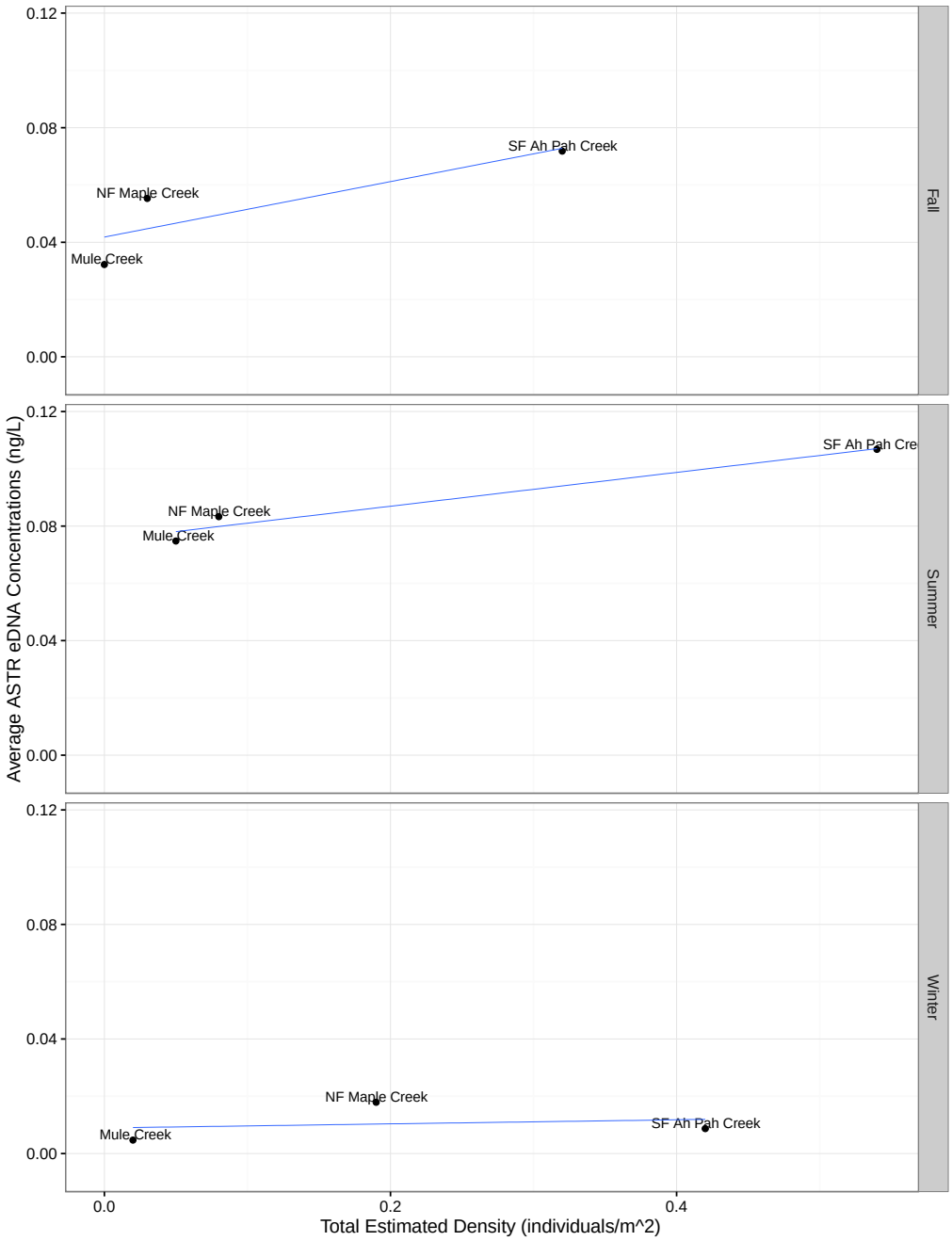

Figure 3. Comparison of average DNA concentration (ng/L) per stream using the ASTR primer set to the total estimated density of each stream (individuals/m^2) during our Summer field season (estimate= 0.06, p-value = 0.13), Fall field season (estimate= 0.09, p-value = 0.34), and Winter field season (estimate= 0.01, p-value = 0.86). The gray shaded curve represents the standard error (se) above and below the mean eDNA concentration.

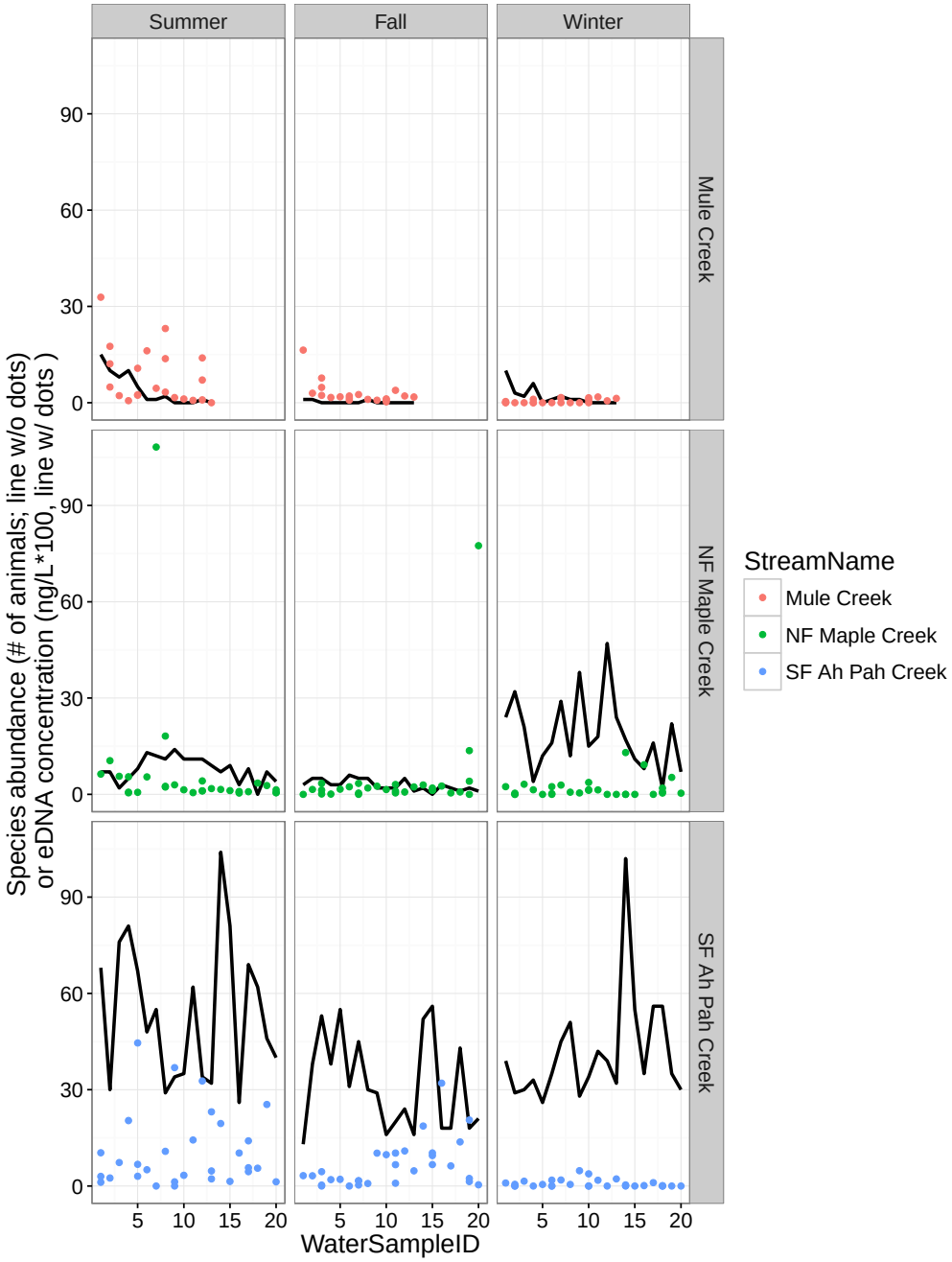

Figure 4. Plot comparing the relative abundance (# of animals; black line w/o dots) with the eDNA concentrations (ng/L * 100; colored line w/dots) observed at sampling locations in each stream during different field seasons. All eDNA concentrations were multiplied by 100 to accentuate the relationship between number of animals and eDNA concentrations. The WaterSampleIDs are numbers that correspond to the sampling locations within each stream.

To investigate if there was a relationship between eDNA concentrations (ng/L) and animal density (individuals/m^2), I plotted all eDNA concentrations as individual points according to the estimated animal density found at the same location (Figure 5). To do this, I created a generalized additive model (GAM) and generated a plot, or GAM visualization, that employs a smooth curve that fits to portions of the data. As a result, a linear curve line was not included and instead the smooth curve fluctuated according to the data. This analysis showed that there is a significant positive correlation between eDNA concentrations and the number of animals observed when stream and season data are combined (p-value = 0.01). Some of the eDNA samples had high DNA concentrations at high animal densities, but several eDNA samples also had high concentrations at very low animal densities. In case the relationship was masked when all eDNA concentrations are grouped together, I created another plot including the smooth curves of three GAMs, each including the animal densities and eDNA concentrations of each stream as individual points and separated by field season (Figure 6). In this plot, there is a significant, positive correlation between animal density and eDNA concentrations for Mule Creek during the summer (estimate = 0.005, p-value = 0.05). There is no significant correlation for any streams during the fall and winter field seasons.

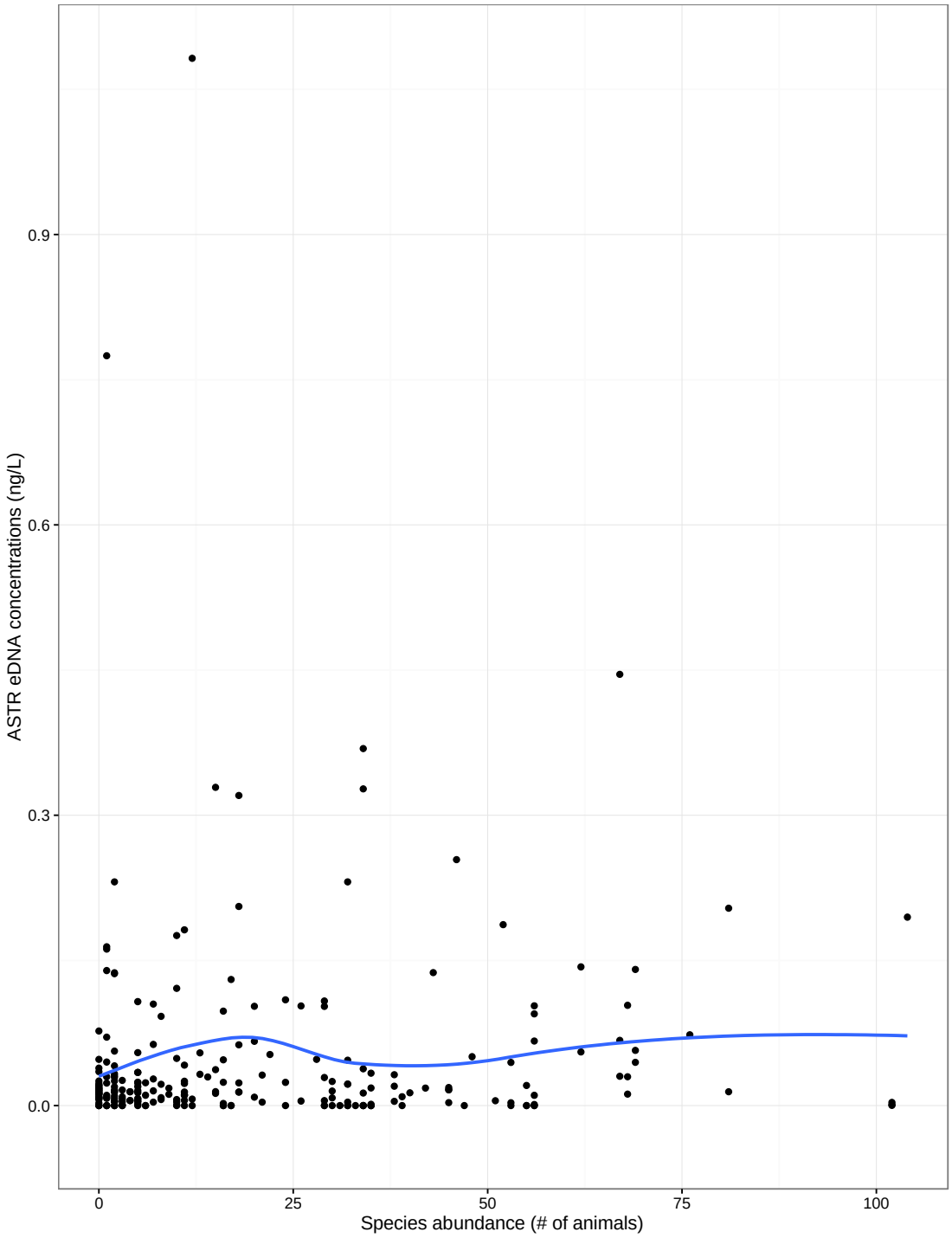

Figure 5. A generalized additive model (GAM) visualization of individual eDNA concentrations (ng/L) and their corresponding species abundance (# of animals) fit with a smooth curve that fluctuates with the data.

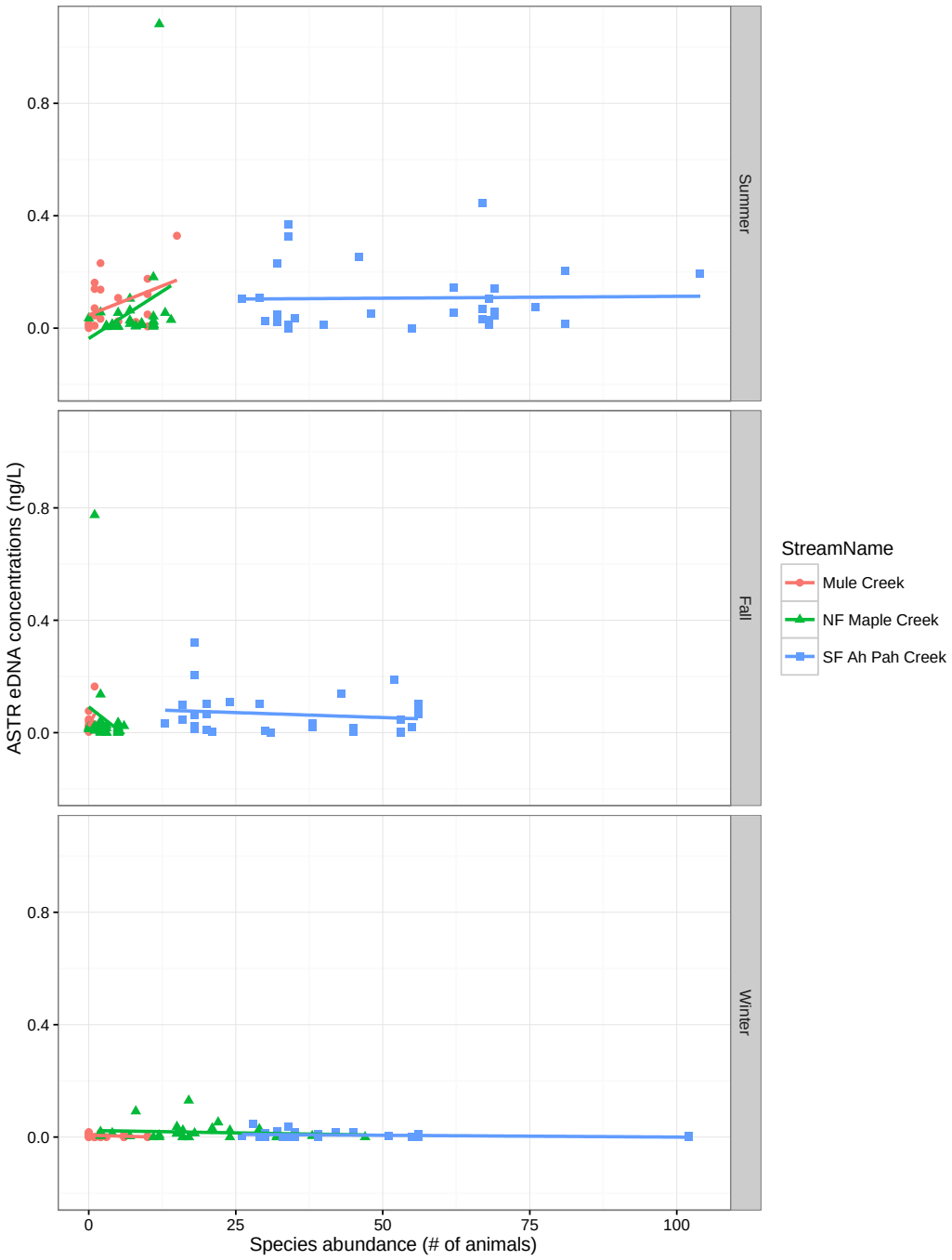

Figure 6. Plot of individual eDNA concentrations (ng/L) and their corresponding species abundance estimates (# of animals) fit with smooth curves created from an individual GAM for each stream and field season. The gap between Maple and NF Maple Creeks and SF Ah Pah Creek during the summer and fall resulted from a large difference in the number of animals observed in these streams.

To assess the effect of near vs. far animals on eDNA concentrations, I created a plot that included each animal observation as an individual point (Figure 7). Based on this graph, there is no pattern between eDNA concentrations and animals found nearby. Instead, most of the high eDNA concentrations were located near the beginning of each sampling site (WaterSampleID).

To observe the effect of distance and animal abundance on eDNA concentrations, I created a plot of all eDNA samples as individual points, aligned on the x-axis according to the number of animals observed within the first 10 meters of each sampling location (WaterSampleID; Figure 8). This plot shows that there is no clear relationship between animal abundance and eDNA concentrations at specific locations in a stream; some samples had high eDNA concentrations when animals were not present while others had high eDNA concentrations when animals were present. However, a positive correlation was revealed when evaluating the estimated species density and average eDNA concentration of the entire stream (Figure 3).

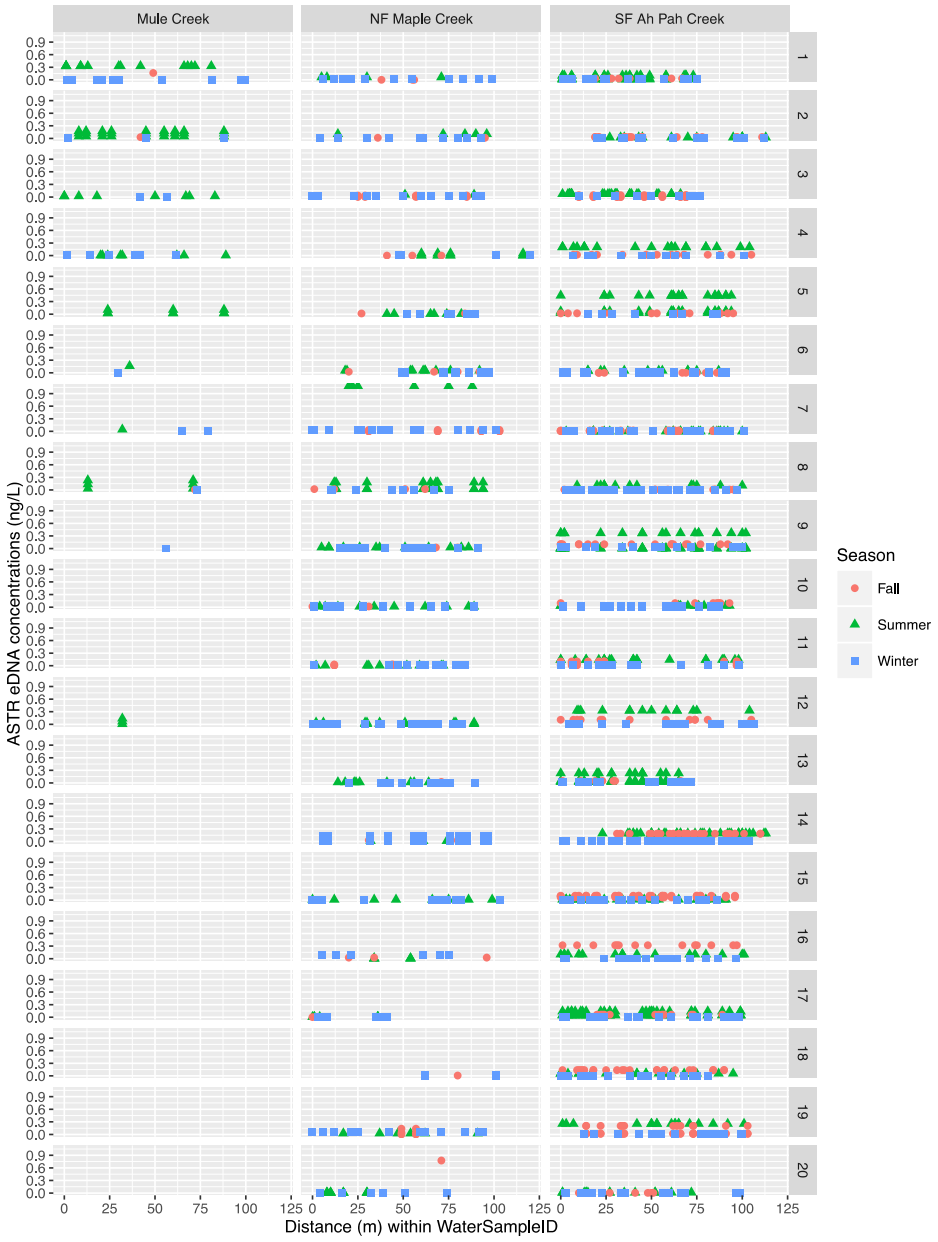

Figure 7. Individual animal observations plotted according to the location within each sampling site (WaterSampleID) that the observation occurred. Animal observations are grouped according to stream and the sampling site (WaterSampleID; y-axis on right). The y-axis on the left represents concentrations of eDNA (ng/L). The location of each point along this y-axis (left) indicates the amount of eDNA found in the eDNA sample taken at that water sampling site. Each animal detection, or individual point, was aligned along the x-axis according to the distance within each sampling site where the animal was observed.

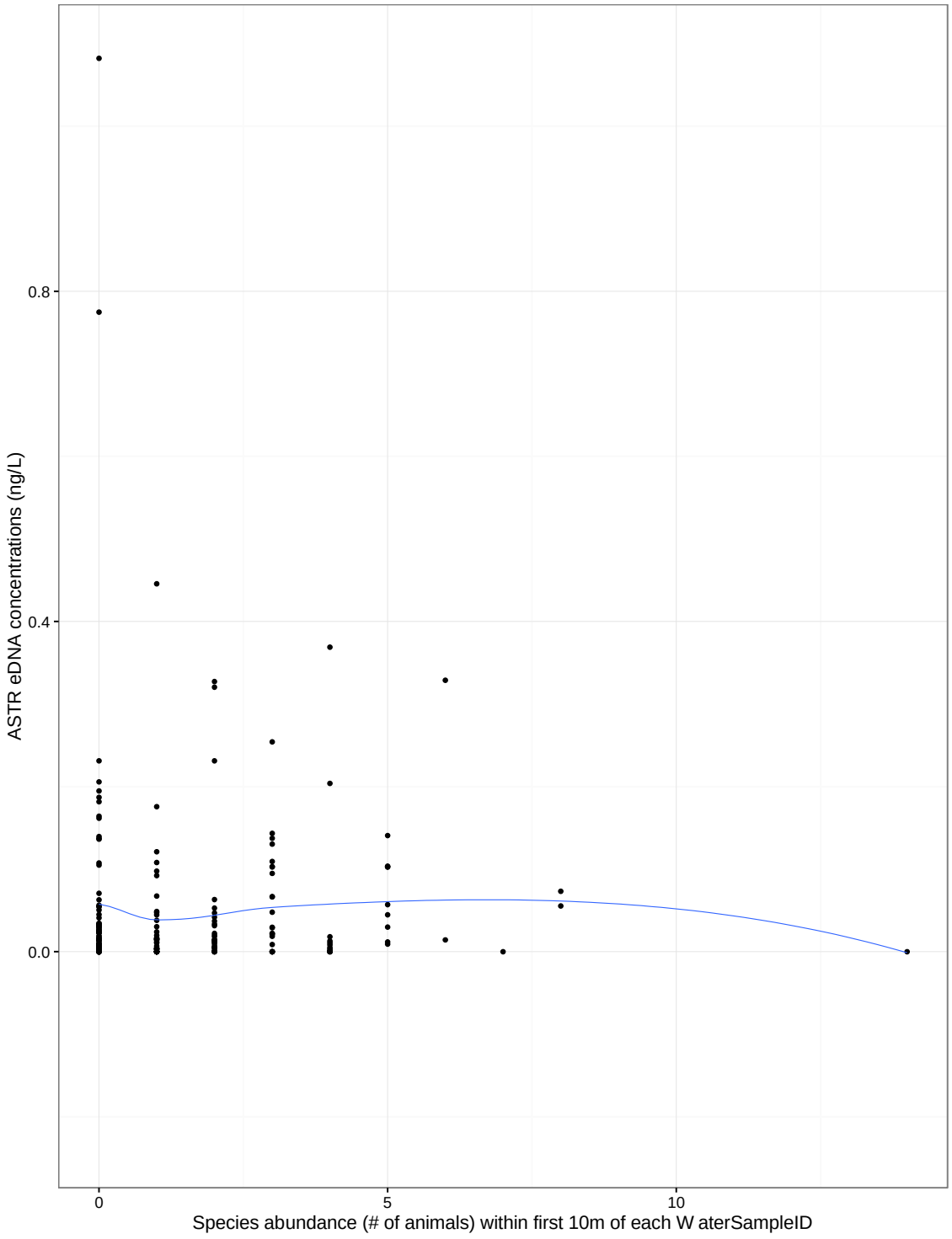

Figure 8. Plot showing eDNA concentrations as individual points plotted according to the number of animals observed within the first 10 meters of each water sample ID.

DISCUSSION

Comparison of Detection Rates of eDNA Sampling vs. Traditional Sampling

This research investigated the effect of season on the ability to use eDNA sampling to detect stream-dwelling amphibians, while also comparing the efficacy of eDNA sampling to a traditional sampling technique. The results of this study suggest that eDNA techniques using species-specific primers can be more effective than rubble-rousing for detecting coastal tailed frogs in fast-moving streams during the summer and fall seasons (Table 2). Our results are consistent with many other previous studies that found that eDNA sampling provided higher detection rates relative to traditional sampling (Biggs et al. 2015, Dejean et al. 2012, Foote et al. 2012, Janosik and Johnston 2015, Jerde et al. 2011, Pilliod et al. 2013, Takahara et al. 2013, Thomsen et al. 2012b). Other studies that included multiple sampling seasons reported that detection rates differed for each season (Deiner et al. 2015, Goldberg et al. 2011). In my study, the detection rate for eDNA sampling fell below the detection rate for traditional sampling during the winter field season. During this time, streams experienced high flow rates and decreased animal densities (Tables 8 and 12). Although animals were frequently detected in the winter using traditional sampling, the number of animals observed was relatively low compared to the summer field season. Thus, the detection rate for traditional sampling during the winter field season remained high, but the low number of animals observed corresponded with low eDNA detection rates and concentrations.

While the ASTR primer set outperformed the ASMO/TR primer set at detecting coastal tailed frogs, their combined purpose was to amplify all haplotypes of the target species. To do this without amplifying DNA from any other co-occurring amphibian species, a large amount of effort needs to be put towards properly developing and optimizing the primers. Primer design is critical to the success of eDNA sampling. The ASMO/TR primer set was a degenerate primer developed to compensate for any haplotypes that may have gone undetected by the ASTR primer set. Its degenerate nature made it less species-specific than the ASTR primer set. The ASMO_TR primer set still provided a higher detection rate than traditional sampling during the fall season (Table 2). Importantly, when detection rates are low when using traditional sampling, the sensitivity of eDNA sampling paired with reliable primers can increase the detection of species.

Previous research, conducted only during a single season, found that eDNA sampling provided higher detection rates than traditional sampling when animals were at low densities (Pilliod et al. 2013). My study found similar results in only two of the three field seasons. Specifically, in my low density stream (Mule Creek) eDNA sampling provided higher detection rates than traditional sampling only during the summer and fall field seasons; during the winter, traditional sampling provided a higher detection rate compared to eDNA sampling (Table 3). Because of the high detection rates observed when using eDNA sampling at low densities, eDNA sampling can be used to monitor population growth and decline as well as review changes in a species' range.

Interpreting the Effects of Environmental Variables on eDNA Concentrations

My analysis showed that the winter field season negatively affected the efficacy of eDNA sampling in all three streams (Table 9). More specifically, I saw the lowest eDNA concentrations during the winter, as well as a decrease in the number of animals observed, relative to the summer field season (Table 4 and Table 12). By contrast, some studies that only took place during the winter or spring months have found higher eDNA concentrations relative to the concentrations found during the winter in my study. In particular, previous eDNA studies to detect fish species like salmonids saw increases in population abundance during the winter and spring due to breeding or spawning events during these seasons (Janosik and Johnston, Sigsgaard et al. 2015, Smart et al. 2015, Takahara et al. 2012). Large spikes in animal abundance during these events caused an increase in eDNA production and detectability despite increased rainfall and colder water temperatures (Klymus et al. 2015, Spear et al. 2015, Thomsen et al. 2012a). My results differed from those of these studies due to differences in the biology of my study species. During the winter, newly metamorphosed tailed frogs have left the streams and moved into the woods (Stebbins et al. 2003), contributing to the relatively low number of animals found in the streams during this season. Also, it is possible that during high winter and spring flows, tadpoles experience decreased metabolism or changes in behavior, such as limited foraging, and therefore have decreased eDNA production (Goldberg et al. 2011). It is clear that using eDNA sampling to effectively assess and

manage aquatic species will rely heavily on an understanding of the biology of the species and the seasonality of the study area.

The water temperature of each stream did not vary significantly relative to each other during each season, but water temperature was lowest during the winter field season in all three streams (Table 5). Water temperature was included in one of my top models, and the summary of this model suggests that warmer water temperatures will increase eDNA concentrations (Table 10). This finding was unexpected because it does not agree with other studies showing that colder water temperatures are optimal for DNA persistence (Strickler et al. 2015). In my study, the winter field season had the lowest eDNA concentrations and streams experienced the lowest water temperatures. It is possible that the actual significance of water temperature in my study was confounded by an association between two correlated variables -- colder water temperatures and low eDNA concentrations -- both of which are characteristic of streams in my study during the winter and spring months.

It seems intuitive that increased turbidity in streams would negatively affect the ability to extract eDNA from water samples and also inhibit the amplification of eDNA during qPCR. Most eDNA studies did not collect turbidity measurements; the few studies that measured turbidity did not analyze its effect on eDNA concentrations (Goldberg et al. 2013, Takahara et al. 2012, Tréguier et al. 2014). In my study, I did not see a correlation between turbidity and eDNA concentrations due to the relatively narrow range of turbidities over which we sampled during each field season. In the future, I

recommend sampling when streams are experiencing a wider range of turbidities, which might reveal a significant correlation between turbidity and eDNA concentrations.

During the winter field season, increased flow rates resulted in decreased eDNA concentrations (Table 11 and Figure 2). These findings support the results of previous research that showed that increased flow rates negatively affect eDNA concentrations due to the "dilution effect" (Jane et al. 2015, Gingera et al. 2016, Laramie et al. 2015). A limitation of my study was that I did not have exact flow rate measurements for each sampling site and instead used predicted flow rates calculated in RStudio. The predicted flow rate measurements appropriately decreased as sampling sites moved upstream towards the headwater, which is typical of a stream system; as such my results likely were not affected by using this approach (Macnab et al. 2006). I anticipated the negative correlation between predicted flow rate and eDNA concentrations shown in the analysis, regardless of the lack of real flow rate values at each sampling site, due to the "dilution effect" of the eDNA samples. In the future, I recommend collecting flow rate measurements from every sampling site to truly analyze the effect of flow on eDNA concentrations at specific locations. Nonetheless, I learned that during the winter months in northern California, the efficacy of eDNA declines due to a combination of environmental variables, but most importantly, the negative effect of flow rate on eDNA concentrations. Consequently, I recommend that eDNA sampling be avoided during seasons of high stream flows.

When the effect of stream size on eDNA concentrations was analyzed, I found that higher eDNA concentrations were observed when eDNA sampling was performed in

the largest stream, SF Ah Pah Creek (Table 10 and Table 11). It is likely that the significance of this correlation is due to the high abundance of animals found in our largest stream. Previous studies have found a positive correlation between animal densities and eDNA concentrations at study sites (Olson et al. 2012, Takahara et al. 2012). This positive correlation has been reported in past studies that used a variety of different methods, indicating that we may one day expand from using eDNA sampling to simply detect presence and absence and instead use eDNA concentrations to monitor population size.

Relationship Between Animal Abundance and eDNA Concentrations

Two major goals of this study were to 1) investigate the relationship between average eDNA concentration and total species abundance found in each stream, and 2) assess the relationship between eDNA concentrations and species abundance at specific locations within the stream. During the summer and fall field seasons, our ability to see a stream-level correlation between animal abundance and average eDNA concentration is most likely due to the accumulation of DNA shed by multiple animals throughout the stream (Table 4 and Table 12). This trend disappears during the winter field season due to environmental conditions decreasing eDNA concentrations, despite increased animal observations relative to the fall field season. Our results were comparable to the findings of multiple other studies that found a stream-level, positive correlation between animal abundance and eDNA concentrations (Erickson et al. 2016, Jane et al. 2015, Klymus et al. 2015, Pilliod et al. 2013, Mahon et al. 2013). However, eDNA techniques executed in

unmanipulated streams (i.e., only including naturally occurring species) have not found a direct correlation between abundance and eDNA concentrations at specific locations within the stream (Baldigo et al. 2017, Pilliod et al. 2013, Spear et al. 2015). By design, this project included a micro-assesment of individual sampling sites to address the influence of mean abundance of animals at particular sampling sites on independent eDNA concentrations. Similarly, my research did not find any correlation between animal abundance and eDNA concentrations at specific sampling sites (Figure 4). The lack of a positive correlation at specific sampling sites is most likely due to the influence of flow rate, which can transport eDNA away from its original source. I recommend collecting multiple eDNA samples to assess animal abundance at the stream-level and avoid focusing on this relationship at specific sampling sites. More extensive eDNA research should be conducted utilizing flume experiments and an analysis of stream hydrology to replicate stream environments in order to better understand the movement of eDNA.

Implications

Overall, eDNA sampling was proven to be an effective technique in northern California for detecting coastal tailed frogs at a range of animal densities during the summer and fall. To date, eDNA sampling has been used to provide presence/absence data across the range of a species of interest. Due to the positive correlation between stream animal abundance and mean eDNA concentration, it may be possible to monitor the growth or decline of known populations of amphibians by using eDNA

concentrations to provide information on population size at the stream-level. Notably, the promise of using eDNA sampling to provide high detection rates, even when animals are at low densities, suggests that it can be used to detect species in sites where their presence is known but traditional surveys could not detect them. This is especially important for addressing shifting species distributions and characterizing the spread of invasive species. Attempts to use eDNA sampling to monitor aquatic amphibians should be avoided during rainy seasons or under other conditions when streams experience increased flow rates. While traditional sampling techniques will remain a vital part of species monitoring by providing real-time identification and life history information, the easy application of eDNA techniques and significant decrease in cost and effort suggest that it can be used as a reliable stand-alone method.

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