Message In A Bottle: A Comparison Of Environmental Dna Detections Across Years For The Endangered Reticulated Flatwoods Salamander (Ambystoma Bishopi).

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MESSAGE IN A BOTTLE: A COMPARISON OF ENVIRONMENTAL DNA DETECTIONS ACROSS YEARS FOR THE ENDANGERED RETICULATED FLATWOODS SALAMANDER (*AMBYSTOMA BISHOPI*)

By

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THESIS CERTIFICATION

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Abstract

Environmental DNA (eDNA) is a non-invasive, cost-effective tool used to monitor elusive and rare species by isolating DNA from water samples and screening that DNA for specific sequences that match a particular species of interest. Environmental DNA was used to monitor and detect a federally endangered salamander species (*Ambystoma bishopi*) in ephemeral ponds on Hurlburt Field, in Florida during the breeding seasons of 2018–2022. We collected water quality measurements at each pond for each month throughout the five years sampled that included depth, pH, water temperature, dissolved oxygen, specific conductivity, and salinity. Principal Component Analysis (PCA) was employed on the fullest data set available, 2021, to determine any relationships between water quality measurements and detection rates. Visual summaries of the results from 2018 to 2022 alongside monthly precipitation levels and a pond's monthly given water temperature were incorporated to assess any changes in detection rates relative to these conditions. Comparisons were made between four ponds across all five years sampled. Restored ponds versus unrestored ponds were compared using Fisher's exact test. Water temperature and pH were found to be the most influential water quality factors explaining detection rates for 2021. There was a significant difference in detection rate of *A. bishopi* between restored and unrestored ponds, suggesting that restored ponds have more suitable habitat than non-restored ponds. Management should continue to restore upland and breeding habitat as well as monitor the quality of water within these breeding wetlands to ensure the survival and longevity of this federally endangered species.

Introduction

Reticulated Flatwoods Salamander

The reticulated flatwoods salamander (*Ambystoma bishopi*, RFS) is a mole salamander endemic to the lower Gulf and Atlantic coastal plains. There are 19 remaining self-sustaining populations of RFSs in the Florida panhandle, and one population in southwestern Georgia. Once occurring in the extreme southern region of Alabama, they have not been observed in Alabama since 1981 (Jones et al 1982, Godwin 2003).

First described in 1950 by Coleman J. Goin, the RFS was categorized as a subspecies of the frosted flatwoods salamander (*Ambystoma cingulatum*, FFS) based on morphological differences and geographic barriers. In comparison to the FFS, RFSs are on average smaller bodied, have fewer costal grooves, have noticeably smaller heads, are all black on the ventral side, and have a net-like (reticulated) appearance that is more distinct on the dorsal side. Although these two species share nearly identical ecologies and life histories, Pauly et al (2007) determined RFSs and FFSs are two separate species based on mitochondrial DNA, morphological differences, and allozymic differences. Pauly et al (2007) described these species as being separated by a large geographic barrier, the Apalachicola River in Florida, where FFSs could be found to the east of the river and RFSs were found to the west. Most references and information on the FFSs were published before Pauly et al (2007), therefore there is no distinguishing these two species from each other before 2007.

Adult RFSs are closely associated with longleaf pine flatwoods and savanna communities. Seasonally saturated wetlands are characterized by poorly drained, sandy, and acidic soil that has low, flat topography. In the past, this type of open pine forest was maintained by frequent fires that were ignited naturally from lightning. Fires typically occurred in spring and

summer with a fire return interval of 1 to 4 years (Clewell 1989). Adult RFSs move to their natal ponds from upland habitats to breed, where these isolated ponds are characterized by acidic, tannin-stained, ephemeral wetlands that can be described as marsh-like depressions (Palis 1997, Safer 2001). Rainy weather combined with cold fronts moving into the area, triggers adults to breed during fall and early winter when ponds flood.

Longleaf pine flatwoods ground cover usually consists mostly of wiregrass (*Aristida stricta*; Kesler et al 2003). Saw palmetto (*Serenoa repens*), gallberry (*Ilex glabra*), huckleberry (*Gaylussacia* spp.), blueberry (*Vaccinium* spp.), beakrushes (*Rhynchospora* spp.), and legumes are a few low-growing shrubs that are also found in the upland habitat of RFS. These low-growing shrubs co-exist with a wide array of grasses and forbs (Sekerak et al 1996). Breeding wetlands usually consist mostly of pond cypress (*Taxodium ascendens*), blackgum (*Nyssa sylvatica*), slash pine (*Pinus elliotti*), and longleaf pine (*Pinus palustris*). Occasionally, sweetbay magnolia (*Magnolia virginiana*), sweetgum (*Liquidambar styraciflua*), red maple (*Acer rubrum*), pond pine (*Pinus serotina*), and loblolly bay (*Gordonia lasianthus*) can be found in breeding habitats. The midstory, which can vary in density, consists of myrtle-leaved holly (*Ilex myrtifolia*), St. John's-worts (mostly *Hypericum chapmanii* and *H. fasciculatum*), bamboo-vine (*Smilax laurifolia*), fetterbush (*Lyonia lucida*), titi (*Cyrilla racemiflora*), sweet pepperbush (*Clethra alnifolia*), and vine-wicky (*Pieris phillyreifolia*). Dominated by mostly graminaceous species, the groundcover in breeding wetlands mainly consists of beakrushes (*Rhynchospora* spp.), sedges (*Carex* spp.), panic grasses (*Panicum* spp.), longleaf three-awned grass (*Aristida palustris*), hatpins (*Eriocaulon* spp.), plumegrasses (*Erianthus* spp.), jointtails (*Coelorachis* spp.), nutrush (*Scleria baldwinii*), and rosette grasses (*Dicanthelium* spp.). There are also carnivorous species of plants found in both the breeding wetlands and upland habitat like

bladderworts (*Utricularia* spp.) and pitcher plants (*Sarracenia* spp.). RFSs typically lay their eggs in pipewort species within the breeding wetlands, while RFS larval refugia are usually associated with *Rhynchospora* species.

RFSs can also persist in habitats that are less than ideal. Historically, ponds that were larger and deeper usually provided more suitable habitat for RFSs but were more likely to have experienced fire suppression and therefore be overgrown with shrubs. When a pond has not been restored by mechanical removal of vegetation and/or prescribed fire, extensive accumulation of hardwood litter and duff can occur, which creates a matrix of debris that is difficult for adult RFSs to travel through. In flatwoods uplands and ephemeral ponds, fire suppression also leads to greater overstory canopy closure (Bishop and Haas 2005, Gorman et al 2009, Gorman et al 2013) which in turn decreases the diversity of herbaceous ground cover. Lower herbaceous diversity has been shown to support less diversity and therefore lower abundances of invertebrate prey in the wetlands (Chandler et al 2015). Without prescribed fire or naturally occurring wildfires, longleaf pine may become outcompeted by slash or pond pine in the uplands. An accumulation of woody vegetation may lead to a reduced hydroperiod or length of time these isolated wetlands contain standing water (Jones et al 2018).

In this study, restored ponds generally consist of ponds that have had extensive mechanical removal of vegetation and in some cases, have been restored with fire after initial mechanical vegetation removal. Mechanical removal of woody vegetation requires a large crew to manually remove unwanted vegetation and apply herbicide throughout, which is all a very labor-intensive process. Mechanically removing this vegetation allows for more open-canopied wetlands which are more suitable for the RFS (Gorman et al 2013). Once mechanical removal of vegetation has occurred, emergent herbaceous vegetation needed within the pond basin can begin

to grow in which is needed for the reproductive cycle of the RFS. Although, emergent herbaceous vegetation responds more quickly when the pond basin is prescribed fire because without fire, an accumulation of organic debris within the basin reduces or prevents germination of herbaceous vegetation (Gorman et al 2013). There may be other factors influencing the presence of the RFS in ponds, including but not limited to: timing of prescribed fires and removal of vegetation, and whether the pond basin contained standing water during the prescribed fire. These could ultimately confound our results of the differences between restored and unrestored ponds.

There are several physical water properties that are important for the health and survival of many salamander species. The amount of dissolved oxygen (DO) available in a pond has been shown to have a significant influence on the hatching success of other similar mole salamander species in restored wetlands (Sacerdote and King 2009). In addition to DO, pH has also been shown to affect the growth and survival of mole salamander species in which very low or very acidic pH levels can be lethal (Freda 1986, Anderson and Johnson 2018). pH levels of 5 were shown to negatively affect the growth of salamanders, with salamanders exposed to higher pH levels of 6 and 7 showing significantly larger size differences (Anderson and Johnson 2018). Salinity has been shown to affect other salamander species such as the Anderson's salamander *Ambystoma andersoni* (Ahumada et al 2018). Salinity levels higher than ten percent are not tolerable for newly hatched *A. andersoni* larvae (Ahumada et al 2018). Lastly, water temperature can play a role in the survival of eggs and larvae throughout many aquatic systems (Albers and Prouty 1987, Grant et al 2014). A certain range of lower water temperatures are optimal for the egg development and larval phases of the RFS life cycle (Albers and Prouty 1987, Grant et al 2014).

Out of the 20 known populations of the RFS throughout Georgia and Florida, roughly half occur on public land. Four of these populations live on United States Department of Defense lands in Florida including Eglin Air Force Base and Hurlburt Field. The largest RFS population is located on Eglin Air Force Base. The population of RFS on nearby Hurlburt is restricted to several breeding ponds, which support at least one metapopulation. Hurlburt may have a metapopulation that overlaps with part of Eglin's population, but further DNA analysis is needed to determine how interconnected these populations are. Using microsatellite nuclear DNA markers on Eglin Air Force Base, Wendt (2017) found that salamanders within isolated wetlands less than 1 km from each other were genetically divergent populations. Each breeding wetland was found to function as essentially a semi-independent local population at that site (Wendt 2017). An average dispersal distance of 230 m was measured and no evidence of connectivity beyond 1.5 km was found (Brooks, Smith, Frimpong et al 2019). These results support the idea that in the Florida panhandle, there are multiple metapopulations. For RFSs to remain resilient in the fragile longleaf pine habitats that they occupy, genetic connectivity between populations is essential (Whitely et al 2015). Thus, longleaf pine habitat and sufficient hydrology should be promoted by appropriate associated ecosystem processes (Semlitsch et al 2017).

The RFS has a complex life cycle. RFSs first start off in an egg stage, then hatch and live in an aquatic larval stage with gills, and finally metamorphose to live out a terrestrial juvenile and adult stage with lungs. RFSs migrate to and breed in ephemeral wetland areas during late fall and early winter caused by cold-front rains where females will lay eggs in small groups. Herbaceous ground cover, specifically on top of and within rosette-forming herbs and grasses, and moisture-retaining microhabitats play an important role in the breeding cycle of RFSs, particularly to aid in egg development (Palis 1995, Anderson and Williamson 1976, Palis 1997).

Developed eggs hatch into larvae after 22–36 days of inundation within the wetland basins if water levels are adequate (Anderson and Williamson 1976). Sometimes filling of ponds may be inadequate for larvae to develop, or if they do develop, it is possible larvae may metamorphize. After an 11- to 18-week larval period, metamorphosis can occur between the months of March and May (Palis 1995). After metamorphosis, juvenile salamanders usually disperse from the ponds into the upland pine flatwoods habitat, but occasionally will stay near the pond during seasonal droughts (Palis 1997). Both juveniles and adults are fossorial, utilizing root channels, crawfish burrows, or burrows of their own making until they return to their natal wetland to breed during the fall (Petranka 1998, Powell et al 2015, Brooks, Smith, Gorman et al 2019). Males reach sexual maturity after one year while females may reach maturity between 1 and 2 years (Petranka 1998). Based on captive animals, Palis et al (2006) found that RFSs lived for approximately four years, although the exact lifespan of this species is unknown. A few individuals have been witnessed to live 9–12 years (George Brooks, Virginia Tech, 2019 pers. comm.).

The RFS is faced with several threats as larvae and adults, with the most significant threat being loss of habitat and habitat fragmentation, which creates isolated ephemeral breeding ponds. Pine flatwoods, used by juveniles and adults as non-breeding habitat, have been reduced to approximately 18% of their original extent, accounting for now only 5.6 million acres (2.27 million hectares) of what used to be 32 million acres (12.8 million hectares) (Outcalt 1997). Pine flatwoods have experienced extensive damage from agriculture, silviculture, and urban development. These threats are presumably the cause of extirpation in Alabama and near-extirpation in Georgia (Ashton Jr. 1992, Outcalt 1997). Conversion of forests to intensively managed pine plantations render the habitat incompatible with RFS. Pine plantations often have

no natural or prescribed fire regimes and become close-canopied as a result. Pine plantations typically have significantly less understory herbaceous vegetation than natural longleaf pine forests (Hedman et al 2000); most importantly, herbaceous vegetation is needed for RFSs to deposit eggs and for larval development sites (Palis 1997). Longleaf pine forests require a natural fire regime to reduce woody competition. With a prescribed fire regime implemented, a diverse herbaceous understory is also promoted. Without fire or with improper burn management, chemical and physical changes can occur on the landscape and the ecosystem will become unsuitable for RFSs (Palis 1997). Current management practices prioritize restoring remaining and historical habitat by implementing growing season prescribed fire and restoring breeding wetlands through removal of hardwood midstory vegetation. Hardwood midstory vegetation can cause shading out of the understory, specifically emergent vegetation in the wetlands and understory vegetation in the uplands.

Aside from human threats like urbanization and intensive silviculture, climatic events can impact RFSs. Occasional droughts are naturally occurring events, but the RFS has been extirpated from most of its historical range making it difficult for the few remaining populations to replenish themselves when faced with current droughts that result in short hydroperiods. Palis et al (2006) found that immigration rates of adult frosted flatwoods salamanders to breeding ponds steadily declined during a drought period in 1999–2002. Between 1999 and 2014, Chandler et al (2016) found that Eglin Air Force Base experienced, on average, a 0.8–month shorter hydroperiod than any other time since 1896. Droughts often do not leave enough time for paedomorphic salamanders to metamorphose into adults (Palis and Hammerson 2008). Thus, a dramatic drop of the water table would not allow for RFS eggs to be properly inundated (Palis and Hammerson 2008). Protecting clusters of breeding sites with ponds that have different

hydrologic regimes is imperative to guarding RFS from continued population declines (Palis et al 2006). However, the conversion of ephemeral ponds into permanent water bodies may render the ponds unsuitable for the reproductive cycle of the RFS (J. Palis pers. obs.). This is because in permanent water bodies, fish species quickly become established and prey on amphibian eggs and larvae. Additionally, invertebrate or vertebrate predators may become established in ponds if they are filled too early, giving aquatic predators the opportunity to feed on RFS eggs before hatching (Taylor et al 2005). Out of the thirty known mole salamander species, about half are stable throughout their range. The reticulated flatwoods salamander is one of three ambystomatids that are listed as vulnerable by the International Union for Conservation of Nature (IUCN 2011). Under the federal Endangered Species Act and Florida's Endangered and Threatened Species Rule, the reticulated flatwoods salamander is protected as an endangered species (73 Federal Register 47257). Species are designated as Threatened or Endangered under the Endangered Species Act and have recommended recovery actions, established Federal protections, and certain actions and practices are prevented from occurring.

Environmental DNA (eDNA)

Methods of accurately monitoring amphibians have become of urgent need. Conventional trapping methods can be challenging or less feasible for species that are difficult to detect, such as rare, small, or cryptic species (Thomsen et al 2012, Kelly et al 2014, Janosik and Johnston 2015, Thomsen and Willerslev 2015, Ota et al 2020). One alternative approach is the use of environmental DNA (eDNA), which involves capturing DNA fragments in a small water sample that are shed by an organism into the surrounding environment (Ficetola et al 2008). DNA traces can be found in feces, mucus, gametes, shed skin, organelles, slime, or extracellular DNA. Extracted DNA can be identified taxonomically through DNA barcoding through amplification

and sequencing (Hebert et al 2003). eDNA can be used for monitoring presence/absence of species, estimating abundance, determining spatial use, and detecting invasive species, all of which are vital for management purposes. Researchers recommend the use of eDNA specifically when densities of the target species are low (Pilliod et al 2013). In addition, eDNA degrades in water within days or weeks (Dejean et al 2011), allowing managers to ensure any positive detections are from recent occurrences in the area. Lastly, we mainly decided to use eDNA to detect RFS to discover information about the status and distribution of this endangered salamander species.

There are a variety of techniques employed for capturing eDNA, but the two most used are the ethanol-sodium acetate precipitation and filtration method. The ethanol-sodium acetate precipitation method involves concentrating and de-salting nucleic acid in the water sample so that the DNA is precipitated out of the aqueous solution. In contrast to the filtration method, the precipitation method is more suitable and efficient when taking small (15 mL) water samples from smaller, more lentic water bodies (Ficetola et al 2008, Dejean et al 2012, Muha et al 2019). Contamination risk is also decreased when using the precipitation method in comparison to the filtration method because of the nature and method in which filtration samples are taken and handled (Muha et al 2019).

Using eDNA in comparison to conventional survey techniques helps to also eliminate the possibility of organisms being incorrectly identified (Stribling et al 2008). Using eDNA was beneficial in this study because it can detect the species even when the species is not directly present. Conventional survey techniques also often involve invasive and harmful methods to the species of interest, such as pitfall and funnel traps, which can harm stress or kill both target and non-target species. In addition, conventional trapping and capture techniques can cause

destruction to the environment in which the species inhabits (Wheeler et al 2004). Additionally, conventional survey methods can be labor intensive. For example, Jerde et al (2011) found that while electrofishing, it took 93 days for species identification experts to identify a rare fish species, in comparison to using eDNA, which only took four hours to detect the same fish species. eDNA as a non-invasive alternative can provide more accurate estimations of a species abundance while also providing higher detection sensitivity (Biggs et al 2015, Valentini et al 2016). As such, eDNA has been demonstrated to be a low-cost, time-efficient, non-invasive standardized sampling approach.

The objective of this study was to analyze and compare eDNA detections across five years from 2018 to 2022 and associated months according to the following conditions: wet versus dry years and restored ponds versus unrestored ponds. We compared various water quality measurements recorded at each pond to understand further which water quality properties influence the average eDNA detection rate across time. We expected wet years to have a higher RFS detection rate because longer hydroperiods allow more time for larval development before metamorphosis to the terrestrial juvenile stage. We hypothesized that restored ponds would have higher detection rates because of greater cover of herbaceous ground cover needed for the egg and larval phases of RFS development and reduced organic layer build up in the pond basin. Lastly, we hypothesized that lower pH and DO levels would increase eDNA detection rates across ponds and time because of the sensitivity salamanders have regarding low acidity and oxygen levels present while breeding and developing. Therefore, we predicted ponds with lower acidities and higher DO levels would yield overall more positive eDNA results.

Methodology

Study Site

Hurlburt Field, located in Okaloosa County in Northwest Florida, is a United States Air Force Installation. Approximately half of the installation consists of wetlands (Integrated Natural Resources Management Plans [INRMP 2015]). The initiatives of the Hurlburt Field Integrated Natural Resource Management Plan include maintenance and restoration of habitats and populations of species of conservation concern. Along the western edge of Hurlburt Field, which shares a border with Eglin Air Force Base, longleaf pine, wiregrass savannas/flatwoods are the dominant ecosystems, and they harbor multiple ephemeral ponds (INRMP 2015).

Field Data Collection

Samples were collected by the U.S. Fish and Wildlife Service and the University of West Florida during January–April for years 2018–2022 on Hurlburt Field (Figure 1). Environmental DNA water samples were collected from four ponds during the years 2018 and 2019, from 16 ponds in 2020, and 23 ponds in 2021 and 2022 (Figures 2–5). Samples were taken once every month to track the status and distribution of the RFS larvae throughout the known breeding season. The 2018, 2019, and 2020 results and statistics came from previous reports, while 2021 and 2022 results and statistics were completed by myself and Dr. Janosik. The table below displays how many samples were taken each month during each year (Table 1). We chose to wait at least three weeks between each sampling round to ensure any previous RFS DNA collected had sufficient time to fully degrade in the aquatic environment (Dejean et al 2011). Locations of sampling sites were not selected randomly, instead we selected sites based primarily on known habitat preferences the RFS uses for egg laying and larvae refugia to maximize detection probability.

Table 1. Yearly sampling dates and number of samples collected in each month.

Year	Sampling Dates	Samples Collected	Number of Ponds
2018	January 30	29	4
	February 21	30	4
	March 29	25	4
2019	February 1	32	4
	February 28	32	4
	April 17	26	4
2020	January 16, 17, 21	83	15
	February 18, 21, 22, 23	82	16
	March 21, 26, 27, 28	75	13
2021	February 4, 5, 8	117	22
	March 9, 11	120	23
	April 11, 14, 18	122	23
2022	January 13, 14, 16, 17	80	16
	February 15, 17, 18	101	15
	March 21, 22	46	9
	April 12, 13, 14	121	23

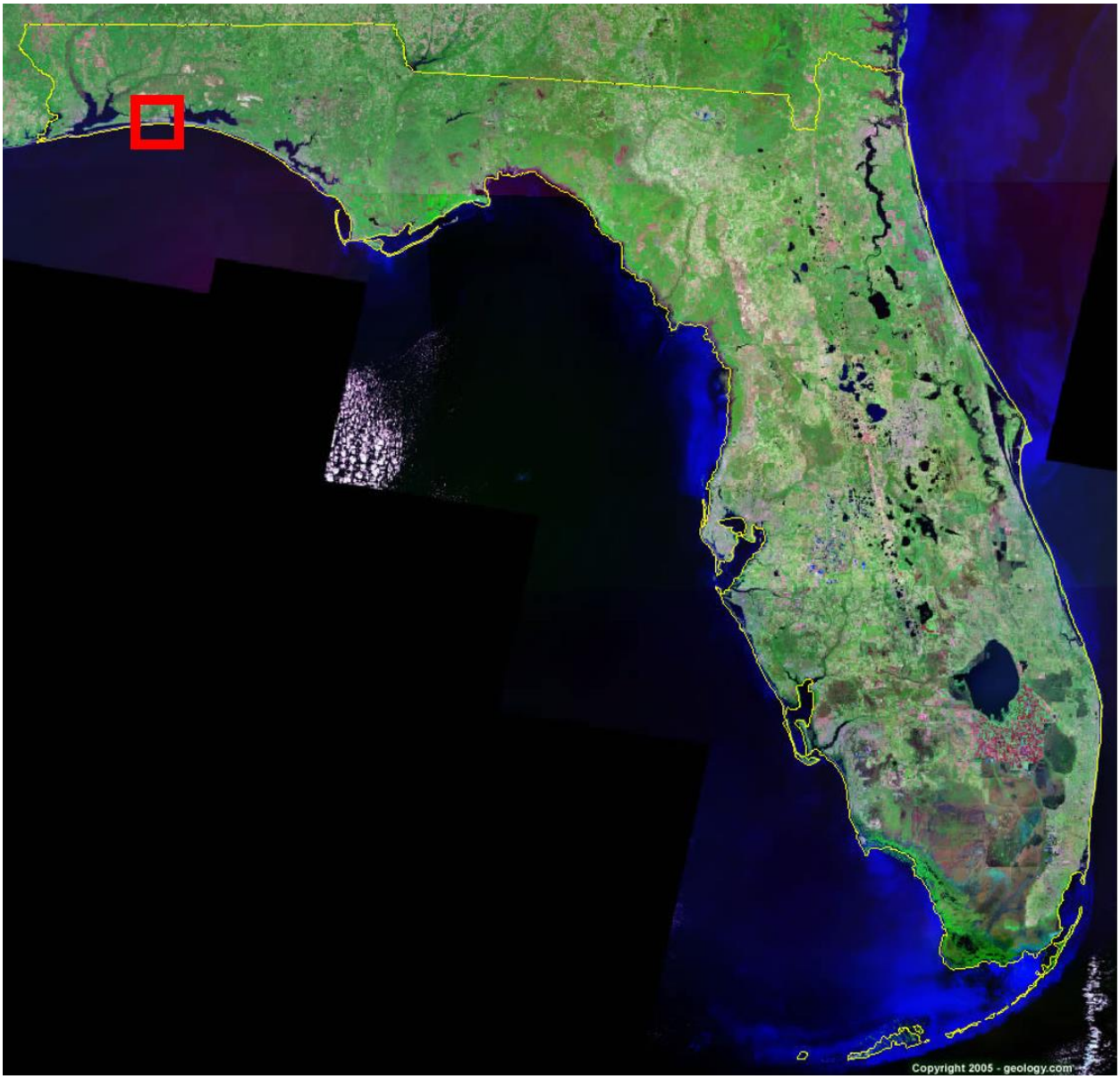


Figure 1. Map of Florida with study site, Hurlburt Field, outlined with a red box.

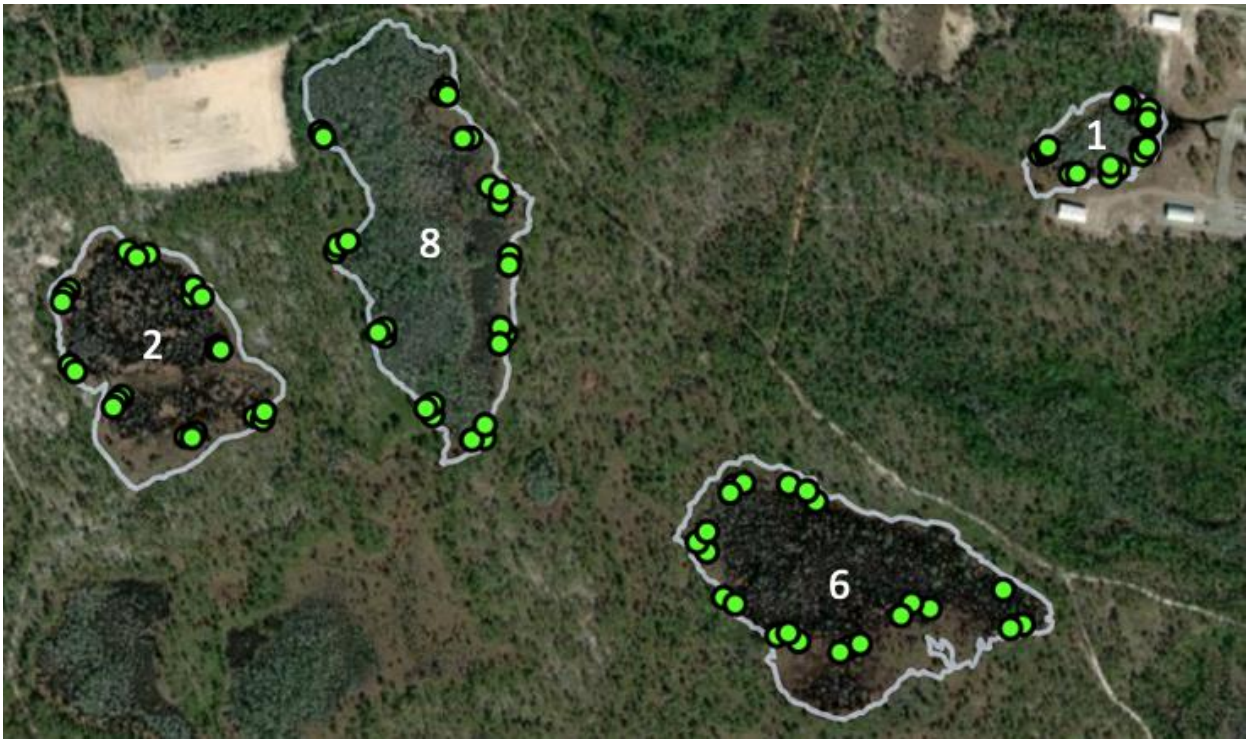


Figure 2. Green dots represent locations of sites where eDNA water samples were collected from years 2018 and 2019.

Figure 3. Teal dots represent locations of where eDNA water samples were collected in 2020. All ponds are outlined with a gray border.

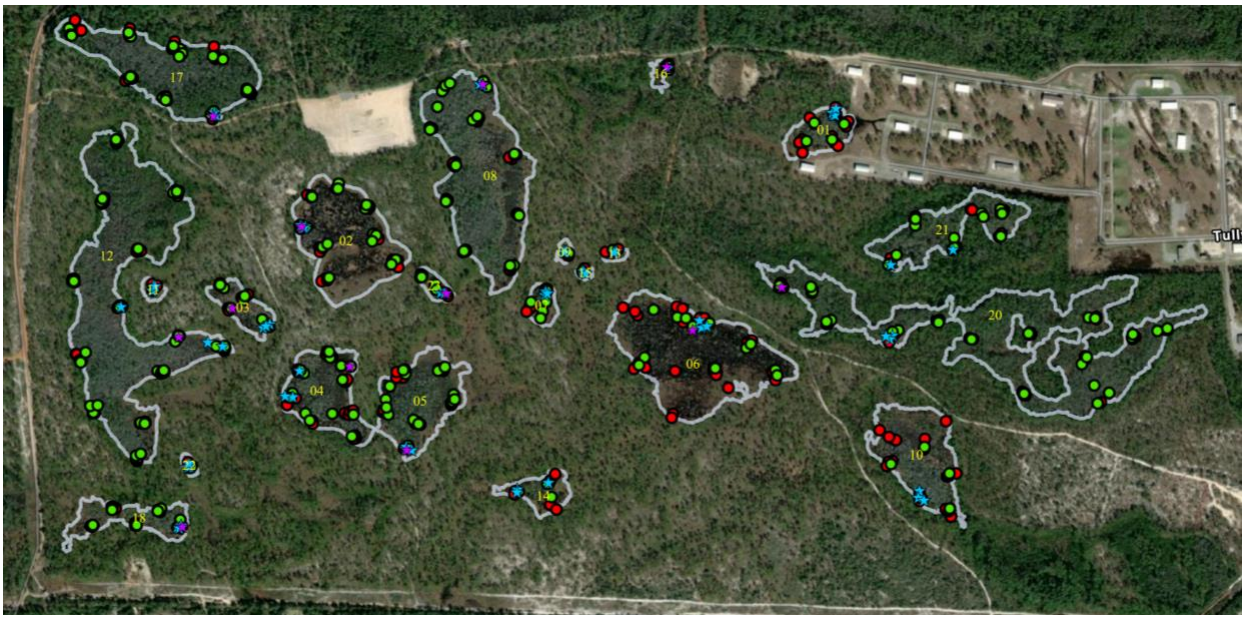


Figure 4. Red dots represent locations of where eDNA water samples were collected in 2021. Green dots represent locations of where eDNA water samples were collected in 2022. Blue stars indicate locations of water quality measurements for each pond taken in 2021. Purple stars indicate locations of water quality measurements for each pond taken in 2022. All ponds are outlined with a gray border.

Figure 5. Map of all 23 ponds on Hurlburt Field with pond 19 zoomed in. Red dots represent locations of where eDNA water samples were collected in pond 19 in 2021. Green dots represent locations of where eDNA water samples were collected in pond 19 in 2022.

Surface water samples were taken in 15mL triplicates for each site and preserved immediately in 50mL sterile conical tubes by adding the sample to a solution of 33.5 mL of 200 proof ethanol and 1.5mL of 3M sodium acetate (pH 5.2). Field boots were sterilized between sites by decontaminating them with a 10% bleach solution between ponds to prevent cross contamination. Water quality data taken at each pond including water temperature in degrees Celsius (T), dissolved oxygen as mg/L (DO), specific conductivity as uS (SC), pH, salinity as a percentage (S), and depth of the sensor (on the YSI instrument) in meters (D). All sample collections took place before dip netting of larval salamanders. In the field, samples were stored

on ice in a sterile cooler. Also in the field, we avoided walking through a pond as much as possible while also collecting samples before trapping of larval salamanders occurred to avoid DNA potentially being transported around the pond. Until the DNA samples were extracted, they were stored in a cool, dark location. Field collection controls of deionized water, sodium acetate, and ethanol were included and extracted for each day sampling occurred in the field to ensure contamination in the field did not occur.

Lab Work

To further separate nucleic acids from the solution after precipitation, preserved samples were centrifuged for thirty minutes at 3500 g and 4° Celsius (Ficetola et al 2008). This resulted in a small pellet of DNA at the bottom of the conical tube. The DNA pellet was then lysed, washed, and purified so that the product was relatively pure DNA. This was all completed using the DNeasy Blood and Tissue Kit (Qiagen). DNA was amplified using polymerase chain reaction (PCR). Molecular primers (Amcin F GGC CCG TCA ACT TTC CTC TAA and Amcin R TGG TCC AGG TAA ATC AAT TGC A) that were designed to amplify a 132 bp region of the D-loop control region were previously published for RFS and employed in this study (McKee et al 2015). PCR reactions consisted of 17.6 microliters (µl) of deionized water, 2.5 µl of 10x DreamTaq Buffer, 2.5 µl dNTP, 1 µl of each primer (forward and reverse), 1.2 µl of bovine serum albumin (BSA), and 0.3 µl taq polymerase (Qiagen, Valencia, CA). PCR amplification took place with the following protocol: initial incubation at 94°C for 3 minutes, 34 cycles of 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute, followed by a final extension of 72°C for 10 minutes (Pfleger et al 2016). Extraction and PCR samples also had deionized water control samples to ensure contamination did not occur. PCR products were visualized under a UV light, and a 1% agarose gel electrophoresis was used to ensure the adequate base pair length

of the target DNA was amplified. Finally, the positive bands were bi-directionally sequenced by the University of Arizona Sequencing Facility for species verification.

Data Analysis

Sampling site information and water quality measurements (YSI, ProQuantro) were recorded in the field using ArcGIS FieldMaps on a tablet and saved into a file geodatabase to later be exported and integrated into ArcGis Pro (version 3.0.3, ESRI 2022) to create map visualizations. Across the five years of eDNA sample collection, ponds 1, 2, 6, and 8 were sampled each year and percent detection for each sample month was compared across each year. Total percent eDNA detections per month were compared to total rainfall per month for each sampling year. Precipitation data obtained from National Oceanic and Atmospheric Administration (NOAA) has oversight from the National Centers for Environmental Information (NCEI). Daily summaries were requested that displayed daily precipitation totals and temperatures from a weather station located in Niceville, Florida (30.52436°N, -86.49238°W). Additionally, average water temperature across ponds for a particular month was compared to percent of eDNA detection across each month and year.

Using the program *Statistix* (version 10, Analytical Software), a variety of statistical tests were performed including multiple linear models including stepwise linear regression, analysis of variance (ANOVA), and two sample *t* tests. A two-sample *t* test was run on the 2018 eDNA detection data and water quality measurement data by grouping ponds with an overall detection rate above zero in one category and ponds with a detection rate equal to zero into another category. An ANOVA was run on the 2019, 2020, and 2021 detection rates and water quality parameters for each pond by month. Detection rates were calculated by taking an individual pond's positive detections for a given month and dividing that number by the total number of

samples taken for that pond in that given month so that the end value is a percentage. Fisher's Least Significant Difference (LSD) procedure was used to test for which water quality parameters had different means from each other by month. Principal component analysis (PCA) was also used on the 2021 water quality measurements to determine relationships with detection rates. For this PCA, detection rates were calculated for each pond across each of the three months. If no data were available for a pond, neither detection rate nor water quality measurements, then the pond and water quality measurements for that particular month were not included in the PCA.

For restored ponds versus unrestored ponds, Fisher's exact test was used to determine whether there was a correlation between restored or unrestored ponds and RFS detection rates. R Studio was used to run several of these tests (R Core Team 2022). Years 2018 and 2019 were not included in the restored versus unrestored analyses. In general, ponds 1–9 and 13 were categorized as fully restored ponds because they had either been partially cleared, the edge was cleared, or the entire area was cleared (Table 2). Whereas ponds 10–11 and 14–23 were categorized as unrestored ponds (Table 2). If an unrestored pond was restored by having the edge cleared or partially cleared, or the entire area was cleared after the 2020 or 2021 season, the restoration status of the pond was altered from unrestored to restored for the next breeding season. Each sampling site within a pond was categorized in a restored or unrestored category. Pond 12 was excluded from the analysis because delineation of restored or unrestored status is unknown until after the breeding season in 2022. Specifically, most ponds on Hurlburt Field have recorded histories of when each pond has had any form of restoration work completed as shown in the table below, except for pond 12. Pond 12 is a large, dynamic pond. The southern portion of the pond does not have as thick of an herbaceous edge layer as other ponds like 20 and

21, but the northern portion of the pond does. Many of the ponds, especially medium to larger sized ones, are restored in pieces, as funding and timing can be very limited. As pond 12 has no recorded history of restoration efforts, this pond was excluded from the analysis.

Table 2. Restoration status of all ponds on Hurlburt Field by year.

Pond	Unrestored	Edge Cleared	Partially Cleared	Entire Area Cleared
1		✓ (2016)		✓ (2020)
2		✓ (1993)		✓ (2017)
3				✓ (2002)
4		✓ (1993)		✓ (2020)
5		✓ (2002)	✓ (2021)	
6		✓ (2002)		✓ (2018)
7				✓ (2020)
8		✓ (2003)	✓ (2021)	
9				✓ (2014)
10	✓			
11	✓			
12			✓ (2022)	
13				✓ (2020)
14	✓			
15	✓			

Table 2. Restoration status of all ponds on Hurlburt Field by year (continued).

Pond	Unrestored	Edge Cleared	Partially Cleared	Entire Area Cleared
16	✓			
17	✓			
18	✓			
19	✓			
20	✓			
21	✓			
22	✓			
23	✓			

Results

Yearly Detection Rates

Per the 2018 report, eighty-four eDNA water samples were collected in 2018 from four ponds (Janosik and Whitaker 2018). Ten (12%) of these samples had positive detections of RFS DNA, all of which occurred in January (Janosik and Whitaker 2018) (Figure 6). January had a total of 29 eDNA samples taken, February had a total of 30 samples taken, and March had a total of 25 samples taken (Janosik and Whitaker 2018). No positive detections occurred in February or March of 2018 (Janosik and Whitaker 2018). When broken down by pond over the three months, one sample out of 14 was positive (7%) in pond 1, two out of 25 samples were positive (8%) in pond 2, four out of 25 samples were positive (16%) in pond 8, three out of 20 samples were positive (15%) in pond 6 (Janosik and Whitaker 2018). The overall detection rate for January

was 21% (6 out of 29) (Janosik and Whitaker 2018). When broken down by month for only January, there was one positive detection out of four (25%) for pond 1, two out of eight (25%) in pond 2, three out of eight (38%) in pond 6, and four out of nine (44%) in pond 8 (Janosik and Whitaker 2018).

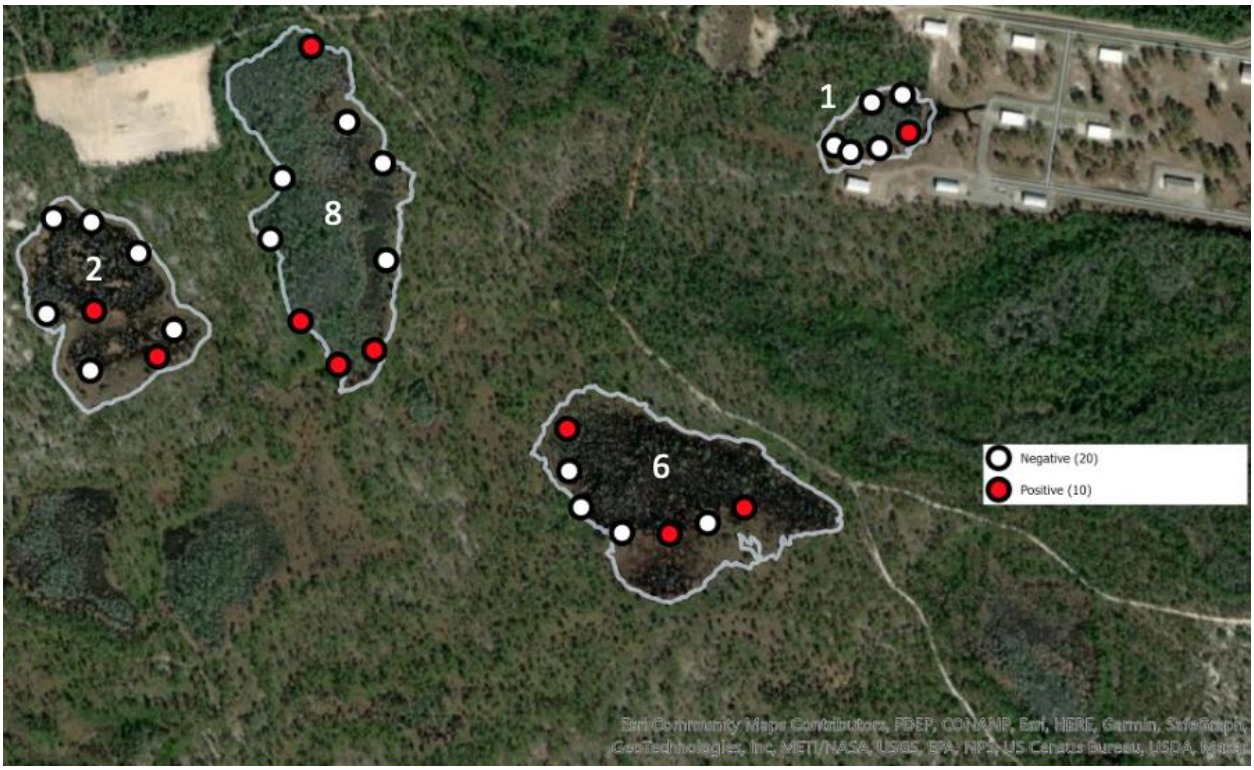


Figure 6. January 2018 map of sampling sites and eDNA detections. White circles indicate negative detections while red circles indicate positive detections.

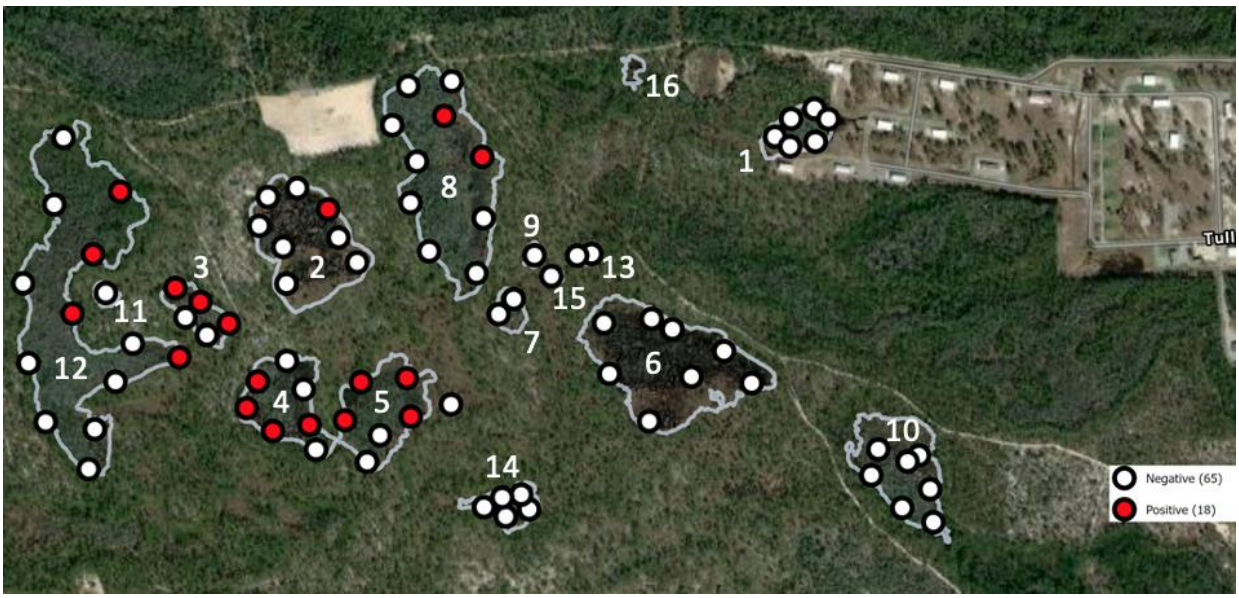
Per the 2019 report, ninety eDNA water samples were collected in 2019 from four ponds, of which 17 (19%) samples were found to have positive detections of RFS DNA (King et al 2019) (Figure 7). February of 2019 had the highest occurrence of positive detections at 25% (8 out of 32), followed by March with a rate of 22% (7 out of 32), and April with a rate of 8% (2 out of 26) (King et al 2019). Two out of 15 samples in 2019 were positive for pond 1, both occurred in February (King et al 2019). Three out of 20 samples were positive for pond 2, two of which occurred in February and one in March (King et al 2019). Four out of 19 samples were positive for pond 6, one occurred in February, two occurred in March, and one in April (King et

al 2019). Lastly, eight of 19 samples were positive for pond 8 of which three occurred in February, four in March, and one in April (King et al 2019).

Figure 7. February–April 2019 combined map of sampling sites and eDNA detections. White circles indicate negative detections while red circles indicate positive detections.

Per the 2020 report, 238 eDNA water samples were collected from 16 ponds, of which 82 (34%) samples were positive for RFS DNA (King et al 2020) (Figure 8). January had 18 out of 64 (22%) positive samples for RFS, February had 30 out of 82 (37%) positive samples, and March had 34 out of 73 (47%) positive samples for 2021 (King et al 2020). Positive detections were recovered at 46 sites in eight ponds (King et al 2020). Specifically, there were four positive detections in pond 2, six positive detections in pond 3, sixteen in pond 4, thirteen in pond 5, eight in pond 6, ten positives in pond 8, four in pond 10, and twenty-one in pond 12 (King et al 2020).

A.


16
1
8
9
2
13
3
15
11
7
12
6
4
5
10
14
Negative (65)
Positive (18)

B.


16
1
8
9
2
13
3
15
11
7
12
6
4
5
10
14
Negative (52)
Positive (30)

C.

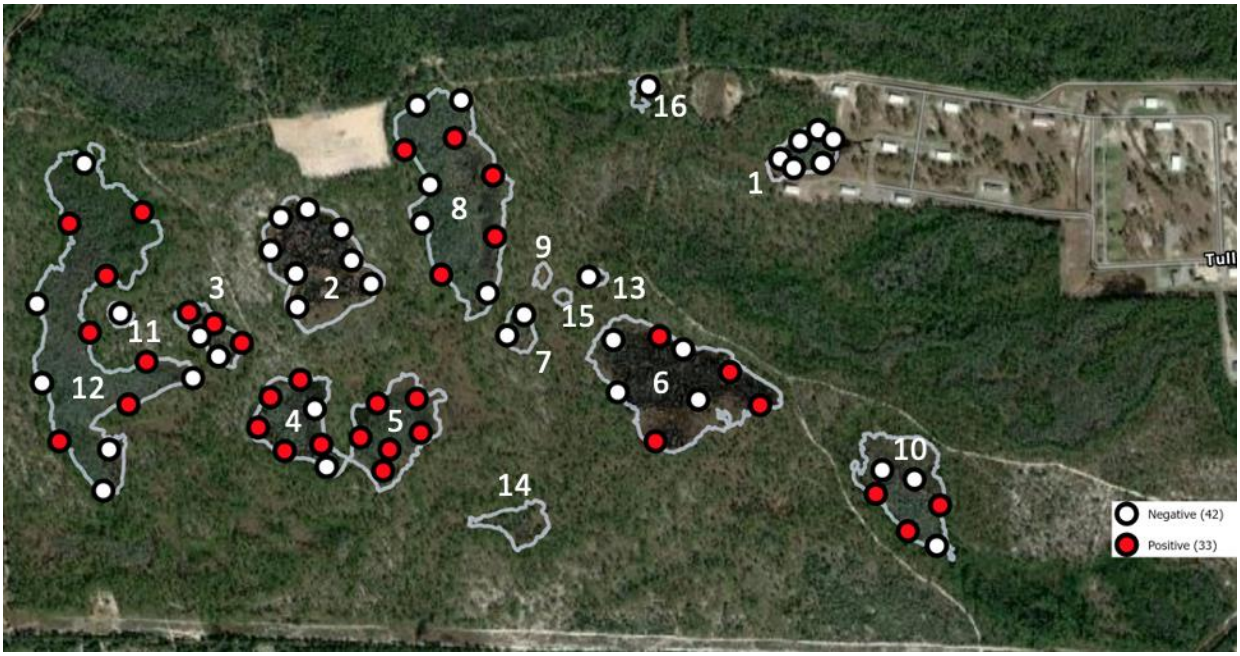
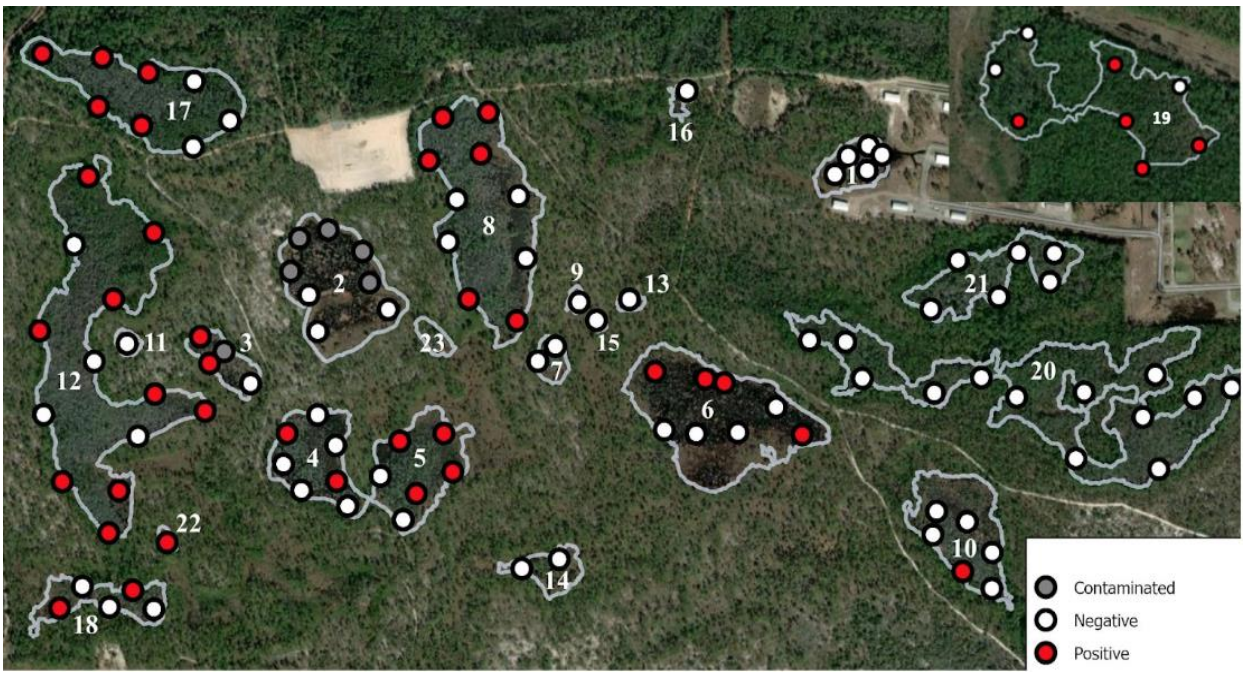


Figure 8A–C. January–March 2020 maps of sampling sites and eDNA detections. White circles indicate negative detections while red circles indicate positive detections.

In 2021, 338 eDNA samples were collected from 23 ponds, of which 83 samples (25%) were positive for RFS DNA (Figure 9). Forty-one (37%) positive detections occurred in February out of 112 samples, 35 (33%) occurred in March out of 105, and seven (6%) occurred in April out of 121. Positive detections were made at 64 sites and 13 ponds throughout all three months sampled for 2021.

A.


17
16
19
1
8
2
9
13
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7
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6
4
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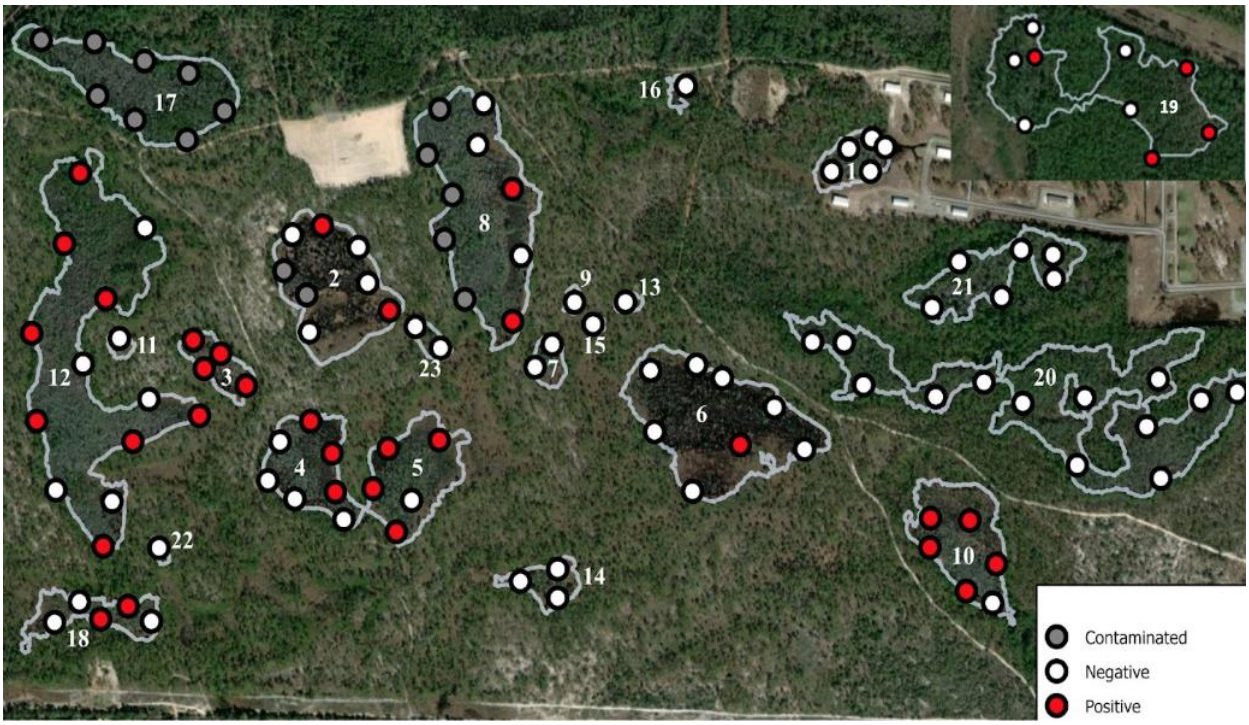
22
10
14
18
Contaminated
Negative
Positive

B.


17
16
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23
7
12
3
20
6
4
5
22
10
14
18
Contaminated
Negative
Positive

C.

Figure 9A–C. February–April 2021 maps of sampling sites and eDNA detections. White circles indicate negative detections while red circles indicate positive detections. Pond 19 is displayed in the upper right corner of each map.

In 2022, 348 eDNA samples were collected. Positive detections of RFS DNA were made for 42 samples (12%, Figure 10). Four (5%) positive detections occurred in January out of 79 samples, 16 (16%) positive detections occurred in February out of 98, six (13%) positive detections occurred in March out of 45, and 16 (13%) positive detections occurred in April out of 119. Positive detections were made at 40 sites and 12 ponds throughout all four months sampled for 2022.

A.


17
16
1
8
2
9
13
21
11
3
23
15
7
12
20
6
4
5
10
22
14
18
Pond border
Detection
Negative
Positive

B.


17
16
19
1
8
2
9
13
21
11
3
23
15
7
12
20
6
4
5
10
22
14
18
Detection
Negative
Positive

C.

D.

Figure 10A–D. January–April 2022 maps of sampling sites and eDNA detections. White circles indicate negative detections while red circles indicate positive detections. Pond 19 is displayed in the upper right corner of each map (except for in January when samples were not taken from pond 19).

Detection Rate Comparison across Four Ponds for Years 2018–2022

Across the five years of eDNA sample collection, ponds 1, 2, 6, and 8 were the only four ponds sampled every year for at least three continuous months (Table 3). RFS DNA was detected continuously in ponds 6 and 8 (Figure 11). Pond 1 only had detections in January 2018 and February 2019 then had zero detections in 2020, 2021, and 2022 (Figure 11). Pond 2 had detections from 2018–2021, 2022 did not have any positive eDNA detections within January, February, March, or April (Figure 11). The year 2022 had a significantly lower yearly detection rate (12%) than all other years, which is reflected in the percent detection rate of zero for pond 2 in 2022. Pond 6 had positive detections from 2018 to 2022 but had quite a bit fewer positive detections throughout 2022 than when compared to 2018, 2019, 2020, and 2021 (Figure 11). Pond 8 had positive detections throughout 2018–2022 (Figure 11). When summing the ponds altogether throughout all five years, most positive detections occurred in February and March (Figure 12). Ponds 2, 6, and 8 all had detections every year except for pond 2 in 2022.

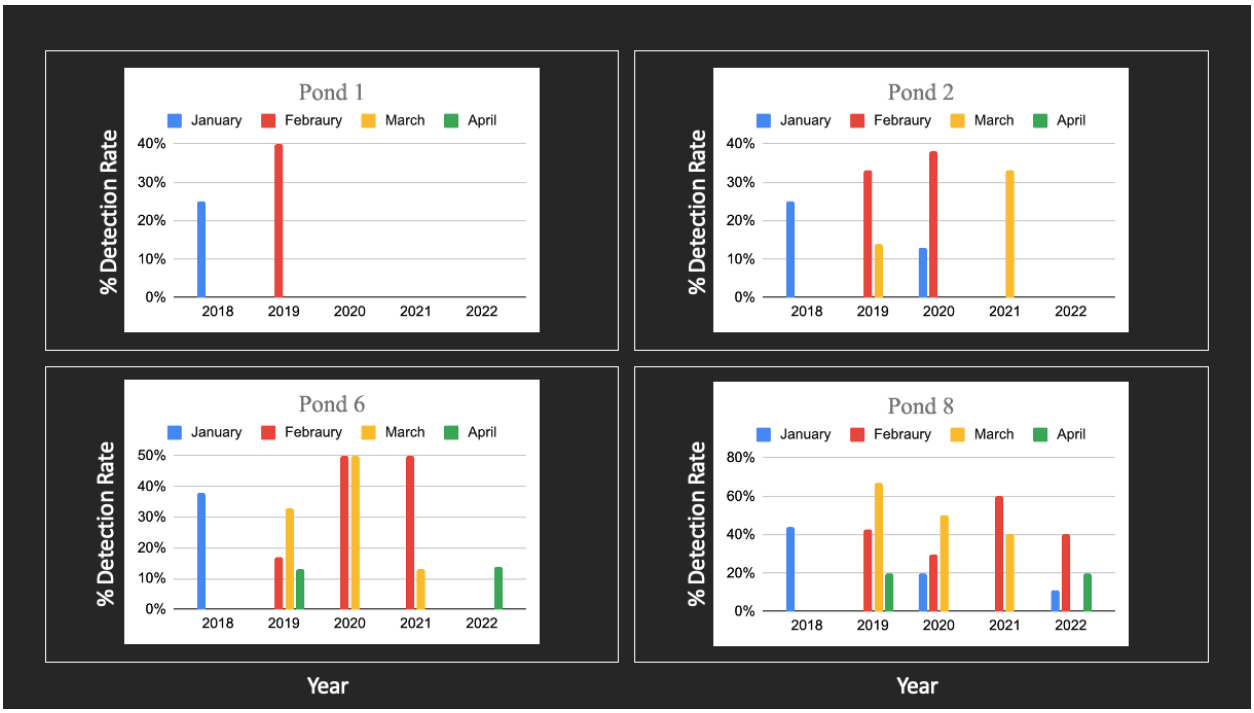


Figure 11. Graphs displaying the positive detection rate of RFS DNA for four ponds by month for years 2018–2022.

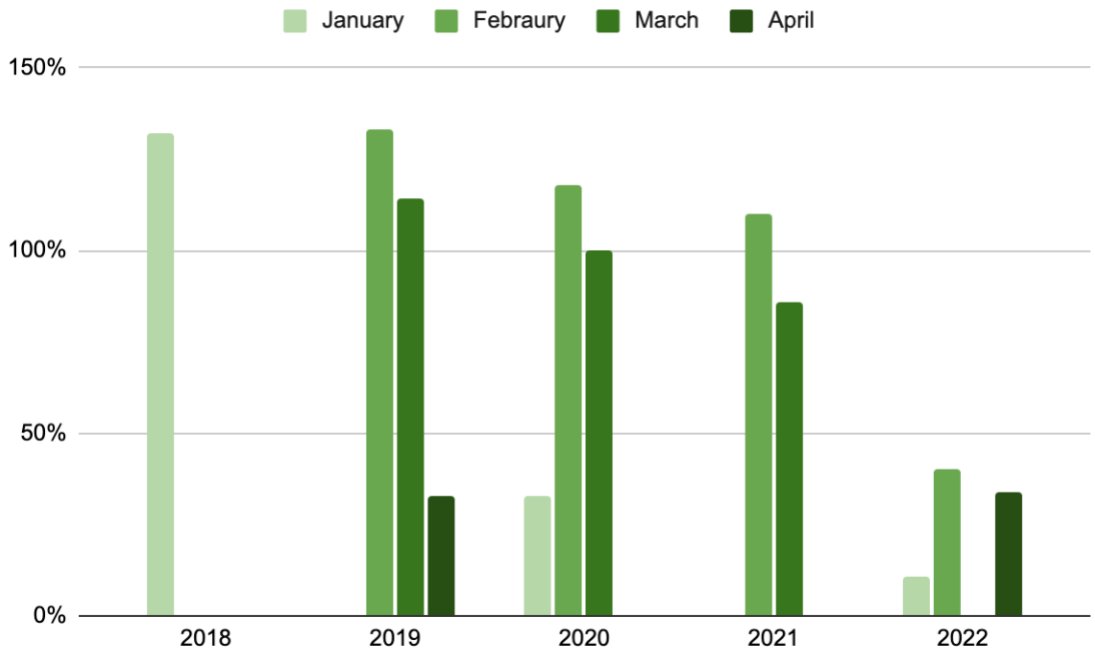


Figure 12. Graph displaying the sum of positive detection rates for RFS DNA in ponds 1, 2, 6, and 8 by month for years 2018–2022.

Table 3. Comparison of eDNA sampling efforts and positive detections of RFS DNA each year from 2018–2022 in four ponds, 1, 2, 6, and 8. Asterisk (*) indicates that a particular pond for that year was not included in the final analysis.

Pond	Sample	eDNA Positives	Positive Detection Rate	Year	Restoration Status
1	14	1	7%	2018	Restored*
1	17	2	12%	2019	Restored*
1	18	0	0%	2020	Restored
1	15	0	0%	2021	Restored
1	10	0	0%	2022	Restored
2	25	2	8%	2018	Restored*
2	23	3	13%	2019	Restored*
2	24	4	17%	2020	Restored
2	17	2	12%	2021	Restored
2	24	0	0%	2022	Restored
6	20	3	15%	2018	Unrestored*
6	23	4	17%	2019	Restored*
6	24	8	33%	2020	Restored
6	24	5	21%	2021	Restored
6	21	1	5%	2022	Restored
8	25	4	16%	2018	Unrestored*
8	27	8	30%	2019	Unrestored*
8	30	10	33%	2020	Unrestored
8	25	8	32%	2021	Unrestored
8	29	7	24%	2022	Restored

Detection Rates and Precipitation

During the months of October–April 2018 received 89.46 centimeters of rain, 2019 received 96.24 centimeters, 2020 received 77.32 centimeters, 2021 received 78.92 centimeters, and 2022 received 72.75 centimeters (NCEI). During the fall of 2019 leading up to the beginning of winter for 2020, rainfall was abundant, especially in December of 2019 with NOAA recording 25 centimeters of rainfall at the Eglin Air Force Weather Station (Figure 13). As rainfall decreases throughout late fall and early winter, detection rates spike in the beginning months of the year. 2018, 2020, and 2021 were all considered wet years, as their yearly precipitation rates were all above the average for a given year. 2019 and 2022 were therefore considered dry years since their averages were below the yearly average. With 2022 being an abnormally dry year, the overall yearly detection rate of 12% reflects the lack of rainfall. In Florida, the year 2022 was the 5th hottest year on record since 1895, 1.3 degrees Celsius above the average while 2021 was 1.2 degrees above average (Powell 2022, Powell 2023, NCEI). The year 2020 was 1.71 degrees Celsius above average, 2019 was 1.67 degrees above average, and 2018 was 1.16 degrees Celsius above the historical average of 21.17 degrees Celsius (Brouillette 2019, NCEI).

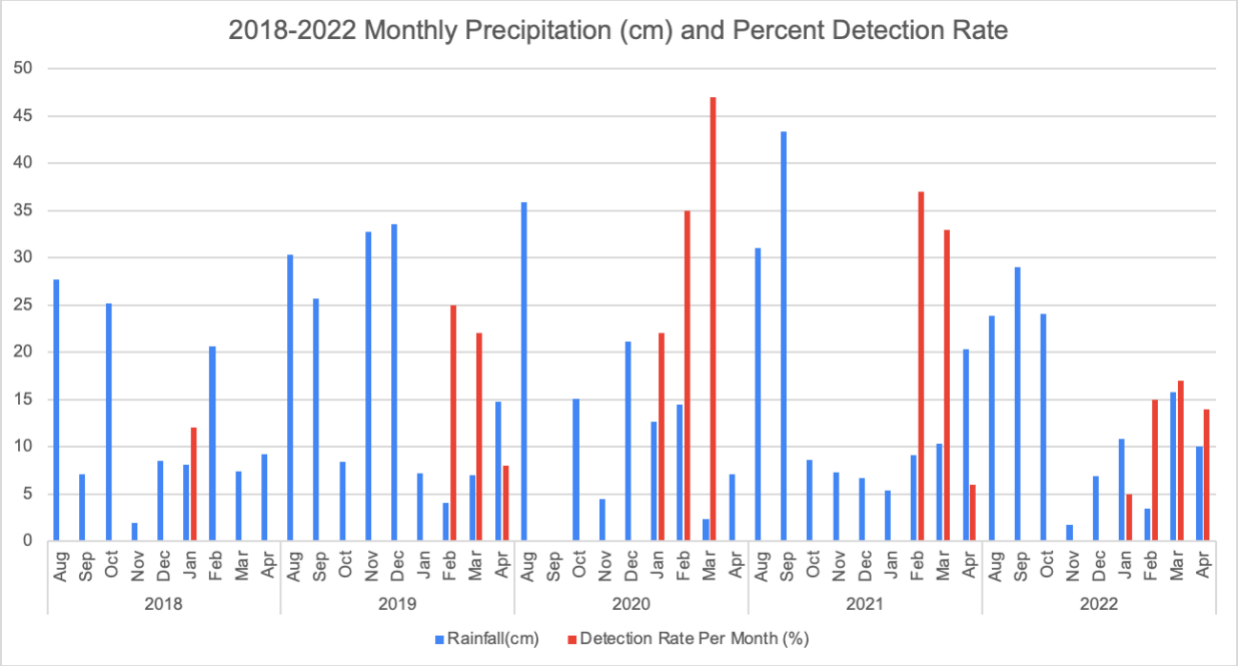


Figure 13. Monthly precipitation counts (cm) and eDNA detection rates of RFS DNA for each year sampled.

Detections Rates and Water Temperature

Water temperature was generally noticeably lower in January than other months. January 2018, February 2019, and February 2021 all had the highest detection rate per month for these years while also having the lowest water temperature per month for each year. March 2020 does not follow this pattern as March 2020 had the highest detection rate and the highest water temperature for that year. An average monthly water temperature was measured across the available ponds for a given year and graphed alongside the positive eDNA detection rate for that month (Figure 14).

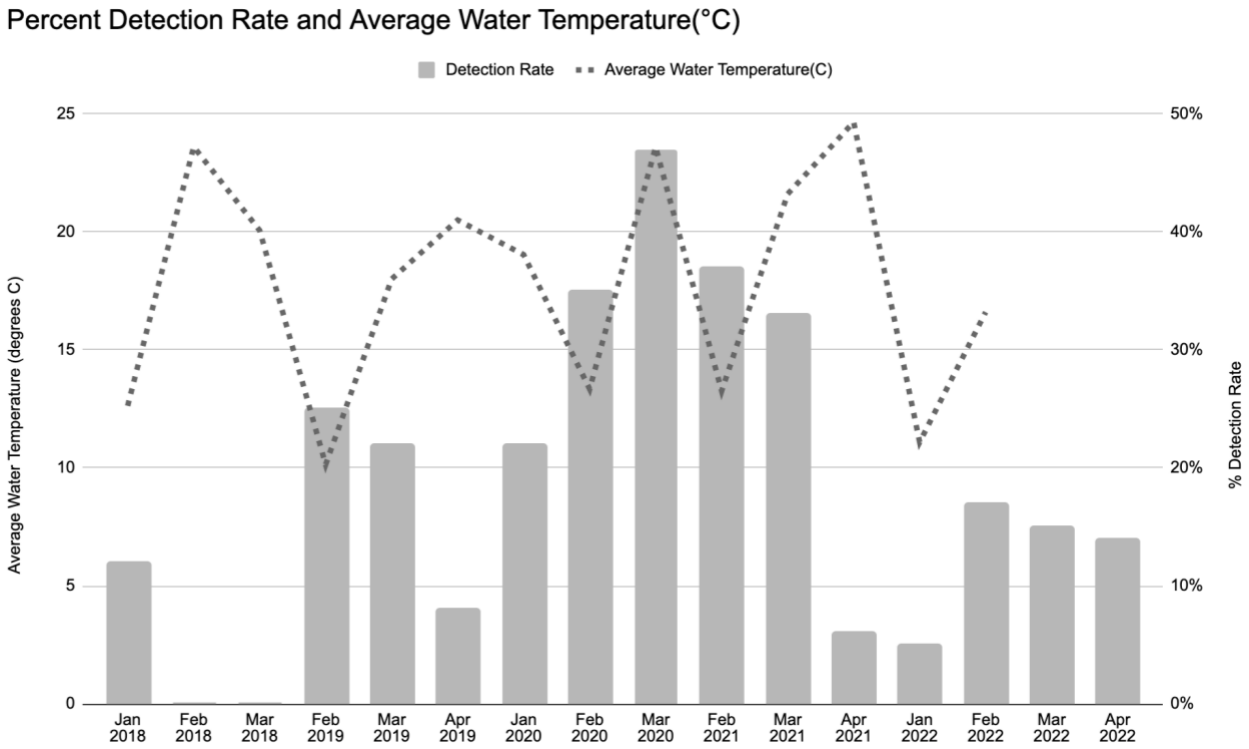


Figure 14. Graph displaying positive detection rate of RFS DNA in light gray bars and the average monthly water temperature in dotted gray line taken across years 2018–2022.

Detection Rates and All Water Quality Parameters

All raw water quality measurements are listed in supplementary tables at the end of this study (Tables 5–8). When a two-sample *t* test was performed on the 2018 data, DO, pH, SC, and depth (D) did not have any major influences on the eDNA detection rate. Water temperature of a pond for a given month in 2018 did have a significant pooled p-value ($p=0.0013$) (Table 4). When an ANOVA was performed for the 2019 detection rates and water quality parameters for each pond by month, significant p-values were only recovered for water temperature ($p<0.0000$) (Table 4). Detection rate, DO, Dosat, SC, pH, and depth were all not significant ($p>0.05$), meaning that these means did not differ from each other across months. When an ANOVA was performed on the 2020 data, significant p-values were recovered for pH ($p=0.0088$), water

temperature ($p<0.0000$), depth ($p=0.0062$), DO ($p<0.0000$), and Dosat ($p<0.0000$) (Table 4). Detection rate and SC were not considered significant ($p>0.05$), so the means did not differ across months for these factors. When an ANOVA was performed on the 2021 data, significant p-values were recovered for detection rate ($p=0.0062$), pH ($p<0.0000$), water temperature ($p<0.0000$), DO ($p=0.0001$), and Dosat ($p=0.0062$) (Table 4). Contrastingly, SC, depth, and salinity were not considered significant ($p>0.05$) for 2021. Fisher's Least Significant Difference (LSD) procedure was used to test for which water quality parameters had different means from each other by month. Most measurements were similar enough to each other except for water temperature, in which all three groups (February, March, and April) had means significantly different from one another. When a stepwise linear regression of detection rate was performed on the 2021 data, the only significant variables found were pH ($p=0.0033$) and water temperature ($p<0.0000$) (Table 4).

Table 4. Significance of certain water quality parameters across multiple statistical tests from 2018–2021.

Statistical Test	**Significant ($p<0.05$)**	**Not significant ($p>0.05$)**
2018 two sample *t* test	Water temp.	DO, Dosat, pH, SC, depth
2019 ANOVA	Water temp.	DR, DO, Dosat, pH, SC, depth
2020 ANOVA	Water temp., pH, DO, Dosat, depth	DR, SC
2021 ANOVA	DR, water temp., pH, DO, Dosat	SC, depth, salinity
2021 stepwise linear regression	Water temp., pH	DO, Dosat, SC, depth, salinity
2021 PCA	Water temp., pH	

Restored Versus Unrestored Ponds

Fisher's exact test was used to determine whether there was a correlation between restored or unrestored ponds and RFS detection rates. Without pond 12 included, 2020 had a p-value of 0.0003819, 2021 had a p-value of 0.2034, and 2022 had a p-value of 0.1081. When the years 2020–2022 were combined, there was a p-value of 0.01493. With these results, the p-values for the year 2020 (p=0.00038) and the combined three-year (p=0.01493) analysis were significant. The year 2020 had 57 positive detections in restored ponds while only four positive detections in unrestored ponds. Mosaic plots are displayed to visualize proportions between each category (Figure 15).

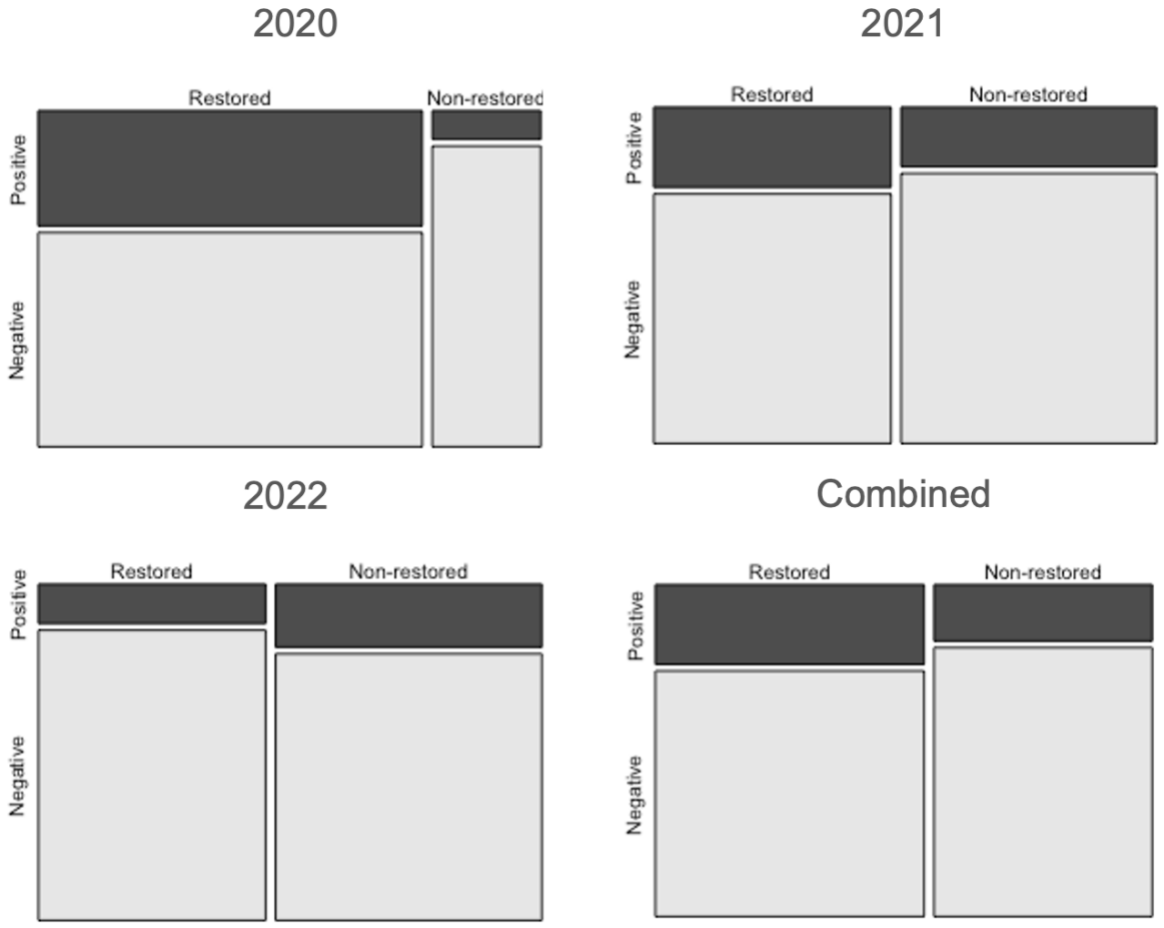


Figure 15. Mosaic plots displaying positive and negative percentage detection data for RFS DNA from restored versus unrestored ponds over three years (2020–2022) and a combined three-year analysis.

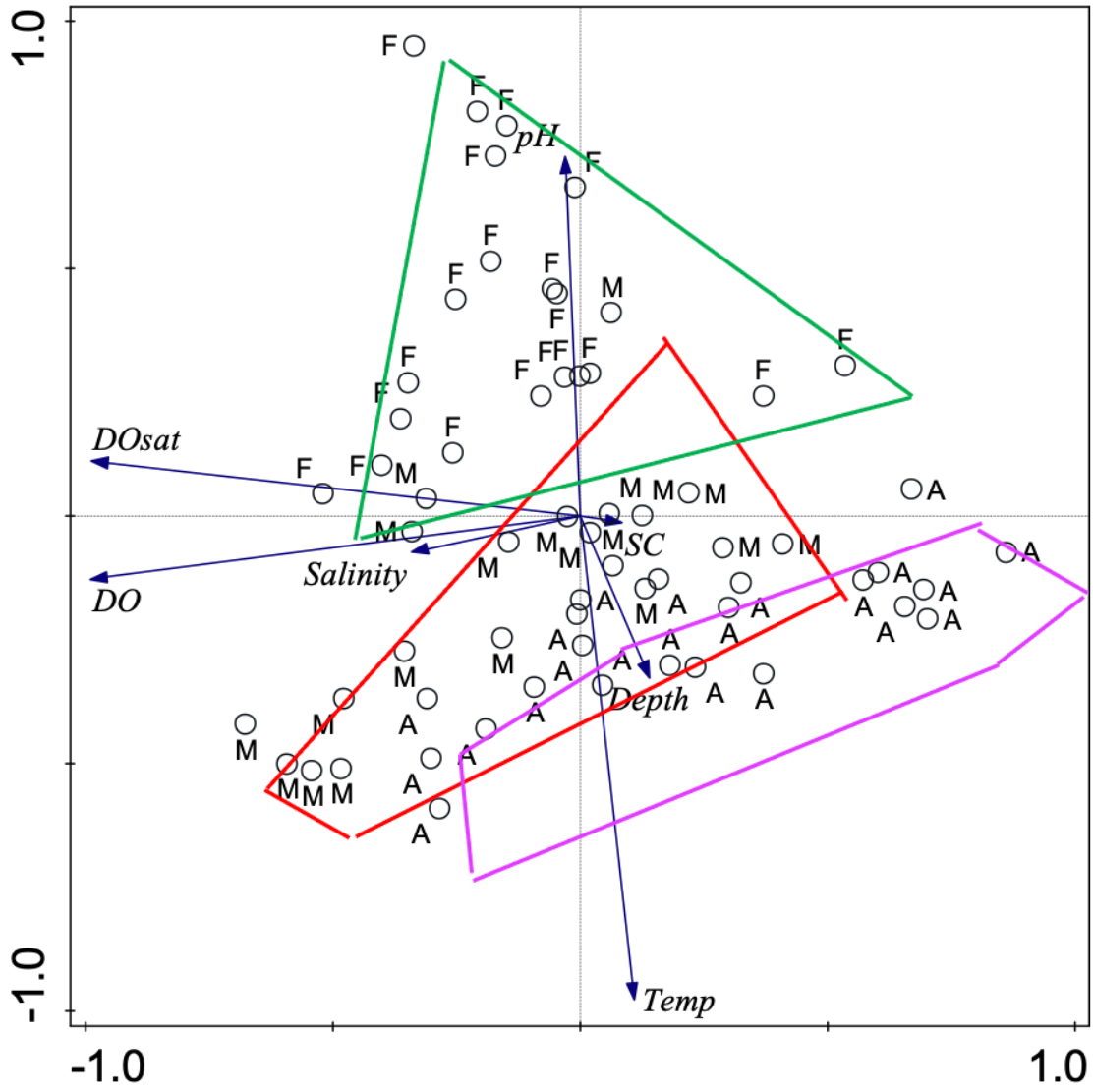
Principal Component Analysis by Year

Principal Component Analysis was performed for 2021 (Figure 16). In this PCA, there was a strong correlation between detection rate and axis 2 (p-value=0.0002). Nearly aligned with axis 2 are pH and water temperature. As the water temperature decreased, the detection rate increased. As pH increased, the detection rate for a given pond increased. In this particular PCA analysis, there is also a distinct seasonal pattern that occurred with little to no overlap between each month. February water quality measurements for each pond were grouped towards the upper half of the PCA, March water quality measurements were grouped together towards the middle, and April measurements were grouped towards the bottom. This indicates that water quality measurements for each month were very distinct from each other. For example, February 2021 detection rates correlated with higher pH levels and lower water temperatures, while April 2021 detection rates correlated with lower pH levels and higher water temperatures. March 2021 detection rates fall in between February and April. February 2021 had far more detections than April 2021.

The original model was:

$$DR = DO + Dosat + SC + pH + T + D + S$$

with DR representing detection rate by pond and month as a percentage, DO being dissolved oxygen (mg/L), Dosat being the dissolved oxygen saturation percentage, SC being the specific conductivity (uS), pH, T being water temperature of a given pond (degrees Celsius), D being the depth of the sensor (m), and S being salinity as a percentage (when available).



	DR	Case R1	Case R2
DR	1		
p-value	0		
Case R1	0.0525	1	
	0.6852	0	
Case R2	0.4624	0	1
	0.0002	1	0

Figure 16. Principal Component Analysis with axes scores including water quality measurements and 2021 RFS eDNA detection data. Inside green are February water quality measurements by pond, red are March water quality measurements by pond, and purple are April water quality measurements by pond.

Discussion

From 2018 to 2022 RFSs were consistently detected. Specifically, of the ponds that were sampled across all years, RFSs were continuously detected in ponds 6 and 8. Years that had higher precipitation rates (wet years) also had higher yearly eDNA RFS detection rates. Restored ponds were generally found to have higher detection rates than unrestored ponds. Except for one year, higher detection rates were found during the month with the lowest water temperature. These results indicate that water temperature and pH are the most influential factors explaining detection rates across months. Additionally, these results show the importance of certain abiotic factors on the detection rates of ponds.

As rainfall and air temperatures decline throughout late fall and early winter, detection rates spike in the beginning months of the year. Precipitation is the predominant factor known to trigger the reproductive process of RFS; specifically, adult RFS movement is correlated with rainfall, triggering adults to initiate breeding by traveling from their upland habitat to the pond basins (Palis 1997, Greenberg et al 2003, Westervelt et al 2013). The year 2020 had the highest yearly eDNA detection rate out of all five years sampled but did not have the most amount of rainfall according to the precipitation data gathered. The year 2018 had the same yearly detection rate of 12% as the year 2022, even though 2018 had the most amount of rainfall while 2022 had the least amount of rainfall. Therefore, our hypothesis that wet years would have higher yearly eDNA detection rates than dry years was not supported. Precipitation, although important, may not be the only factor initiating the reproductive process of RFS, as ambient air temperatures most likely also play a role (Semlitsch 1985, Palis 1997). The total amount of rainfall for each breeding season throughout each year may not be the best predictor for the duration of the hydroperiod on the landscape. In addition, more accurate rainfall data for each individual pond

and hourly precipitation rates would be very useful to further investigate the length of hydroperiod within each pond and the timing and depths at which the ponds fluctuate between. This could be accomplished by installing a well in every pond to track rainfall or by collecting on-site rainfall data.

When considering climate change, it is important to consider the direct effects rising temperatures will most likely cause to the hydrology within pond basins for salamander reproductive success and eDNA detection. Even slight decreases in the amount of precipitation that could occur in this area could cause population declines from the lack of water in pond basins and more dramatic reductions can cause population extirpation (Westervelt et al 2013). With temperatures rising, evaporation rates increase and therefore also play a role in reducing the amount of water in ponds. In the next few to several decades, the overall reproductive success of these amphibians is projected to decrease due to the effects of climate change on wetlands. The number of years these ephemeral ponds have suitable hydroperiods will be reduced (Chandler et al 2016). In contrast, heavy rainfall events may cause flooding which could introduce fishes and other predators to these normally ephemeral isolated ponds (Walls et al 2013).

The year 2022 had detection rates across ponds 1, 2, 6, and 8 all noticeably lower than years 2018–2021, which is likely due to the significantly reduced hydroperiod observed in these years. During March of 2022, amid the juvenile development period, there were 14 completely dry ponds. Even one of the larger ponds, pond 8, among others, had not been dry during the month of March in any other sampled year except 2022. Pond water levels and hydroperiods are affected by multiple factors including total annual precipitation, timing of precipitation, the water table, evaporation, and evapo-transpiration. If the amount of inundation occurs outside of the timeframe needed for larval development, and the hydroperiod is too short for development due

to fast evaporation and evapo-transpiration rates, or excessive flooding across the landscape introduces predatory fish, RFS larvae are unlikely to successfully be recruited into the Hurlburt Field metapopulation (Walls et al 2013).

It is important to note that both positive and negative eDNA detections are being affected by multiple physical parameters that ultimately degrade eDNA in these aquatic systems. Environmental DNA degradation will occur more quickly in ponds exposed to higher UV levels, which coincides with warming ambient air temperatures and warmer water temperatures due to climate change (Strickler et al 2015). Warmer water temperatures, as well as aquatic systems that are more neutral or acidic in nature, also degrade eDNA more quickly (Strickler et al 2015, Tsuji et al 2017). Therefore, my results indicating that the earlier months of the RFS reproductive cycle may have higher detection rates are likely because these are time periods where the ponds are generally experiencing less exposure to UV levels, with associated lower water temperatures, and are also less acidic.

When Fisher's exact test was run to test the difference between restored and unrestored ponds, the year 2020 and the combined 3-year analysis had significant p-values. These results indicate that there was a significant difference between restored and unrestored ponds regarding positive and negative detections. Restored ponds generally have more suitable habitat for RFS than unrestored ponds, with these results supporting our original hypothesis stated above. Restored ponds are more generally more suitable because they tend to have more herbaceous vegetation, though that vegetation recovery can occur slowly over extended years post-restoration.

Herbaceous vegetation growth post-restoration is greatly benefitted by implementation of prescribed fire. Anecdotally, I noticed that although none of the ponds I surveyed received

successful basin burns post-restoration, nearly all of them received successful uplands burn with at least some impact to the ecotonal vegetation around ponds. Using prescribed fire to manage this landscape is complex and requires intensive planning. For instance, when ponds are restored by having contractors come in and manually clear vegetation, there is a higher chance prescribed burns on the range will penetrate the pond basins making that particular pond more suitable for RFSs (Bishop and Haas 2005). Whereas, ponds that have not had any restoration work but are hit with prescribed burns may have lower chances of fire reaching the pond basins and therefore leaving more duff build-up (Bishop and Haas 2005). Timing also plays a factor when using prescribed burns to manage these ephemeral wetlands because if a prescribed fire takes place during a time when ponds are still filled with water, the fire will not penetrate the pond basins resulting in minimal or zero impacts to herbaceous vegetation or the duff layer within the pond basins (Jones et al 2018).

Detection rates across ponds 1, 2, 6, and 8 for the five years sampled all differ. Pond 1 was only detected in early 2018 and early 2019 which may indicate poor larval survival in this pond since there were detections early-on but no detections in the later months sampled. Pond 1 is smaller and somewhat isolated, as many of the other ponds are positioned closer to each other (Wendt et al 2021). The nearest known occupied area near pond 1 is pond 6, which is over 450 meters away, Wendt et al. (2021) found that dispersal was extremely limited between ponds over 400 meters away from each other. Pond 1 is also located very close to the easternmost edge of Hurlburt Field, making this location extremely difficult to be properly burned with a prescribed fire and possibly susceptible to edge effects. Edge effects are known to cause adverse microclimate conditions such as increased sunlight and temperatures, decreased humidity levels, and therefore decreased moisture levels among the forest floor and pond basins (Bunyan et al

2012, Hofmeister et al 2019). Pond 2 most likely did not have detections during 2022 because of the reduced amount of rainfall received that year which could have prevented the pond from filling with water sufficiently (NCEI). Other unexpected positive detections like one in pond 14 in April of 2021 may possibly be from a predator preying upon a salamander and tracking the salamander's DNA into a pond (Díaz-Ferguson and Moyer 2014, Jobe et al 2019). Another factor that could be causing positive detections in some areas could be due to the way water flows throughout the entire system, causing DNA to travel from one pond to another.

January 2018, February 2019, and February 2021 all had the highest detection rate per month for those years whilst also having the lowest water temperature per month for each year, except for March 2020. Because water temperature of a pond was negatively correlated with the eDNA detection rate, water temperature may influence when RFS lay eggs and/or more than likely provide an optimal range for salamander egg masses to better develop in (Albers and Prouty 1987, Grant et al 2014). Water temperature could also play a role in triggering larval salamanders to go through metamorphosis when the temperature increases past a certain point. Larvae may metamorphose sooner if water temperatures are higher, whereas when water temperatures are low, larvae may spend more time foraging in their aquatic habitats and metamorphose later at a larger size (Beachy 1995, Michimae 2011).

Our findings in the PCA indicate that RFS eggs and/or larvae have higher eDNA detection rates and therefore assumed higher chances of surviving and persisting, in ponds with lower water temperatures and neutral to low pH levels (Goldberg et al 2017). These findings, in part, support our original hypothesis that pH would be one of the most influential factors in explaining detection rates. DO was the other factor stated in our original hypothesis predicted to be significant, though DO was not considered significant in the PCA and therefore does not

support our original hypothesis. Although water temperature and pH were significant in the PCA, it is important to remember that survival rates in general decrease throughout the breeding season, with lower survival rates occurring later in the breeding season due to predation, health, environmental factors, and many other reasons. Throughout the statistical tests run on the 2018, 2019, 2020, and 2021 data, water temperature constantly varied across months. For 2021, water temperature was found to be a very significant indicator of detection rates, showcasing just how important water temperature can be for the early aquatic life stages of this species. With the stepwise linear regression run on the 2021 data, pH, alongside water temperature, was found to be a significant variable in explaining detection rates. Certain levels of pH can certainly adversely affect many salamander species like RFS throughout their early aquatic life stages (Barr and Babbitt 2002). More specifically, very low pH levels can cause embryos to abort immediately when exposed or can cause lower hatching success rates later at moderate pH levels (Freda 1986). Not only can acidic pond water cause direct mortalities, but it can also cause disruptions in the way amphibians interact with other aquatic organisms, therefore affecting trophic relationships (Freda 1986).

Although levels of salinity were not found to be significant, perhaps a reason for that may be a result of little fluctuation in salinity levels. It is important to note though that salinity levels can increase when hurricanes occur if sea water penetrates the freshwater ephemeral wetlands. This seawater can persist in the freshwater ephemeral ponds and affect the subsequent breeding season for RFS. Although, RFS's ability to tolerate fluctuating salinity levels is unclear, they are most likely more sensitive during their earlier life stages (Walls et al 2019). More information and research are needed to determine RFS's tolerability to varying salinity levels and how varying salinity levels may affect their survival throughout their major life stages.

Appropriate conditions of moisture, ambient and water temperature, pH, food resources, and refuge are all important microhabitat requirements for many species of amphibians (Gorman et al 2013). Minor habitat modifications, for example in the types of vegetation present, can easily disrupt any of these microhabitat requirements. Many amphibians, including RFSs, spend most of their lives in a terrestrial environment and migrate seasonally to a different, aquatic environment to breed. Since RFS has a complex life cycle that involves both different habitats and microhabitats, it is important to appropriately manage this landscape to further ensure the longevity of RFSs. Additional studies that incorporate more accurate precipitation data are needed to further investigate and quantify the influence of this particular factor in the reproductive success of the RFS.

Biodiversity loss is occurring at an alarming rate, faster than any other time throughout human history. The World Wildlife Fund (WWF) and the Zoological Society of London tracked over 5,230 species of animals for over 50 years, in which according to their report in 2022, an average of 69% of these species have experienced population declines between 1970 and 2018 (WWF 2022). Hundreds of amphibian species have gone extinct in the past 50 years; specifically, the IUCN reports 2,490 out of 7,296 (34%) amphibian species are threatened by extinction worldwide (The IUCN Red List of Threatened Species, version 2020–2).

There are several factors threatening amphibians with habitat loss having one of the largest impacts (Gallant et al 2007, Sodhi et al 2008). The major contributing factor to the decline of 63% of all known amphibian species and 87% of threatened amphibian species is some form of habitat loss (Hoffman et al 2008). It has become obvious that methods of accurately monitoring amphibians have become of urgent need. Amphibians can be elusive and difficult to track, especially when there are several fragmented populations or when population

numbers are low. As demonstrated in this study, the use of eDNA provides some resolution and insight into the aforementioned challenges.

Continued monitoring, conserving, and restoring of these vulnerable ephemeral wetlands, their respective water quality measurements, hydrological conditions, and population levels throughout the breeding season is imperative for RFS, and many other species. Multiple techniques to monitor these imperiled amphibians may be needed to have a more comprehensive picture of population dynamics of RFSs. Prescribed fire regimes are highly encouraged to continue, as Hurlburt Field has already established, to help maintain as natural conditions as possible. Diversifying fire-management strategies, by using prescribed fires during the summer months, may also benefit RFS by ensuring these breeding wetlands are properly burned and ultimately could positively contribute to this endangered species' recovery (Bishop and Haas 2005). Longer durations of hydroperiods, low water temperatures, and appropriate pH levels are necessary to ensure proper development of embryos and larvae as well as the continued longevity of this species. If hydroperiods decrease, which will more than likely be the case in the decades to come, other forms of habitat degradation will need to be mitigated. Therefore, maintenance of high-quality breeding ponds is of utmost importance to effectively conserve this species. Previous studies, as well as this study, have shown restored ponds are generally considered more suitable habitat for RFS reinforcing the importance of upland and wetlands management. This study demonstrates the utility and the need for proper management, restoration, and the use of environmental DNA in the detection of species such as the RFS.

Management Recommendations

Based on the results of previous studies as well as this study, there are certain times of the year and environmental conditions in which environmental DNA should be collected to optimize

the surveyor's time and the probability of collecting a positive eDNA water sample. Timing of when samples are collected is one of the most important factors for positive detections. Based on the results within this study, February is the best month throughout the breeding season to collect eDNA water samples with the goal of collecting a higher percentage of positive detections. If samples cannot be taken in February, March may be the next best month based on available results in this study. If possible, sampling more than once could help validate the previous sampling period's positive and negative results.

In particularly dry months or drought years, ponds 12 and 19 should be prioritized, assuming many other larger ponds like ponds 4, 5, and 8 are entirely dry. In years with sufficient rainfall and hydroperiods, ponds 3, 4, 5, 8, and 12 should be prioritized. Ponds 6, 17, and 19 may also be of interest and yield many positive results. Triplicates should be taken at each site to increase the likelihood of capturing RFS DNA. Depending on the goals management is trying to achieve, samples should be collected sometime in February; if another sampling round is warranted, samples should be collected at least 21 days after the first sampling round. This ensures that any DNA detected in the previous sampling period has degraded fully.

If there is a significant amount of water in the ponds and ponds have received large amounts of precipitation prolonging hydroperiods, pH levels will be lower, and samples should be collected in pH levels around 3.75–5. Although, additional studies are needed to determine whether the type of vegetation present in ponds or certain abiotic factors are more influential in explaining the presence of RFSs. If years that have received normal or less amounts of precipitation than the average, pH levels will drastically fluctuate but most likely be higher and therefore samples should be collected with pH levels ranging from 4 through 8. Water temperatures should be below 20 degrees Celsius at the time of sampling. At higher

temperatures, positive eDNA results are less likely to occur based on the results from this study. It is important to note that directly after a heavy rainfall event, ponds will be filled with more water, essentially diluting eDNA samples. Increased amount of water in the ponds will therefore decrease detection rates. To optimize detection rates after a heavy rainfall event, it may be advisable to wait around one week to give larval salamanders time to move around and slough off DNA in the ponds.

For pond 3, the northernmost and easternmost points are more likely to yield positive eDNA results than other sites throughout the pond, although all sites have given positive results. This is because those specific points have yielded positive results more often than the other points. For pond 4 many points are equally capable of yielding positive results except for the southernmost point in the pond. For pond 5, the northeastern two points and northwestern two points are equally capable of producing positive results more so than the southern points. Many, if not all, sites in pond 8 can yield positive results particularly the second sampling site down starting from the north end on the eastern edge of the pond often yields positive results. All the sampling points in pond 12 can yield positive results, although positive results may be more likely on the southern and eastern portions of the pond. The northernmost site in pond 12 is likely to not yield any positive results. Lastly, it is important to note that DNA is often moving throughout the entire pond, being transported by other animals as well as the weather such as wind and rain so the points mentioned in this paragraph may not always hold true.

Supplementary Tables

Table 5. 2018 Detection rates and water quality measurements by month and pond.

Pond	Month	Detection Rate (%)	DO (mg/L)	Dosat (%)	SC (uS)	pH	Water Temp (°C)	Depth (m)
2	January	25	6.37	54.8	0.044	4.61	8.41	0.25
8	January	25	2.9	25.5	0.083	4.16	10.19	0.25
6	January	38	8.47	89.7	0.057	4.53	17.63	0.25
1	January	44	9.56	93.1	0.048	5.98	14.29	0.25
2	February	0	4.21	47.5	0.026	5.39	21.21	0.33
8	February	0	3.51	38.3	0.051	4.35	20.48	0.33
6	February	0	4.33	54.4	0.041	4.7	27.62	0.33
1	February	0	4.86	57.2	0.041	6.25	25.09	0.33
2	March	0	3.85	41.7	0.039	4.44	19.17	0.33
8	March	0	5.48	59.1	0.051	4.21	18.86	0.33
6	March	0	4.05	45.3	0.064	4.45	20.75	0.25
1	March	0	8.28	93.8	0.054	5.78	21.47	0.25

Table 6. 2019 Detection rates and water quality measurements by month and pond.

Pond	Month	Detection Rate (%)	DO (mg/L)	Dosat (%)	SC (uS)	pH	Water Temp (°C)	Depth (m)
1	February	13	12.54	31	0.046	6.36	10.59	0.33
2	February	10	6.25	58.8	0.036	6.22	9.81	0.33
6	February	5	8.47	80.4	0.055	4.3	11.58	0.33
8	February	17	5.73	51.3	0.073	4.39	8.56	0.33
1	March	0	5.43	61.4	0.067	5.68	18.64	0.25
2	March	5	2.93	32.8	0.042	4.62	18.12	0.33
6	March	10	4.31	48.6	0.055	4.32	18.5	0.25
8	March	22	2.72	29.5	0.072	4.1	16.75	0.25
1	April	0	3.45	40.8	0.045	6	20.77	0.25

Table 6. 2019 Detection rates and water quality measurements by month and pond (continued).

Pond	Month	Detection Rate (%)	DO (mg/L)	Dosat (%)	SC (uS)	pH	Water Temp (°C)	Depth (m)
2	April	0	2.95	32.7	0.037	4.73	19.64	0.33
6	April	5	4.16	52.5	0.057	4.34	23.94	0.25
8	April	6	3.67	40.6	0.084	4.15	17.74	0.25

Table 7. 2020 Detection rates and water quality measurements by month and pond.

Pond	Month	Detection Rate (%)	DO (mg/L)	DOsat (%)	SC (uS)	pH	Water Temp (°C)	Depth (m)
1	January	0	2.56	21.1	0.068	5.34	20.66	0.14
13	January	0	2.38	27.8	0.087	4.78	20.89	0.134
9	January	0	1.22	14.4	0.052	4.33	20.16	0.17
15	January	0	1.38	16.3	0.047	4.2	20.74	0.19
7	January	0	2.75	33.6	0.088	3.96	21.67	0.119
6	January	0	3.24	46.5	0.077	3.99	24.2	0.1
10	January	0	4.38	53.7	0.067	4.11	23.58	0.09
2	January	13	3.12	40	0.055	4.48	25.17	0.125
8	January	20	2.97	30.9	0.054	3.8	15.31	0.19
14	January	0	3.46	38	0.071	3.93	15.54	0.095
5	January	57	2.33	25.4	0.068	3.98	16.23	0.113
4	January	57	3.95	42.6	0.048	4.08	17.57	0.093
3	January	60	4.62	52.2	0.057	4.5	18.87	0.125
11	January	0	5.1	56.7	0.063	4.03	19.08	0.08
12	January	31	5.92	50.2	0.053	4.26	5.83	0.13
7	February	0	3.97	40.5	0.68	4.3	13.86	0.191
13	February	0	4.6	48.8	0.048	4.4	15.56	0.189
6	February	50	3.75	41.6	0.066	4.25	17.58	0.138
9	February	0	4.02	39.6	0.042	4.62	15.05	0.139

Table 7. 2020 Detection rates and water quality measurements by month and pond (continued).

Pond	Month	Detection Rate (%)	DO (mg/L)	DOsat (%)	SC (uS)	pH	Water Temp (°C)	Depth (m)
10	February	17	4.16	47.3	0.072	4.33	18.83	0.122
16	February	0	3.78	41.4	0.037	5	16.45	0.146
8	February	30	4.51	45.2	0.057	4.32	13.01	0.197
14	February	0	3.68	37.3	0.059	4.57	13.85	0.125
5	February	43	2.66	27.1	0.06	4.79	13.93	0.098
3	February	0	2.73	28.5	0.057	4.86	14.83	0.128
1	February	0	3.62	37.8	0.09	6.04	14.98	0.203
2	February	38	3.65	38.1	0.053	4.66	15.62	0.136
4	February	88	4.44	41.1	0.47	4.45	14.91	0.126
12	February	77	3.85	38.2	0.049	4.66	14.98	0.152
12	March	54	1.69	22.4	0.054	4.94	23.55	0.07
3	March	60	1.17	13.8	0.08	4.73	21.29	0.1
6	March	50	0.91	12	0.092	4.48	26.45	0.1
16	March	0	1.11	12.6	0.0043	4.95	23.27	0.13
10	March	50	1.17	19.1	0.058	4.62	22.7	0.165
13	March	0	0.99	11.8	0.048	4.88	23.17	0.055
7	March	0	1.03	13	0.077	4.3	25.96	NA
1	March	0	1.22	17	0.19	6.46	28.04	0.125
4	March	86	1.15	13.5	0.04	4.46	22.14	0.09
5	March	100	0.91	11	0.074	4.73	22.66	0.07
8	March	50	0.98	11.1	0.04	4.59	22.85	0.12
2	March	0	1.15	13.6	0.068	4.64	20.99	0.115

Table 8. 2021 Detection rates and water quality measurements by month and pond.

Pond	Month	Detection Rate (%)	DO (mg/L)	DOsat (%)	SC (uS)	pH	Water Temp (°C)	Depth (cm)	Salinity (%)
6	February	50	6.86	59.1	0.106	7.67	7.21	8.8	0.005
10	February	17	5.33	46.8	0.080	7.66	8.58	9.8	0.040
20	February	0	4.41	44.2	0.077	7.80	12.25	9.8	0.040
16	February	0	6.54	66.9	0.059	8.30	13.63	12.8	0.030
1	February	0	7.82	87.1	0.154	8.09	18.17	9.2	0.070
13	February	0	6.48	74.3	0.077	7.35	18.65	6.4	0.040
7	February	0	5.49	60.7	0.098	7.31	17.57	8.0	0.050
4	February	29	3.80	40.9	0.077	7.51	15.45	7.9	0.040
5	February	67	1.92	19.4	0.097	7.47	14.10	14.5	0.050
3	February	67	3.85	42.9	0.079	7.48	14.93	9.0	0.040
2	February	0	6.51	68.1	0.074	7.32	16.31	7.9	0.040
17	February	63	2.42	24.9	0.051	7.39	15.19	12.6	0.030
8	February	60	4.55	41.8	0.073	7.41	14.23	12.0	0.040
22	February	100	5.63	51.0	0.082	7.81	8.54	8.5	0.040
18	February	40	4.37	40.3	0.093	7.56	8.82	15.2	0.040
12	February	69	5.37	49.5	0.075	7.75	8.73	11.4	0.040
11	February	0	0.00	0.0	0.000	0.00	0.00	3.2	0.000
21	February	0	5.82	57.6	0.086	7.67	11.61	12.0	0.040
15	February	0	5.27	51.0	0.063	7.63	12.16	7.6	0.030
9	February	0	4.40	47.0	0.070	7.38	15.72	8.5	0.040
19	February	63	4.28	43.3	0.054	7.61	13.42	7.0	0.030
19	March	44	3.65	37.3	0.058	6.87	14.37	6.3	0.030
11	March	0	0.00	0.0	0.000	0.00	0.00	0.0	0.000
2	March	33	3.89	46.4	0.069	6.86	18.65	11.2	0.040

Table 8. 2021 Detection rates and water quality measurements by month and pond (continued).

Pond	Month	Detection Rate (%)	DO (mg/L)	DOsat (%)	SC (uS)	pH	Water Temp (°C)	Depth (cm)	Salinity (%)
3	March	100	3.70	40.2	0.070	7.07	17.55	15.5	0.040
12	March	62	5.89	66.2	0.057	5.90	18.18	9.0	0.040
4	March	43	5.95	74.4	0.071	6.44	24.54	9.5	0.040
5	March	80	3.23	37.5	0.077	6.96	17.89	14.4	0.040
18	March	40	3.44	41.1	0.076	6.79	20.80	11.5	0.040
14	March	0	3.71	43.3	0.068	6.98	19.39	11.5	0.040
17	March	0	3.26	37.7	0.054	7.00	19.60	18.7	0.030
22	March	0	0.00	0.0	0.000	0.00	0.00	0.0	0.000
8	March	40	4.60	54.3	0.074	6.78	20.02	9.9	0.040
16	March	0	7.16	89.8	0.058	6.90	22.39	19.8	0.030
21	March	0	1.82	26.7	0.093	6.81	21.48	9.2	0.050
13	March	0	7.18	100.7	0.065	6.22	30.50	9.2	0.040
15	March	0	0.00	0.0	0.000	0.00	0.00	0.0	0.000
9	March	0	6.17	69.3	0.077	7.12	19.12	10.5	0.040
7	March	0	7.82	107.0	0.088	6.30	28.80	11.0	0.050
23	March	0	2.90	31.7	0.071	7.14	19.03	10.3	0.040
6	March	13	8.81	117.4	0.096	6.47	27.14	9.9	0.050
10	March	83	2.52	30.0	0.073	6.98	20.13	12.8	0.040
20	March	0	4.55	57.2	0.068	6.71	23.90	10.5	0.040
1	March	0	6.80	93.3	0.149	7.18	28.60	13.1	0.070
16	April	0	3.60	48.1	0.053	7.14	18.35	23.0	0.030
19	April	22	2.86	36.3	0.049	6.84	24.74	6.5	0.030
3	April	0	1.38	16.6	0.058	7.11	21.29	8.0	0.030
18	April	40	2.53	29.4	0.560	6.83	23.27	12.0	0.030
14	April	33	1.44	17.6	0.052	6.95	23.54	14.0	0.030

Table 8. 2021 Detection rates and water quality measurements by month and pond (continued).

Pond	Month	Detection Rate (%)	DO (mg/L)	DOsat (%)	SC (uS)	pH	Water Temp (°C)	Depth (cm)	Salinity (%)
5	April	0	3.69	46.3	0.053	6.88	23.50	10.0	0.030
4	April	0	2.81	49.2	0.052	6.66	25.53	11.0	0.030
22	April	0	2.81	36.6	0.066	6.86	24.52	14.0	0.040
11	April	0	1.42	21.1	0.430	6.90	23.32	10.0	0.030
12	April	15	4.18	53.3	0.044	6.83	24.88	14.0	0.030
2	April	0	2.11	28.1	0.046	6.97	25.65	14.0	0.030
17	April	0	3.73	46.0	0.055	7.05	23.03	14.5	0.030
1	April	0	5.40	72.2	0.079	7.37	27.42	9.5	0.040
8	April	0	2.24	29.0	0.070	6.89	23.87	8.5	0.040
21	April	0	1.60	18.4	0.074	6.76	24.36	9.0	0.040
23	April	0	2.68	33.1	0.059	6.76	26.54	11.0	0.003
7	April	0	4.71	61.4	0.058	6.54	27.66	12.0	0.030
9	April	0	1.53	15.5	0.039	6.99	23.89	13.0	0.020
15	April	0	0.00	0.0	0.000	0.00	0.00	0.0	0.000
13	April	0	5.05	73.1	0.051	6.27	32.06	11.5	0.030
6	April	0	5.55	72.0	0.063	6.87	27.23	16.0	0.040
10	April	0	1.33	16.6	0.059	7.03	23.00	17.5	0.030
20	April	0	1.03	12.3	0.056	6.92	24.12	9.5	0.030

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