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Environmental DNA Monitoring of Non-Native Mudpuppy (Necturus Maculosus) and Transient Rainbow Smelt (Osmerus Mordax)

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ENVIRONMENTAL DNA MONITORING OF NON-NATIVE MUDPUPPY (*NECTURUS MACULOSUS*) AND TRANSIENT RAINBOW SMELT (*OSMERUS MORDAX*)

By

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Thesis Advisor: Dr. Michael Kinnison

An Abstract of the Thesis Presented
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Whether considering an expanding non-native species or a priority native species with a dwindling local population, the monitoring of low-abundance, sporadically distributed, or otherwise elusive populations, can prove difficult. In separate studies, we tested the viability of environmental DNA (eDNA) for monitoring a species in both of the above circumstances, the common mudpuppy (*Necturus maculosus*), a spreading non-native species, and rainbow smelt (*Osmerus mordax*), a declining species of concern. Mudpuppy are fully aquatic salamanders that were introduced to the Belgrade region of central Maine in 1939 and again in 1940. Though they had been present for nearly 80 years when this study began, their ecological impacts and secondary spread have not been well documented. Following a year of trapping through the winter ice, eDNA methods were added concurrently with traditional trapping techniques to demine if detection could be improved in order to better document secondary spread and estimate abundance. Overall, eDNA was helpful in this effort as mudpuppy were detected in all but one waterbody where they were trapped and in two where they were not. Occupancy models were used to estimate survey power and sampling efforts for 95% probability of detection based on our data. Trapping and eDNA showed comparable power at the level of lake regions and

number of sampling holes. However, when looking at the level of technical replicates, trap data required 6.4 replicates (trapping events) while eDNA required 10.9 (qPCR replicates). However, the amount of work and expense to obtain qPCR replicates is likely less than to implement additional days of trapping. Trap and eDNA sampling depth data were also used to gain preliminary insight on environmental preferences. Kologorov Smirnoff tests comparing overall depth distribution and individual mudpuppy caught at a given trap site did not reveal an observable trend in depth preferences. T-tests revealed a modest preference for 4-8m depths, but this was likely due to depths available in study sites as opposed to true biological preference. Overall, the combined results of trapping and eDNA sampling both suggest that the mudpuppy invasion has been relatively gradual, and provided baseline occupancy information for potential future assessments of range expansion.

In the second study of this thesis, we assessed eDNA as a means to monitor anadromous rainbow smelt (*Osmerus mordax*), a species of special conservation concern in Maine. As anadromous fish, rainbow smelt migrate up streams and rivers to spawn during the early spring period when typical nighttime visual surveys can be difficult or even dangerous. As such, the current use of many coastal streams for spawning is poorly known. We hypothesized that eDNA might facilitate improved survey efforts to define smelt spawning habitat. However, the lotic environments and behavior of smelt present potential challenges for eDNA. Rainbow smelt often enter smaller streams at night and depart by morning, such that fish eDNA might be flushed out of the system relatively quickly. By combining daytime eDNA sampling with fyke netting, we confirmed that smelt eDNA could be detected up to weeks following peak spawning events. Indeed, there was some evidence that concentration of eDNA (copies/L) rose over the approximately 8-13 days following spawning events, suggesting developing and hatching smelt

larvae might be the primary source of residual eDNA. Adding to this study, we conducted eDNA surveys in four streams of varying smelt abundance and estimated sampling effort for 95% detection probability using occupancy modelling. Ultimately, results suggested that at the stream with least detections, sampling effort involving collection of three water samples, collected on three days, and analyzed with six qPCR replicates would provide ≥ 95% detection probability. Comparing those recommendations to the sampling design used in this study, the number of qPCR replicates used was the only sampling value below our generated recommendations. These results demonstrate that eDNA methods can be effective for monitoring smelt in lotic systems during their breeding period, particularly with a modest increase in sample processing effort to increase detection probabilities.

DEDICATION

I dedicate this work to my parents and grandparents who have been sources of endless support through my life and academic career.

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CHAPTER 1

ENVIRONMENTAL DNA SETTING THE STAGE: THE IMPORTANCE OF CONTEXT

The overarching theme of this thesis is the use of environmental DNA (eDNA) to detect species that are difficult to observe or capture. Environmental DNA is any DNA leftover in the environment from an organism (e.g. sloughed off epithelium, fecal matter, mucus layer) and from which the presence of a species can be inferred. In recent years, this method of "sight unseen" detection (Jerde et al., 2011) has steadily increased both in overall use and in potential applications. To date, eDNA has been extracted from water samples (e.g. Thomsen et al., 2015), sediment samples (e.g., Turner, Uy, and Everhart 2015), and more recently, from air itself (e.g., Clare et al., 2021). The increasing appeal of eDNA methodologies is largely due to the potential to detect very rare species, as well as the potential to reduce costs relative to traditional survey methods. These benefits are demonstrated widely throughout the literature, but eDNA detection is not equal for all species, and as a relatively new methodology, eDNA still presents challenges that may limit its efficacy. These challenges include, but are not limited to, the risk of false positive or negative results and the need to adapt eDNA approaches to very different organisms and habitats.

False positive and false negative detections are common concerns for any eDNA monitoring. These false detections may occur due to a primer and probe set that was not specific to a target species or from contamination either in the collection process or in the lab. This can be a very significant concern in the use of eDNA as the method is often applied to cases where any detection is consequential, such as determining the range of invasive or threatened species. Trusting eDNA detections without visual confirmation is almost an inherent part of the

methodology in many applications. False negatives tend to happen where eDNA assays or sampling methods are poorly targeted to the focal species, lack sufficient power due to sampling design, or where PCR inhibition from environmental compounds (tannins commonly found in lakes or streams) masks detection of eDNA that is actually present in a sample. Guidelines for developing sensitive assays and overcoming PCR inhibition are now widespread in the eDNA field (Bigs et al. 2015; Turner et al., 2015; Hunter et al., 2019) and universal to most organisms. By contrast, issues surrounding adequate sampling methods and survey designs are much more unique to particular organisms and habitats. The rate at which different taxa shed eDNA, where and when they shed eDNA relative to their behaviors and life cycle, and the prevailing environmental conditions acting on that eDNA (e.g., dilution, degradation, deposition), all play a likely large role in relative species detectability (Barnes et al., 2014; Turner, Uy, & Everhart, 2015; Troth et al., 2021). However, these taxonomic and habitat challenges are not insurmountable, rather they are context-dependent limitations that can often be specified and overcome through power analyses and refinement of survey effort (Wilcox et al., 2016; Wilson et al., 2016; Stoeckle et al., 2017; Hunter et al., 2019; Jerde, 2021).

To put some of the context factors into relevance for the current thesis, consider the sampling tradeoffs inherent to detecting organisms inhabiting lentic (lake) versus lotic (stream) systems. In lentic systems, it is relatively easy to collect eDNA samples with less concern for timing as eDNA will remain suspended in the water column for upwards of weeks as long as the species makes adequate use of the water column when shedding eDNA. In contrast, in lotic systems, running water can quickly flush eDNA from the system within hours or days, which may limit eDNA use for some highly transient species. Conversely, where one samples may be less challenging in some lotic systems than in lentic systems, because eDNA can be transported

large distances (kms) downstream, whereas the water in deeper areas of lakes in seasons such as winter may experience little mixing or transport making the location and number of sampling sites very important. Although it might seem counterintuitive, this thesis addresses the more difficult set of these eDNA sampling contexts: a transient fish in small streams, and winter deep water sampling for an invasive salamander. The reasoning being, if eDNA can work well in these challenging contexts, it can likely work well in many others.

Chapter 2 applied eDNA in lentic systems in order to detect the common mudpuppy (*Necturus maculosus*). This is a fully aquatic salamander species that was introduced to the Belgrade region of central Maine in 1939. Since initial introduction, there have been reports of mudpuppy in several additional waterbodies throughout Maine. Their ecological impact to this point is largely unknown, but their relatively large size (up to 40cm) and broad-carnivorous diet are potentially problematic for a number of co-occurring species of conservation concern. In response, the Maine Department of Inland Fisheries and Wildlife (MDIFW) began assessing relative abundance and secondary spread throughout the state in 2017, utilizing standard minnow traps and minnow traps modified to increase diameter. Trapping was conducted through the ice during winter. Preliminary trapping results from 2017 indicated that mudpuppy had spread beyond their initial watershed, but did not confirm positives at several lakes likely to contain mudpuppy based on proximity to the introduction site. This suggested high potential for false negatives in the initial results, making these waterbodies a priority for future study. In 2018 and 2019 we began working with the MDIFW to assess secondary spread of mudpuppy by incorporating an eDNA study along with trapping surveys. Environmental DNA was considered potentially beneficial due to the typically cryptic nature of the species and the high effort required for winter ice trapping. As eDNA is potentially suspended in the water column of lentic

systems for long periods of time, it was likely that eDNA might be detected from water samples taken well after the local occurrence of individuals at specific survey sites. The previous trap data allowed for added context in the study in that we could be more selective in our selection of survey sites. This allowed for greater certainty in our results by accounting for potential eDNA false negatives using trap data. Utilizing the two methods together also permitted an assessment of optimal sampling effort required for mudpuppy surveys conducted with either eDNA or trapping.

In Chapter 3 of this thesis, we assessed the potential to employ environmental DNA to determine if and when anadromous rainbow smelt (*Osmerus mordax*) use coastal streams for spawning. As previously mentioned, lotic systems present a challenge as the time eDNA spends in the system is greatly reduced. In this particular study, we targeted rainbow smelt breeding streams as 47% of 279 potential spawning locations were listed as unknown by Maine Department of Marine Resources in 2012 (Enterline et al., 2012). Adding to the challenge of the lotic system itself, rainbow smelt spawn in early spring, during high water conditions, and are nocturnal breeders that generally exit small streams by day. These streams are difficult and potentially dangerous to visually survey for a potentially small number of fish or eggs. Hence, there was interest in whether smelt eDNA might provide an alternative. This required an initial eDNA study pairing eDNA with fyke net surveys in order to ascertain the sampling window for detecting known smelt spawning events. This work suggested that smelt eDNA could be detected for multiple weeks following smelt spawning, likely as a byproduct of egg and larval development. A follow up study took place in four streams, two of which were known to have a high smelt run and two of which were low or uncertain in smelt abundance. These sites were sampled throughout the window of potential smelt spawning to conduct a hierarchical occupancy

model assessment of survey power and to provide survey design recommendations for future smelt surveys.

Together the next two chapters demonstrate the efficacy of eDNA for detecting challenging organisms in difficult environments. At the same time, we demonstrate the importance of temporal and environmental context in designing eDNA surveys.

CHAPTER 2

MONITORING OF NON-NATIVE MUDPUPPY IN MAINE: EDNA AND TRADITIONAL METHODOLOGIES

Introduction

Environmental DNA (eDNA) has become a widespread method for the detection of many aquatic and terrestrial taxa (Jerde et al., 2011; Thomsen et al.,2012; Takahara et al., 2013; Wilcox et al., 2013; Laramie et al., 2015; Sigsgaard et al., 2015). Although the method has purported benefits for detection and quantification of many species, it has a particularly powerful role to play in the monitoring of species that are otherwise difficult to document due to their cryptic habitat use (Sigsgaard et al., 2015; Sakai et al., 2019), low abundances (Thomsen et al.,2012; Gasparini et al., 2020, Dougherty 2016), and lack of familiarity by the general public. This includes many non-native species that may substantially colonize new regions before being widely documented and before their ecological impacts are recognized (Takahara et al., 2013, Dougherty et al., 2016, Thomas et al., 2020). Invasive aquatic species are considered one of the most significant threats to indigenous aquatic species (Strayer, 2010; Havel, J.E., et al., 2015), which themselves often face a lack of sufficient monitoring. However, while eDNA shows much promise for monitoring the colonization and range expansion of low abundance and cryptic invasive species, detection and quantification capacity vary widely for different taxa, habitats, and seasons, making some eDNA surveys prone to uncertainty, especially for interpreting negative samples that may or may not reflect true absence from a site. Pairing eDNA with other traditional sampling methods, such as netting, electrofishing, angling, or visual surveys, can improve our ability to evaluate where and when it may be more or less effective for monitoring purposes. Here we compare the utility of winter eDNA and trapping to document the range of a

cryptic, non-native amphibian in Maine (USA) lakes, the common mudpuppy (*Necturus maculosus*).

The common mudpuppy is a North American, neotenic salamander that lives its entire life in lakes, streams or rivers. While their precise native range is subject to debate, mudpuppy naturally originate from along the Mississippi and Ohio River drainages, and as far northeast as Lake Champlain (Conant and Collins, 1991). Mudpuppy are not native to Maine, likely due to the region's history of glaciation and drainage isolation. However, due to its use in zoological research and teaching, the species was accidentally introduced in the Belgrade Lakes region of Maine in 1939 via escapes from holding pens owned by a Colby College professor (Crocker 1960). As a non-native species, mudpuppy have several characteristics that make them a potentially problematic invader. First, they are tolerant of a wide range of environmental conditions, including the harsh winter conditions characteristic of Maine. Second, mudpuppy are large- and long-lived, with a potential lifespan of over 30 years (Matson 2005). They can grow to lengths of 41 cm. and have broad-carnivorous dietary habits (Chellman et al 2017; Cathy Bevier, unpub. data). Their size allows them to escape predation by many aquatic predators, and to consume diverse food items, such as aquatic insects, crustaceans, mollusks, small fish, fish eggs, and even other amphibians, that are often not consumed by native amphibians (Bishop 1941; Crocker 1960; Gibbs et al., 2007). Maine's State Wildlife Action Plan 2015-2025 (SWAP), includes multiple species of greatest conservation need (SGCN) that potentially fall within this diet breadth. Finally, mudpuppy are a highly cryptic species. They tend to associate with complex benthic habitat for concealment, such as rocky bottoms of rivers and lakes (Murphy et al.,2016; Chellman et al., 2017). In Maine lakes, mudpuppy are likely to occupy deeper waters

(up to 9.8m – Craig et al. 2015), particularly during winter ice cover months. Because of this, mudpuppy are not frequently encountered by the general public, or even by professional biologists surveying for other species.

Zanden and Olden (2008) proposed a 3-component framework for assessing risks tied to secondary spread in invasive species. Briefly the include risks of:

1. *Introduction*: Can a species get to new sites from the original colonization site?
2. *Establishment:* Can the species sustain itself in the new location?
3. *Impact:* Will there be undesired consequences?

Maine Department of Inland Fisheries and Wildlife (MDIFW) is in the process of assessing these factors in understanding Maine's mudpuppy secondary spread. Given their hardy nature and indiscriminate eating habits, it is likely that condition two is met by most lake habitats in Maine, but condition three remains uncertain given limited knowledge of mudpuppy species interactions in the region. In unpublished data by a collaborator, Dr. Cathy Bevier, it was discovered that mudpuppy in Maine primarily ate amphipods, followed distantly by crayfish. Further taxanomic analysis must occur to refine that data, but as of now, their dietary habits suggest they are not a significant threat to species of concern and may serve to curb invasive crayfish populations (Cathy Bevier, unpub. Data). Given uncertainty in environmental impact and evidence that establishment is likely as long as a waterbody is accessible, component 1 has become MDIFW's initial priority. As a primarily aquatic species one would expect that unassisted spread of the species should only occur via waterways connected to their initial site of introduction. Nonetheless, in recent decades a modest number of public reports have accumulated suggesting that mudpuppy have spread beyond their drainage of introduction (Crocker 1960; Collins 2003; Sarnecki 2019). These reports indicate the potential for outside

agents, such as predatory birds (e.g. bald eagle, osprey, great blue heron) or humans, as vectors for the species. Anglers and boaters in particular are thought to be major vectors of aquatic species invasions via human-mediated jump dispersal (Havel et al 2015, Padilla and Williams 2004, Smith et al 2020, Zanden and Olden 2008). In an effort to better understand the range and impacts of non-native mudpuppy, the MDIFW began trapping the species in 2017. Their trapping method employs baited, modified minnow traps deployed during the winter through frozen lake ice along multiple lake transects. However, the probability of capture with this trapping method is unclear, as mudpuppy have not been captured in all waterbodies with prior public reports (albeit of variable confidence). In order to bolster these efforts, winter trapping methods by the MDIFW were supplemented with winter eDNA sampling in 2018 and 2019.

eDNA in the water column of a lentic system derives from multiple sources, including cells sloughed off epithelium, fecal matter, and carcasses (Wotton et al., 2001; Jerde et al., 2011, Merkes et al 2014,). It is possible to identify the presence of a target species by analyzing water or sediment samples for this shed eDNA that may remain dispersed in the water column for days to weeks (Wilcox et al., 2013; Pilliod et al., 2013). In this fashion, eDNA can increase the odds of detection by being less reliant on the temporal and spatial odds that an organism is immediately present at a site during the time it is being surveyed (Wilcox et al., 2013, Barnes et al., 2014, Bedwell and Goldberg 2020). However, there is emerging consensus that sampling appropriate seasonal habitat is important for optimizing eDNA detection (Ostberg et al., 2018; Roussel et al., 2018 Wacker et al., 2019; Troth et al., 2021). For mudpuppy in Maine, this would imply sampling water from near lake bottoms during the winter ice period. Relatively few eDNA studies have attempted winter sampling of eDNA through lake ice (but see Lawson et al., 2019; Bulte et al., 2020). This period offers potential benefits and tradeoffs. Surface ice can offer a

stable platform for precision sampling near the lake bottom, which might be more difficult from a boat. Environmental DNA might also persist longer due to reduced microbial metabolism. However, eDNA might not be as widely dispersed because of reduced lake mixing (Little Fair et al., 2020).

Given a desire by natural resource officials to document the current range of introduced mudpuppy in Maine, and to develop more optimal survey approaches that might be employed for future monitoring of mudpuppy and native amphibians, we sought to answer the following questions:

1. Is winter eDNA sampling effective for detecting mudpuppy in lakes?
2. How do winter trapping and eDNA methods compare for their power and effort to detect mudpuppy?
3. How might future eDNA or trap studies be optimized to provide higher efficiency of trap or eDNA detection?
4. What is the current range and habitat occupancy of mudpuppy in Maine, and how might that relate to natural or anthropogenic spread of the species?

Methods

Development of Primer and Probe Set

We designed a TaqMan MGB-NFQ qPCR assay specific to common mudpuppy by targeting sequence variation for primers and probe within a 73 bp region of the mitochondrial CO1 gene. Sequences for mudpuppy and seven other regional salamanders were aligned using the Benchling software (Benchling [Biology Software] 2018). Primers and probes were designed based on a 60℃ target annealing temperature and at least 4 primer bp differences between mudpuppy and both red-backed salamander (*Plethodon cinereus*) and tiger salamander (*Ambystoma tigrinium*) (Table 2.1), with particular attention to mismatches at the 3’ ends. The other salamanders were added to the alignment following primer and probe development.

Following initial design, primers and probes were in silico tested by BLAST against the NCBI Genbank database (Genbank, www.ncbi.nlm.nih.gov) to exclude potential amplification of other non-target taxa, and primers were tested for species-specific amplification of mudpuppy under lab conditions. A mock gene standard was synthesized (GBlock) to match the target mudpuppy qPCR target region and serve as a quantification standard and positive amplification control.

Table 2.1: Mudpuppy Sequence Alignment: Mudpuppy CO1 TaqMan MGB-NFQ qPCR Primer-Probe Set (developed by G. York) versus homologous gene regions for other New England salamanders. Mudpuppy (MUD) sequences are at the top. Highlighted in red are known mismatches between mudpuppy and other NE salamanders. Species represented: MUD = Mudpuppy (Necturus maculosus), RED= Red-backed Salamander (Plethodon cinereus), TIG=Tiger Salamander (Ambystoma tigrinium), BLU=Blue Spotted Salamander (Ambystoma laterale), ERN= Eastern Newt (Notophtalamus viridescens), NRD=Northern Dusky Salamander (Desmoganthus fuscus), NTL=Northern Two-lined salamander (Eurycea bislineata) SPR= Spring Salamander (Gyrinophilus porphyriticus).

Species	5' Forward 3'	5' Probe 3'	5' Reverse 3'
MUD	aactgtttatcctcctcttgcc	gcccatgcaggtgcctctgtaga	gccggagtctcttcaattcttggc
RED	gacagtatatcccccttttagcc	gcacacgccgggagcctcagttga	gcgggagtatcatctatcttgggt
TIG	aactgtatatcccccacttgca	gcccatgccgggagcttcagttga	gcaggtgtttcatctatcctaggt
BLU	aactgtctatccaccacttgct	gctcatgctggtgcttcagttga	gcaggtgtctcgtctatttttaggt
ERN	gactgtatatcccccactagct	gctcatgcaggggcctctgttga	gcaggggtgtcttcaatttttaggg
NRD	aacagtatatccgccccttagcc	gctcatgcgggagcctctgtaga	gccggagtgtcatctatttttaggc
NTL	aactgtttatccccccccttgca	gctcatgccggagcctctgtaga	gctgggtgtatcctctatccttgga
SPN	aacagtatatcccccccccttgca	gcccatgctggagcctctgtaga	gccgggtgtctcttctatcctcgga

Experimental Procedure

In total, thirteen lakes were sampled using both modified minnow traps and eDNA during the winters of 2018-2019. A pilot trapping season in 2017 occurred prior to the addition of eDNA methods. For the most part, sites where mudpuppy were captured in a given year were not trapped again in subsequent years as once was enough to confirm local presence of the species.

The exception, North Pond, acted as an annual control for trapping and eDNA repeatability, as it had been trapped for mudpuppy prior to the addition of eDNA sampling and was trapped for both years during the eDNA study.

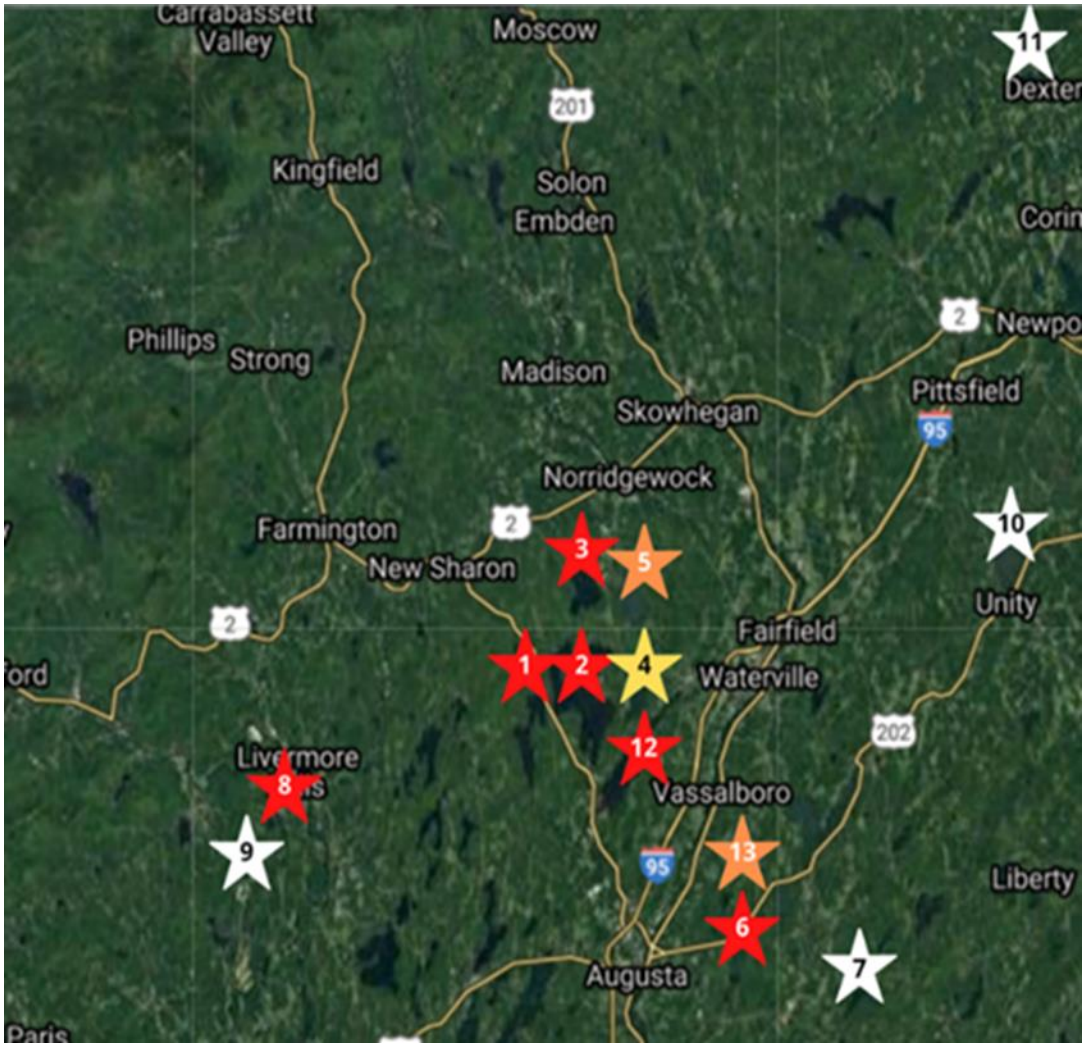

Figure 2.1: Map of Ponds Sampled: Combined mudpuppy survey results for winter trapping (2017-2019) and eDNA sampling (2018-2019). Detection Key: Red Star=Both Methods. Orange Star= eDNA Only, Yellow Star= Trap Only, White Star= Never Detected. Waterbody Key: Long Pond Rome:1, Great Pond:2, North Pond:3, Salmon Lake:4, East Pond: 5, Togus Pond:6, Long Pond Somerville:7, Long Pond Livermore:8, Brettun's Pond:9, Unity Pond: 10, Lake Wassookeag:11, Messalonskee Lake:12, Spectacle Pond:13.

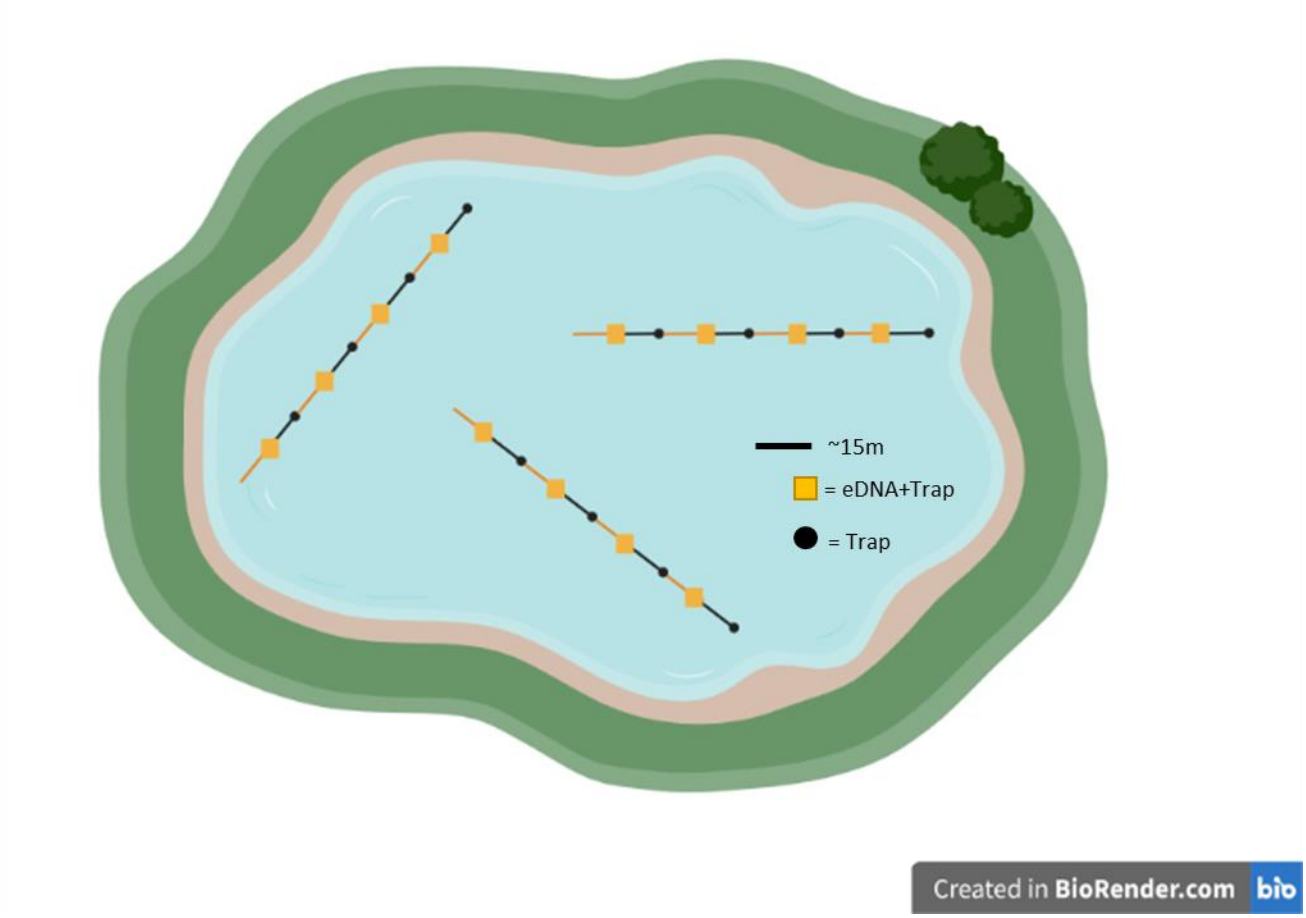

Figure 2.2: Transect Layout: On each lake (2018-2019) eight traps and four eDNA samples were taken. Points on the transect were set approximately 15m apart.

Each lentic system was trapped along three transect lines set in different regions of a lake to improve chances of detection (Fig 2.2). A gas-powered ice auger was used to create 8-10 sampling holes along the transect, spanning approximately 123 meters spacing depending on lake morphometry. In 2017, each transect was trapped at ten holes using a combination of regular minnow traps and minnow traps modified with a larger trap opening. Based on this initial survey year, trapping in 2018-2019 occurred at eight holes per transect using only the modified minnow traps. All traps were baited with a combination of dog kibble and crushed minnows.

Traps were checked two days after being initially set, and were immediately baited and redeployed for an additional two days, for a total of two replicate trapping events per hole.

eDNA samples were collected on the same transects traps were placed. To avoid contamination from trap placement, eDNA was only collected on one date per year per lake, immediately prior to initial minnow trap deployment. For each lake sampling event, the ice auger was sprayed with 10% bleach solution prior to use, and a test hole was drilled away from the transect to rinse the blades of potential surface contamination. Similarly, our PVC eDNA water sampler (See Appendix A), and any reusable field gear, were soaked or scrubbed in bleach solution between waterbodies, and UV sterilized between sampling days. Prior to taking the first sample at a given waterbody, an equipment negative control ("cooler blank") was collected by pouring 2L of water into the PVC water sampler and recollecting that water back into sample bottles for subsequent testing. Water samples were taken at every other hole on the transect for a total of four, two-liter samples per transect. Samples were collected into previously unopened 500 ml Nestle PureLife water bottles that were emptied on site at the time of eDNA collection to maintain eDNA sterility (Wood et al. 2020). Water samples were collected at a standardized 1m from the lake bottom. Upon bringing the PVC sampler to the surface, the water was emptied from the sampler into a wide-mouth jug to facilitate pouring the sample into the collection bottles. Water bottles comprising a sample were in turn sealed in a Ziploc bag and placed in a cooler for transport back to the lab for storage and filtering.

Samples were either filtered directly after returning from field collection or were frozen for no more than two weeks (Great Pond 2019) at -20℃. In the event extraction could not be accomplished in two weeks, samples were stored at -80℃ to prevent further degradation of DNA. Samples and equipment controls were filtered via vacuum pump through Whatman 1.5

micron glass microfiber filters. The eDNA filter apparatus was sanitized with 10% bleach solution and rinsed with DI water between samples. Filtering spaces were sanitized before and after use with a combination of bleach solution and UV light.

The protocol for eDNA extraction followed the DNeasy Blood and Tissue Kit method of Qiagen (Qiagen Inc). Use of an internal positive control (TaqMan TM) showed that PCR inhibition affected some 2018 samples. As a result, an inhibition clean-up step was added during extraction of 2019 samples, and most 2018 samples were re-run following inhibition cleanup (ZYMO Research OneStepTM – PCR Inhibitor Removal Kit D6030).

Quantitative PCRs of samples were conducted on a Bio-Rad CFX96 Real-Time System thermocycler in a 96-well PCR plate format using the thermal profile in Table 2.2. Four replicates of each sample and control were run with the following chemistry: 10µl Taqman Environmental Master Mix2.0 (Applied Biosystems), 5µl of nuclease free water, 2µl of primer ,probe, and nuclease free H2O mix, and 3µl of extracted template DNA, for a total of 20µl. This assay was conducted at concentrations of 10µm primer and 5µm probe. A no-template control was similarly replicated on each plate, substituting Nuclease-free water (Qiagen) for the extraction template. A dilution series of Gblocks (10, 50, 250, 1250, 6250, 31250 copies/µl) was run on a separate set of plates to provide a standard curve for estimating starting copy numbers of eDNA. These controls used similar chemistry to the samples, but the amount of template was reduced to 1µl and nuclease free water was increased to7µl.

Table 2.2: Mudpuppy eDNA Thermocycler Settings.

Action	Time	Temperature	Cycles
Enzyme Activation	10 min	95° C	1
Denaturation	10 sec	95° C	47
Annealing	30 sec	60° C	47

Analysis

Efficiency curves were estimated by analysis of covariance of log of the synthetic gene fragments of the dilution series against their corresponding quantitation curve (Cq) value. Efficiency was calculated as

$$E = -1+10^{(-1/slope)}$$

where the slope of the standard curve generated should be equal to -3.32 for 100% efficiency (Ginzinger et al., 2002).

Hierarchical occupancy analyses were conducted in R using *eDNAoccupancy: An R package for multi-scale occupancy modeling of environmental DNA* (Dorizo and Erickson 2018). This package utilizes a space-state model composed of two main equations to estimate occupancy probabilities at three different nested levels of a sampling scheme. The first equation models a binary occupancy state (i). The second equation models a second occupancy level (j) dependent on the original binary occupancy state (i). The second equation is in turn applied once again for a third tier of modelled occupancy (k) dependent on the occupancy state of the prior two models (i and j). In this study these equations were applied analagously to trapping and eDNA detection data with the primary difference being form of technical replication at the third modelled tier. For eDNA this involved modelling qPCR replicates, whereas for trapping it involved sampling event replicates. The hierarchical model functions are provided below.

1. $Z_i \sim$ Bernoulli (ψ_i)for i=1,2,…N
2. $\mu_{ij}| Z_i \sim$ Bernoulli (θ_{ij}) for j=1,2,… V
3. $y_{ijk}| \mu_{ij} \sim$ Bernoulli (P_{ijk}) for k= 1,2,…S

For trap data: Ψ= lake transect, θ= sampling location (trap hole) on a transect, P= 48 hour trap events. For eDNA data: Ψ= lake transect, θ= sampling location (eDNA hole), P= qPCR replicate.

For each tier, cumulative probability was calculated for a power analysis of the sample design. Cumulative probability was calculated as

$$x^*=1-(1-x)n \text{ where } x= \psi, \theta, \text{ or } P \text{ and } n=i, j, \text{ or } k,$$

depending on the tier of the hierarchy.

A Chi-square test was conducted to determine whether positive eDNA detections were spatiotemporally associated with actual captures of mudpuppy in traps, as might be predicted if eDNA detection is based on primarily local sources of eDNA as opposed to eDNA mixed throughout waterbodies. Only waterbodies/years with positive results for each method were considered.

We sought to better understand the dynamics of mudpuppy range expansion by assessing whether mudpuppy relative abundance over the combined trap interval at a given waterbody, was negatively correlated with distance from the initial introduction site. To do this, abundance was inferred from catch per unit effort (CPUE), here defined as the total number of animals captured divided by the number of trap nights since last checked. A gradually spreading invasion is expected to show highest abundances near its introduction sites and lower abundance in more distant sites due to the combined effects of most individuals dispersing relatively locally and time lags for abundances to increase at the edges of the expansion. Alternatively, a rapidly spreading invasion might be expected to show less evidence for a relationship between distance and CPUE due to a greater role of jump dispersal and rapid population growth in new colonization sites. Ice holes for eDNA sampling and trapping were placed with the goal of spanning different depth zones in multiple locations at each lake. As a whole dataset, a wide range of trap depths were available to quantify mudpuppy depth distributions, though each individual lake did not offer that same range. We assessed mudpuppy depth preferences by

comparing the overall depth distribution of trap sites with the distribution of successful sites and mudpuppy captures using one sample t-tests and a Kolmogorov-Smirnov test was used to assess potential finer-level depth distribution patterns.

Results

Primer-Probe Set and Assay Use

In Silico testing of our mudpuppy eDNA assay showed thirteen to twenty base-pair differences (Table 1) across primers and probe sequences (19-29% sequence differences), confirming the genetic uniqueness of mudpuppy in the region and specificity of our assay. Assay efficiency was determined to be 100.7% (95% CI of 94.8-107.5%). consistent with full efficiency of the eDNA assay (Fig. 2.3).

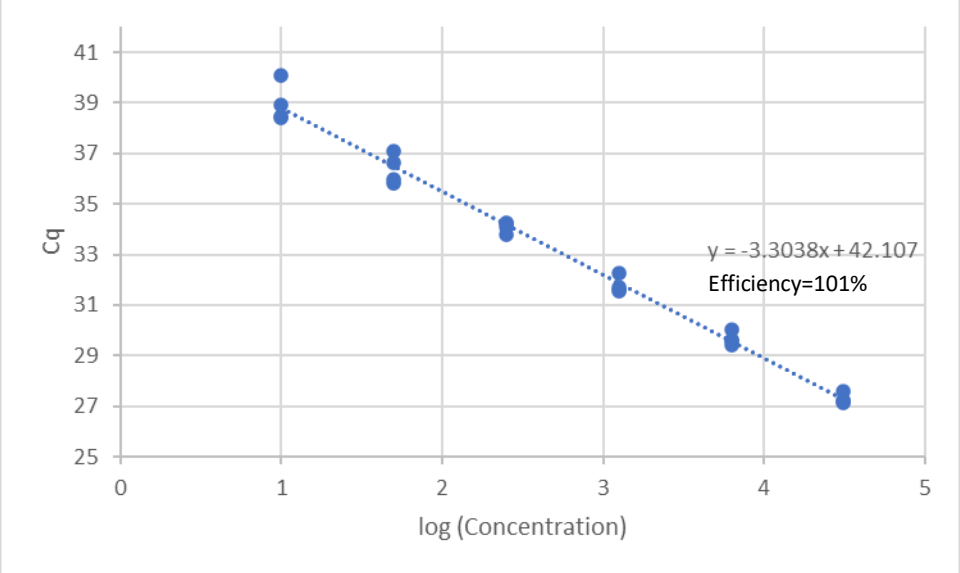

Figure 2.3: Mudpuppy eDNA Standard Curve: The standard curve for synthetic gene fragments of known concentrations. Cq values are plotted against the log of the synthetic gene fragments.

Analysis of cooler blanks (negative field controls) provided evidence of possible sample contamination at a subset of eDNA sites (Table 2.3), likely associated with trace remnant eDNA

on the PVC deep-water sampler and collecting container, despite bleach and UV treatment. Such contamination is not uncommon in eDNA field research, and to address its presence we set a conservative threshold for a positive qPCR replicate of >1 Cq lower than the lowest positive cooler blank value for any sites where such positive blanks were detected. This equates with a positive qPCR replicate having at least twice the estimated eDNA concentration of any known contamination. We also required that at least two independent water samples (as opposed to replicates) be positive for any given waterbody to conclude mudpuppy presence via eDNA alone.

Summary of Detections

Table 2.3: Summary of all trap and eDNA Survey Efforts (2017-2019): Trap years with captured mudpuppy are in red. Trap catch per unit effort where CPUE was measured as the total number of animals captured divided by the number of trap nights since last checked. At eDNA positive waterbodies, both eDNA year and corresponding quantitation curve (Cq) range are in red. Cq values in red represent putative positive detections based on our contamination and repeatability criteria. Cq values not in red represent amplifications that did not meet our criteria and thus, not counted as a positive detection in our results.

Waterbody	Towns	Year(s) Trapped	Avg CPUE	eDNA Year(s)	eDNA Cq Range(s)	Negative Control Cq
Long Pond Rome	Belgrade/Rome	2018	1.45	2018	(39.59-45.56)	NA
Salmon Lake and McGrath Pond	Belgrade/ Oakland	2017	0.90	2018	40.59-NA	NA
North Pond	Smithfield/ Rome	2017, 2018, 2019	0.18	2018, 2019	(40.85-42.19) , (38.23-42.81)	NA, 42.76
Great Pond (North)	Belgrade/Rome	2018	0.32	2018, 2019	(31.12-42.57), (35.52-44.73)	39.27, NA
Messalonskee Lake	Belgrade/Sydney	2017	0.32	2019	38.64- 41.84	NA
Togus Pond	Augusta	2018	0.29	2018	39.46-43.35	NA
Long Pond Livermore	Livermore	2017	0.02	2018, 2019	(39.44-40.33), (40.55-NA)	NA, 39.96
East Pond	Smithfield/ Oakland	2017, 2019	NA	2018, 2019	NA, (40.61-41.07)	NA, NA
Brettun's Pond	Livermore	2017, 2019	NA	2018, 2019	NA, (37.6-40.16)	31.51,NA
Long Pond Somerville	Somerville/ Windsor	2018, 2019	NA	2018, 2019	(40.13-NA), (42-NA)	NA,NA
Unity Pond	Unity/ Burnham	2018, 2019	NA	2018, 2019	NA,NA	39.27,NA
Lake Wassookeag	Dexter	2018, 2019	NA	2018, 2019	NA, NA	NA, NA
Spectacle Pond	Vassalboro/ Augusta	2019	NA	2019	39.04-44.37	NA

Mudpuppy presence was confirmed at seven of our thirteen surveyed water bodies via traps and at eight out of thirteen waterbodies via eDNA. Overlap between the two survey methods was very high, with only one waterbody confirmed via traps but not eDNA (Salmon Lake), and two waterbodies confirmed with eDNA but not trapping (East Pond and Spectacle Pond). Two waterbodies, Salmon Lake and Brettun's Pond, had nominally positive eDNA detection in a single sample each. However, the single Salmon Lake detection did not pass our criterion for having two or more independently positive samples. That said, the single positive sample in that lake is likely a true positive given that mudpuppy were confirmed in that system via trapping. By comparison, although multiple qPCR replicates were positive for a single sample in Brettun's Pond, none of these were high enough to surpass our contamination threshold and thus this site remains inconclusive.

Occupancy Modeling

Occupancy modelling was conducted for each waterbody and year that confirmed mudpuppy through traps or met the previously mentioned conditions for eDNA amplifications (Fig 2.4 A-F). Though parameter estimates vary somewhat by waterbody, a more general estimate can be obtained as the average of the probabilities at the ψ (Transect level), θ (Hole level), and p (Technical replicates = trap events or qPCRs), informing survey power for trapping or eDNA methods at comparable scales of the sampling process. The average trap effort to achieve an approximate 95% probability of capturing a mudpuppy at each sampling scale in a positive waterbody was as follows: Transects=2.2, Holes=3.2, and Trap Events= 6.4 (Table 2.4). For eDNA, the corresponding hierarchical parameter means were: Transects=2.3, Holes=4.2, and qPCR Replicates=10.9 (Table 2.4). Rounding to the nearest whole integer sampling effort, we

found comparable numbers in recommended transects and holes, but more technical replicate effort would be required per hole for eDNA (qPCR replicates versus trap events).

Table 2.4: Occupancy Model Results Summary: The recommended sampling efforts to reach a 95% probability of detection for each mudpuppy confirmed waterbody (Fig 2A-2F). NA= No sample effort that site/year. NA=No positive results to model in that site/year.*

Waterbody	Trap: Recommended Transects	Trap: Recommended Traps	Trap: Recommended Trap Events	eDNA: Recommended Transects	eDNA: Recommended Samples	eDNA: Recommended PCR Replicates
Long Pond Rome 2018	2	2	2	2	3	12
Salmon Lake and McGrath Pond 2017	2	3	2	NA	NA	NA
Salmon Lake and McGrath Pond 2018	NA	NA	NA	NA*	NA*	NA*
East Pond 2019	NA*	NA*	NA*	3	5	20
North Pond 2017	2	4	4	NA	NA	NA
North Pond 2018	2	2	5	3	5	21
North Pond 2019	2	2	17	2	3	3
Great Pond 2018	2	4	4	2	5	4
Great Pond 2019	NA	NA	NA	2	2	3
Messalonskee Lake 2017	2	5	4	NA	NA	NA
Messalonskee Lake 2019	NA	NA	NA	2	5	6
Togus Pond 2018	4	2	4	2	2	9
Long Pond Livermore 2017	2	5	16	NA	NA	NA
Long Pond Livermore 2018	NA	NA	NA	3	5	21
Long Pond Livermore 2019	NA	NA	NA	NA*	NA*	NA*
Spectacle Pond 2019	NA*	NA*	NA*	2	7	10
Average	2.2	3.2	6.4	2.3	4.2	10.9

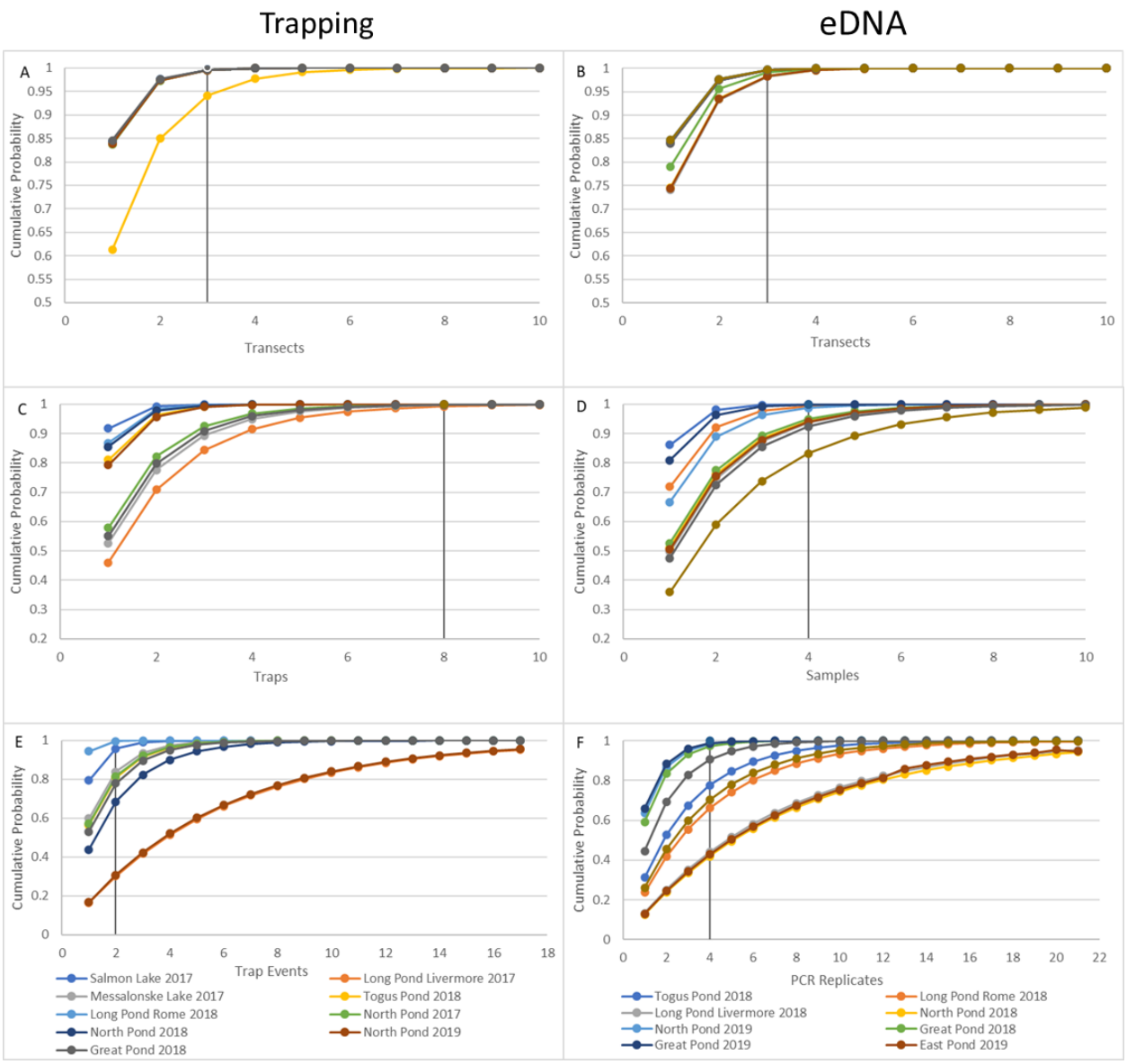

Figure 2.4 A-F: Mudpuppy Cumulative Probability Functions: Traps (left) and eDNA(right). A-B= transect (ψ), C-D= trap set or samples taken (θ), and E-F= trapping events or qPCR (p) hierarchical levels. The x-axis indicates how much effort, in terms of transects, holes or technical replicates are associated with a given expected detection probability (occupancy). Vertical lines represent actual survey efforts from the current study. This only includes lakes with trapped mudpuppy, or detected eDNA, due to requirements for occupancy estimation.

Trap-eDNA Relationship

At a very local spatial scale (sampling holes), Chi-square analysis of positive and negative eDNA detections of mudpuppy at n= 80 trapping locations (also with or without subsequent mudpuppy captures) showed that detection was spatiotemporally non-random ($p = 0.04$; $X^2 = 4.379$, 1 d.f.). The pattern was particularly strong for non-detections, with the majority (n=30) of negative eDNA samples (n=46) coming from negative trapping sites, which is expected if eDNA provides a spatiotemporally larger, albeit locally probabilistic (i.e., due to dilution), detection window compared to trapping. Providing further evidence, there was a slight majority (n=18) of positive eDNA detections at successful (n=34) trapping sites.

Of the nine waterbodies where mudpuppy were detected by either trapping or eDNA, eight were located within 25 km (straight-line estimate) of the initial introduction site (hatchery stream leading from Salmon Lake to Great Pond). Long Pond Livermore, was the only positive lake with no connection to the drainage network of ponds associated with the introduction site. The four lakes without positive detections by either method were all greater than 30km (straight-line estimate) from the introduction site. An apparent relationship of mudpuppy abundance (CPUE) versus linear distance from the initial introduction site was not statistically significant ($p=0.27$; $R_2 = 0.24$) (Fig 2.5), but this is likely due to power associated with only six datapoints.

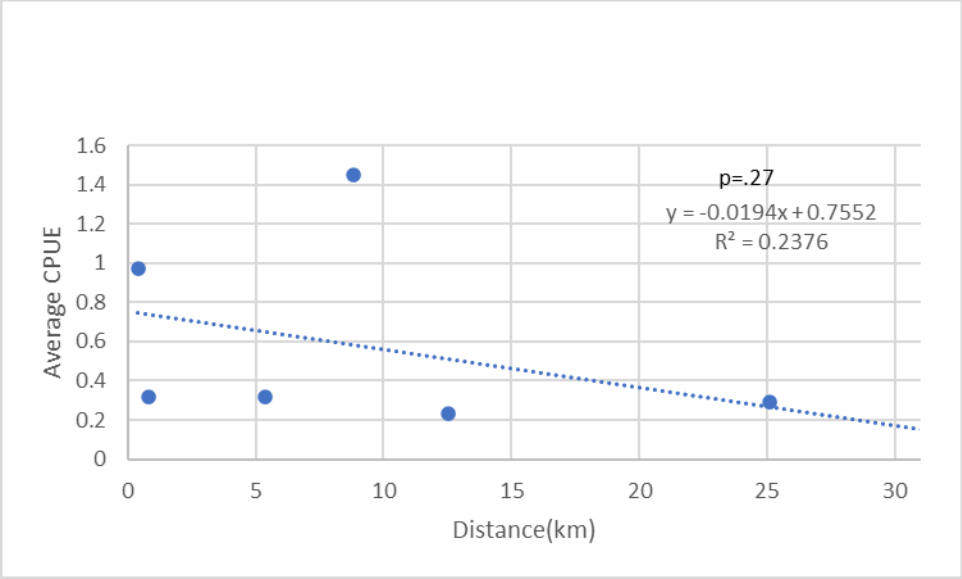

Fig 2.5: CPUE vs Distance: The relationship between average catch per unit effort and distance (km) from the mudpuppy introduction site.

Depth Analysis

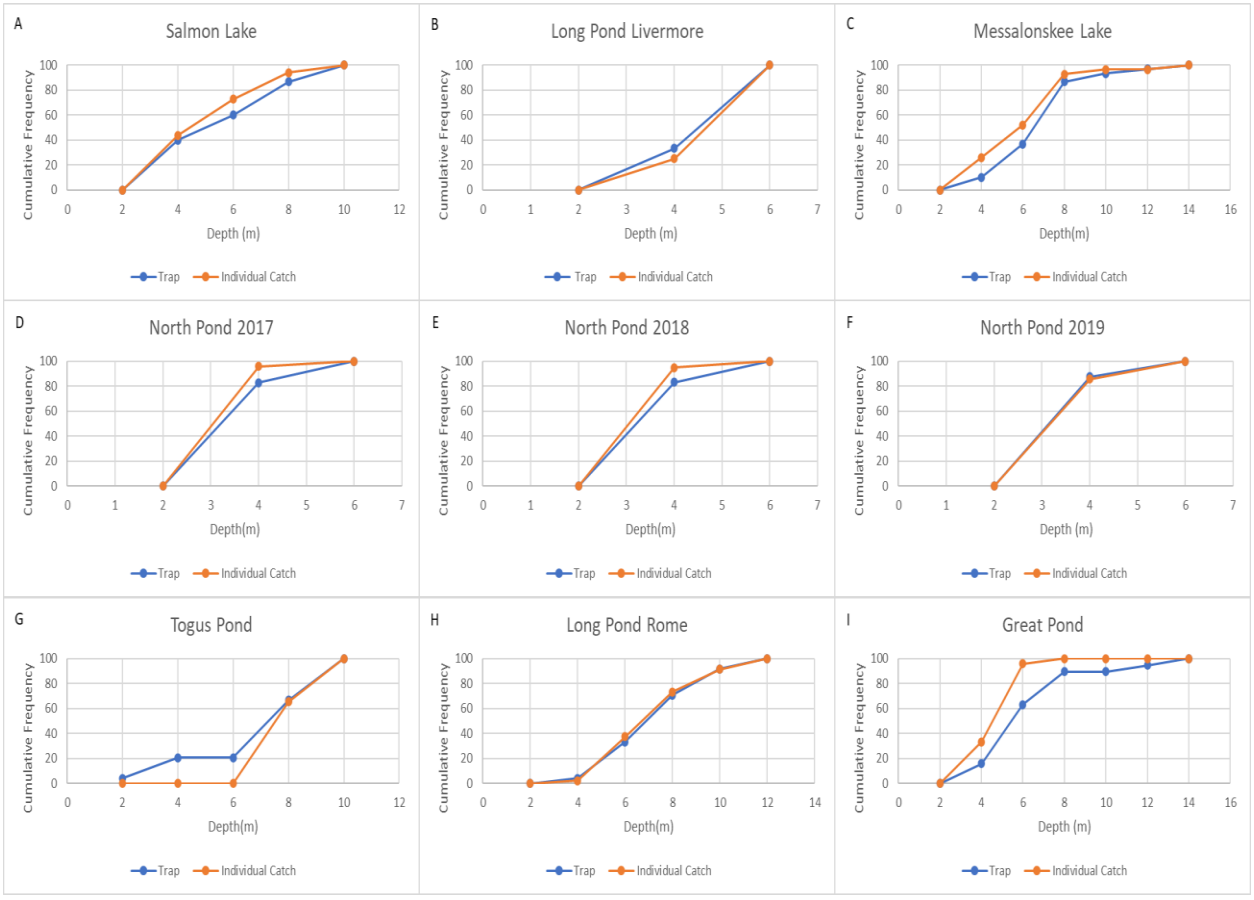

Fig 2.6: Cumulative Overall Depth Distribution: Traps (blue) plotted against cumulative proportion of mudpuppy catches (orange) at 2m depth intervals in each pond.

Cumulative depth frequency analysis suggested that depths between 2-8m account for 85.02% of individual mudpuppy catches (Fig 2.6) while 88.93% of traps were set in that interval. For the most part, mudpuppy were captured in a pattern largely conforming to trapping depth effort, suggesting they do not show strong depth preferences in winter. Kolmogorov-Smirnoff tests revealed only two lakes with a significant difference in proportion of individual catches relative to trapping depth effort, Togus Pond (D= 0.21, Crit value=0.18) at the (2-4m] and (4-6m] depth intervals, and Great Pond (D=0.33 Crit value=0.22) at the (4-6m] interval (Fig 2.6). However, the deviations at these lakes were in opposite directions. At Togus Pond fewer mudpuppy were caught at shallow depths (2-6m] than anticipated; whereas, more mudpuppy were caught than anticipated in the (4-6m] interval at Great Pond.

Discussion

The goals of this study were to determine the viability of winter eDNA sampling for surveying invasive mudpuppy in Maine lakes, compare winter eDNA sampling with winter trapping of mudpuppy in the same waterbodies, provide insights into how to optimize future survey efforts for mudpuppy, and use our combined eDNA and trapping data to map the current invasion range of mudpuppy and assess potential habitat associations. To accomplish this, thirteen Maine waterbodies were surveyed based on proximity to the known site of mudpuppy introduction and information the MDIFW gathered from anecdotal citizen reports and, in some cases, purported photographic evidence. Nine of thirteen sampled waterbodies were confirmed to have mudpuppy by at least one survey method. Trapping and eDNA provided largely complementary data findings, though mudpuppy were detected in more sites via eDNA while requiring less field effort during a difficult season for aquatic sampling. Further, finer-scale analysis revealed that eDNA did predict the local (within lake) presence and absence of

mudpuppy at the level of sampling holes. From a habitat and invasion perspective, analyses suggest that mudpuppy are widely distributed across water depths in lakes during winter, and all but one of the nine confirmed waterbodies were within approximately 25km of the introduction site between Salmon Lake and Great Pond. Notably, one sampled waterbody within that range, Brettun's Pond, remains inconclusive for colonization. An implied weak but negative relationship between mudpuppy CPUE and distance from the initial introduction, is consistent with mudpuppy spreading slowly through waterways, likely via their own dispersal rather than via frequent and extensive jump dispersal facilitated by humans. Likewise, inability to detect mudpuppy by either method at four out of five lakes over 35 km from the introduction site is supports a relatively confined and slow spreading invasion.

Both trapping and eDNA were effective methods for detecting mudpuppy during the winter ice cover season in Maine, as inferred from the fact that mudpuppy were most often detected by both methods and at sites that would be expected based on anecdotal reports. Mudpuppy eDNA was detected in most lakes where the species was captured with trapping. However, future studies applying eDNA alone would not necessarily have the benefit of knowing mudpuppy presence for comparison, and detections in a single sample would be more ambiguous for interpretation in such contexts, particularly given the potential for some level of gear contamination. As such we applied a more rigorous criterion for determining site positivity with eDNA, based on at least two positive samples at eDNA concentrations more than 1 Cq lower than any observed contamination. By this more rigorous criterion, eDNA sampling would have "missed" mudpuppy presence at Salmon Lake, despite having a single positive sample at that lake. However, the risk of such false negatives could be remediated by establishing a protocol that entails rigorously resampling any waterbodies with even a single positive qPCR

replicate in the future.

Some amount of field contamination is common in eDNA studies employing specialized gear for sampling (e.g., deep water sampling), despite extensive efforts to remove such contamination from surfaces through processes like bleaching, UV exposure, and careful packaging for transport (Thomsen et al., 2015; Ficetola et al., 2016; Wilson et al., 2016). Indeed, it may often be impractical to fully eliminate such low-level contamination without incurring large expenses associated with use of fully disposable sampling equipment. However, this does not mean that eDNA cannot be applied under such circumstance, but rather sampling must appropriately account for such contamination potential in providing data interpretations. The foremost method for this is likely to involve rigorous application of negative field equipment controls. Merely opening and closing a control water bottle, without exposing that water to the sampling equipment, would not be effective for detecting possible contamination on our PVC deepwater sampler, even though that method is common in eDNA surface water sampling. Our approach of pouring sterile lab water into the sampler, and then collecting that exposed water for testing, provides the necessary representative control. Likewise, it is important to apply a comparable (or more stringent) level of sample processing rigor to negative controls, such as by analyzing control samples with a comparable number of qPCR replicates. We suggest this level of rigor is particularly important if one is to apply an empirical Cq cut-off threshold for distinguishing positive samples, as we did here.

Regardless of technique employed, surveys that apply insufficient sampling effort are apt to experience a high rate of false negatives, reducing the effectiveness of monitoring and management (Moyer et al., 2014; Wilcox, 2016). For that reason, we applied a hierarchical sampling and analysis design that allowed the estimation of our survey power at each of the

hierarchical levels of the survey process – transects, holes, and technical replicates. Within this framework, transects may be considered to represent the number of lake sections needed to ensure at least one section is sufficiently colonized by mudpuppy. Holes account for the spatial heterogeneity of occupancy within these lake regions, recognizing that individuals are potentially sparsely distributed due to low abundance. Finally, the technical replicate level, represented by number of trap events or qPCR replicates, represents the local site- and lab-specific required effort to detect mudpuppy assuming they are present in that immediate vicinity but do not always enter a trap or shed sufficient eDNA for collection. Based on our hierarchical occupancy modelling, across all successful trapped locations, the average sampling effort required to achieve 95% chance of detection (capture) with baited traps was 2.2 transects, 3.2 traps on each transect, and 6.4 trapping events (48 hour trapping periods) per pond (Table 2.4). For eDNA surveys, the mean required efforts was 2.3 transects, 4.2 samples per transect, and 10.9 qPCR replicates.

Our actual sampling effort met or exceeded these estimates at the levels of transects and holes, suggesting our spatial coverage of sampling was well suited to detection of mudpuppy. The fact that both trapping and eDNA provided approximately similar estimates of required transect and hole effort might be expected on the grounds that sampling effort at these scales is apt to be largely determined by the spatiotemporal heterogeneity of the target species, not the ability to capture or detect the species with a particular tool. However, our sampling effort at the level of technical replicates, whether that was number of trap events or qPCR replicates, was only about a third to half the estimated effort required for 95% probability of detection. As such, it is feasible that each survey method missed detecting mudpuppy in some waterbodies where they were present. The fact that we applied both sampling methods simultaneously likely offset

this shortcoming of the separate methods and reduced the odds that we missed mudpuppy in many locations. Nonetheless, the limited number of technical replicates for both methods could explain our single eDNA amplification at Salmon Lake (definitive false negative; based on prior year trapped specimen evidence) and failure to trap mudpuppy at East Pond and Spectacle Pond (potential false negative; still lacking specimen evidence).

Although mudpuppy were detected at more lakes with eDNA, the difference in number of lakes is not significant within bounds for random sampling error. At a finer level, we examined the correspondence between trapping and eDNA detections at the level of local sites (holes) within lakes. We found support that eDNA detection and non-detection was associated with physical detection and non-detection by traps, consistent with other eDNA studies. This suggests eDNA is an effective, if often imprecise, proxy for spatiotemporal variation in species presence or abundance (Dougherty et al., 2016; Sutherland et al., 2020), though our data shows more correspondence at the negative values. Trapped individuals must actually enter the survey gear to be detected, whereas shedding and dissolution from a mobile source makes eDNA detection a coarser grained process. The potential for eDNA to be dispersed from a source is expected to often be greater during open water seasons where wind, thermal turnover, and ectotherm activity levels are greater. However, this may not apply well to mudpuppy in lakes due to their habit of remaining concealed for long periods below rocks or wood during brighter summer periods. Nonetheless, we suggest that a future study assessing eDNA detection during open water periods in Maine lakes would be worthwhile for comparing temporal efficacy of eDNA.

We chose to sample mudpuppies with trapping and eDNA during winter months because this is a period of somewhat higher reported encounters in Maine,where mudpuppy are occasionally caught by anglers on baited ice fishing gear, and because the winter period

facilitates deploying and retrieving baited traps at specific locations. Once again, it should be noted that sampling eDNA during the winter ice period is relatively rare among eDNA surveys (but see Lawson et al., 2019; Bulte et al 2020). Winter sampling adds special challenges to eDNA work, including freezing conditions that limit use of pumps, absence of wind-driven circulation to disperse eDNA over larger areas, and potentially lower eDNA shedding rates of ectotherms. Nonetheless, winter eDNA sampling was effective, likely in part because mudpuppy are active during this time of year in accordance with their mating season (Craig et al., 2015). However, other studies have had success with mudpuppy eDNA surveys under different conditions. In the Detroit River system, Sutherland et al., 2019 found that all successfully trapped (minnow traps or setline) sites were also positive for eDNA. Environmental DNA also had the highest success rate of the three methods employed in that study.

Mudpuppy are known to prefer shallow areas (~2m) with rocky cover in lotic systems (Chellman 2011, Craig et al 2015, Sutherland et al 2019), but this may reflect seasonal behavior and available habitat in some types of systems. While the Kolmogorov Smirnoff suggests some possible idiosyncratic depth preferences at both Great Pond and Togus Pond, they did not demonstrate a clearly preferred depth range among the dataset as a whole (Fig 2.4). During open water season, cooler surface temperatures of around 5°C have been shown to greatly increase detection probabilities in lotic systems (Sutherland et al., 2020). In a frozen lake, roughly comparable temperatures are found over a wide area of lake bottom. Thus, we recommend that future studies spread their sampling effort widely, potentially targeting lake regions in the vicinity of rocky reefs where mudpuppy might shelter during other periods of the year.

Ultimately, the choice of survey method, trapping or eDNA, may come down to the relative needs of the sampling effort and constraints on time and resources. Trapping provides

physically verified presence, the ability to measure features of the captured individuals (e.g., size, sex, diet), and ability to remove invasive individuals from waterways. However, based on our findings, 95% confidence in detection by winter trapping would require five or more visits to each lake, transect, and trap hole during a time of year when outdoor work is physically demanding and apt to be disrupted by weather or poor ice conditions. The added time spent trapping could strongly constrain the number of ponds surveyed in a season. By comparison, our findings suggest that comparable detection power can be obtained with a single eDNA sampling event per pond. Although increasing qPCR replicates does increase laboratory consumables costs, even doubling these costs (e.g., from 4 to 8 replicates) would be less expensive than more than doubling field crew time, and ultimately only adds minutes to lab sample processing. This is consistent with other research suggesting the cost effectiveness of eDNA (Goldberg et al., 2016; Wilcox et al., 2016; Deiner et al., 2017, Spear et al., 2021).

Despite reported and demonstrated efficacy, it is important to acknowledge the limitations and outliers for eDNA within our study. Whereas there was a relationship between sites with captured mudpuppy and sites with mudpuppy eDNA, it was not as strong as expected in some places, the most notable of which is Salmon Lake. This lake had the second highest CPUE of 0.897, but it was not counted as a positive site based on our stringent eDNA criteria. This site did have a reasonable amplification in one sample (Cq=38.42), and review of initial 2018 runs, prior the inhibition cleanups showed some weak detections in another sample (Cqs at 48.19 and 45.05). Hence, it was our relatively conservative study criteria of only using the post-cleanup 2019 data and having two or more independently positive samples that excluded us from considering this a definitively positive site via eDNA. Despite that these detections were very likely real given that mudpuppy were captured in that location. However, we felt it was

important to follow these rigorous eDNA criterion because future eDNA-only studies would more realistically operate blind without trap data for comparison and without running samples both with and without inhibition cleanup. It is also worth mentioning how sampling years and time might have factored in the outcome for Salmon Lake. This lake was destructively trapped in 2017, eDNA sampled in 2018, and, due to re-running all samples with ZYMO inhibition removal, the PCR data used here was not obtained until 2019. We might have encountered more amplifications had eDNA been sampled at the same time as the positive trapping and had inhibition removal occurred immediately in 2018 to avoid a year of possible sample degradation. There were also outliers worth discussing in the trapping data.

At both North Pond (2019) and Long Pond Livermore (2017) we saw a high number of trap events (48 hour trapping periods) recommended in our occupancy model with seventeen and sixteen respectively. At North Pond destructive sampling may have played a role as 2017 and 2018 held steady at .21 and .22 CPUE respectively, while 2019 dropped to about half that at a CPUE of 0.11. Very low catch, if any, was expected at Long Pond Livermore simply due to distance (~34km) from the introduction site. Since the goal of this study was fine-tuning sampling methods to reach sensitivity where mudpuppy can be detected at low abundances, these outliers were included in the dataset to further inform on these situations. The very different detection rates and efforts across years at the same site nonetheless suggest that trapping success can be very subject to chance encounters, and false negatives. Trapping multiple years, or pairing trapping with eDNA sampling, may often be needed to reliably detect mudpuppy with realistic survey efforts.

Mudpuppy were unintentionally introduced to Maine in 1939 when research animals escaped holding pens in a tributary leading from Salmon Lake to Great Pond. In the intervening

years it has become clear that mudpuppy are now well established in the region, with many reports of captures, particularly during winter months. However, there have also been numerous scattered reports of the species in waters well outside this drainage area, albeit these generally lack physical specimens or photos for confirmation. The combination of trapping and eDNA in this study supports that mudpuppy still have a restricted range in Maine, at least in reasonably detectable numbers, with the greatest implied abundances in closer proximity to the introduction site. This suggests that most of the mudpuppy range expansion in Maine has likely occurred relatively slowly, and through mostly their own gradual dispersal via stream networks rather than through jump dispersal facilitated by human activities like collection as temporary pets or for bait. This hypothesis could in part be evaluated by eDNA and trapping studies in the relatively slow sloughs and streams that link the region's lakes, similar to the aforementioned eDNA studies in lotic systems. The importation and introduction of mudpuppy (or any other non-native wildlife) into Maine waterbodies is prohibited under Maine law, but the law is less clear about the inadvertent or purposeful transfer of resident non-native wildlife from one site to another. In any case, the public is often unaware of these restrictions and additional public education efforts might be beneficial for limiting rare but potentially impactful translocations among distant drainages. Indeed, the detection of mudpuppy in Long Pond Livermore (Androscoggin River drainage) suggests human or wildlife-assisted dispersal has played a role in establishing population(s) in at least one novel watershed that is hydrologically disconnected from the watershed of first introduction (Kennebec River drainage).

In this study we were able to determine lake occupancy of mudpuppies in Maine, test the efficacy of trapping and eDNA methods for winter surveys of mudpuppy, and provide recommendations of sampling efforts and water depths for targeted trapping and eDNA

sampling. The research we conducted has also established a baseline understanding of current mudpuppy distribution and catch per unit effort that will aid future surveys to track the range of this non-native species. Those future efforts are likely to be made more feasible and cost effective through the use of eDNA approaches. Targeting future eDNA or trapping surveys on new waterbodies with public reports, or on waterbodies with suggestive eDNA detections that do not meet our strict criteria for confirmation, may be the most cost-effective way to expand our knowledge of the mudpuppy secondary spread in the near term. Additionally, engaging local lake associations and ice angler groups in eDNA surveys could provide for a more comprehensive and expansive monitoring network, while simultaneously collecting eDNA water samples that might be screened for other exotic species of potential ecological concern, of which there are many in the Kennebec watershed of central Maine (e.g., Chinese mystery snail, rusty crayfish, walleye, and others).

CHAPTER 3

ENVIRONMENTAL DNA DETECTION OF ANADAROMOUS RAINBOW SMELT IN LENTIC SYSTEMS

Introduction

Documenting habitat occupancy is challenging for many organisms because of their behaviors, life histories, crypsis, habitat conditions, or rarity. One particularly challenging case is where organisms transiently occupy difficult to observe habitats for relatively short periods of time. For example, some migratory aquatic organisms may occupy breeding habitats like streams for a few days or weeks out of an entire year. Traditional survey methods, such as trapping or visual surveys may prove ineffective, inefficient or expensive for detecting such organisms. Environmental DNA, or eDNA for short, is quickly emerging as a sensitive and specific means of detecting many hard to survey species (Thomsen et al., 2012; Takahara, 2013; Laramie et al., 2015; Sigsgaard et al. 2015), but its utility for detecting some transient or ephemeral organisms is unclear. Here we assess the utility and optimal survey effort for eDNA detection of a highly transient stream breeding migratory fish, the anadromous rainbow smelt (*Osmerus mordax*).

Rainbow smelt are small anadromous or landlocked fish inhabiting northern temperate and arctic regions of North America. This study focused on the anadromous life history form, which live in marine habitats for most of their lives, but spawn in small coastal streams. Historically, smelt are important commercially and culturally as food, and ecologically important as a forage fish for other species (Chase et al., 2019). The range of anadromous rainbow smelt formerly extended along the East Coast of the United States of America as far south as the Chesapeake Bay. However, that range has diminished to as far north as Buzzards Bay, MA

(Enterline et al., 2012). Some suggested sources for this decline include overfishing, habitat degradation, dams, and climate change (Enterline et al., 2012). Even within their remaining range, anadromous smelt appear to be in decline along the east coast. However, quantifying that decline is difficult, with 47% of 279 potential smelt spawning sites being listed as "uncertain" by the Maine Department of Marine Resources in 2012. Resolving this gap in knowledge is easier said than done due to challenges of detection. Low population abundances, difficult life history and behavior of anadromous smelt, and environmental conditions are all factors in this challenge.

Smelt migration and spawning events typically take place over just a few nights in a given stream, during spring months (March-May), when rains and runoff make water conditions relatively high and turbid (Sirois and Dodson, 2000; Enterline et al., 2012). Smelt are nocturnal spawners and adults typically depart coastal spawning streams by early morning, so visual surveys for adults may be constrained by needing to be in the right place at the right time, with the right lighting and water conditions. Many surveys for smelt instead look for their eggs left behind on rocks, which have a one week to one month developmental window before larvae hatch and immediately emigrate (Chase et al., 2019). However, low abundance smelt populations are apt to leave behind relatively few eggs in relatively few places in the steam system, and the eggs are not always easy to visually confirm (Chase et al., 2019). We hypothesize that eDNA can provide an opportunity to improve smelt spawning habitat monitoring by providing increased detection sensitivity and a longer detection window by targeting the DNA "leftovers" from spawning activity or from newly hatched smelt fry. eDNA takes advantage of the aquatic environment's propensity to suspend and distribute DNA shed from organismal tissues, fecal matter (Wotton et al., 2001), and carcasses (Merkes et al., 2014), for easy collection via water samples (Jerde et al., 2011).

Traditional visual surveys for smelt might be improved upon by eDNA approaches, because eDNA allows for "sight unseen" detection (Jerde et al., 2011). This especially applies when a target species, like smelt, is relatively rare in space or time, making it a valuable tool for detecting species of concern or those establishing non-native populations (Thomsen e al., 2012; Wilcox et al., 2013; Deiner et al., 2015; Laramie et al., 2015, Sigsgaard et al. 2015). However, there are settings which are more limiting when applying eDNA approaches. Specifically, eDNA has a limited period of availability once shed into a system due to processes like current transport, dilution, settlement from the water column, and degradation (Pilliod et al., 2013; Barnes et al., 2014; Turner, Uy, & Everhart, 2015; Wilcox et al.,2016). Rivers and streams can present a particular challenge for eDNA detection because the flow in such systems is known to quickly transport and dilute eDNA from a point source, with some estimates of detectable eDNA persisting only hours or days after removal of a source (Wilcox et al., 2016). This might seem to strongly limit the application window of eDNA for transient stream breeding organisms. However, while the breeding organisms might not be present in streams for very long, breeding activities like deposition of fertilized and unfertilized gametes, abrasion of tissues during nesting, or deposition of carcasses, may provide for an increased window of opportunity (Tillotson et al 2018).

To assess and refine the utility of eDNA for the monitoring of transient stream breeding rainbow smelt, we address the following questions. 1. Given the highly ephemeral nature of breeding smelt in steams, can they be detected using eDNA? 2. What is the window to detect smelt spawning beyond their active spawning window? 3. What sampling design would be most effective for detecting low abundance smelt breeding in coastal streams, in terms of number of events (sampling days), samples, and qPCR replicates? Addressing these concerns, we

performed two field studies. The first compared smelt eDNA detection to fyke net catches, in two streams, which provide the answers to questions 1 and 2. The second field study built on the first using streams with known smelt spawning populations to answer question 3 via hierarchical occupancy modeling of empirical detections.

Methods

Development of Primer and Probe Set

Table 3.1: Smelt Sequence Alignment: Smelt NAD5 TaqMan MGB-NFQ qPCR Primer-Probe Set (developed by G. York). Highlighted at the top in yellow is rainbow smelt (OSM). Highlighted in red are desired mismatches between our target and the competitors which indicate the sensitivity of the assay. OSM = rainbow smelt (Osmerus mordax), ARC = arctic char (Salvelinus. alpinus), ATL = atlantic salmon (Salmosalar), BKT = brook trout (Salvelinus fontinalis), LKT = lake trout (Salvelinus namaycush), RBT = rainbow trout (Oncorhyncus mykiss), BNT = brown trout (Salmo trutta), LWF = lake whitefish (Coregonus clupeaformis), CP = chain pickerel (Esox niger), NP = northern pike (Esox lucius), LMB = largemouth bass (Micropterus salmoides), SMB = smallmouth bass (Micropterus dolomieu), BC = black crappie (Pomoxis nigromaculatus).

Species	5′ Forward 3′	5′ Probe 3′	5′ Reverse 3′
OSM	CACAACCATCCACCGCTCTCTC	TGGGCCAAGCTATCGCGAGCCA	TTGCTGCTGGGAGGTGAATAGC
ARC	TGCCGTCGTCCACCGATTAGCC	TAGGACAAACTATTGCCAGCCA	TGACTATGGGCAGGTTAGTTGA
ATL	AGCCATTATCCACCGATTAGCC	TAGGACAAACCATTGCCAGCCA	TGACCATAGGTAGGTGAGTGGA
BKT	TGCCATCGTACACCGATTGGCC	TAGGACAAACTATTGCCACCCA	TAACTATAGGGAGGTTAGTTGA
LKT	TGCCGTCGTCCACCGATTAGCC	TAGGACAAACTATTGCCACCCA	TGACTATGGGCAGGTTAGTTGA
RBT	CGCCATCATCCACCGATTGACC	TAGGACAAACCATCGCCAGCCA	TGACTATTAGGGCAGGTGAGTTG
BNT	AACCATCATCCACCGATTGGCC	TGGGACAAACCATTGCTAGCCA	TGACCATAGGGCAGGTGAGTTGA
LWF	CGCCATTGTCCACCGACTAGCC	TCGGTCAGGCCATTGCCAGCCA	TAATTATGGGCAGATGGGTGGA
CP	ATCAATTATCCATCGGATAATT	TGGGTCAAAAAATTGCTAGCCA	TAATAAGGGGTAGATTAATTGA
NP	ATCAATTATTCATCGCTTAACC	TCGGCCAAAAAATTGCCAGCCA	TAATTATGGGGAGGTTGATGGA
LMB	AGGGGTTTTCCACCGCCTCATG	TTGGCCAAGCAATTGCCAGCCA	AAGCTAAGGGGGTATTGAGGGA
SMB	CATAGTTTTCCACCGCCTTATG	TTGGCCAAGCAATTGCCAGTCA	AAACTAAAGGGGTGTTAAGGGA
BC	AATAATCACTCACCGCTTCATT	TAGGCCAGTCAATTGCTAGCCA	TAATTAAGGGGTGTTGAGGGA

We targeted the mitochondrial NAD5 gene for our primer and probe design because of the high copy number of mitochondrial genes and taxonomic specificity of this locus (Wilcox 2013). Sequence data for rainbow smelt was obtained from (Genbank, www.ncbi.nlm.nih.gov) and aligned using the Benchling software (Benchling, benchling.com) with homologous sequences for 12 other freshwater fishes that overlap in stream or lake habitat in Maine. There are no other osmeriform fishes in Maine waters, and salmonids would be the phylogenetically closest lineage. Based on these alignments we identified a 134bp amplicon for development of a TaqMan MGB-NFQ qPCR assay. This assay resulted in a minimum of 8 bp

mismatches for the forward and reverse primer and 5 in the probe when compared to the off-target species (Table 3.1). This amplification was further tested for other off-target amplification using BLAST against all available sequences in the NCBI database.

Following in silico design and testing, lab testing was conducted using DNA extracted from fin clips of smelt as well as tissue extracts other common Maine fish species. Tissue samples were extracted using DNeasy blood and tissue kits (Qiagen), and amplification was initially tested with standard PCR under the following conditions: 95°C for 7 min, (95°C 30 second, 60°C 30 sec,72°C for 90 sec) x 30 cycles, 72°C for 7 min.

Sites and Sampling

As noted above, the goal of our first study component was to assess whether smelt eDNA could be detected, even when fish were not directly in the stream, and over what post-spawning time window. For this purpose, we paired smelt eDNA sampling with fyke net surveys that characterized the spawning run dynamics of smelt. The fyke netting portion of the survey, conducted by the Wells National Estuarine Research Reserve (WNERR), is detailed in the report "An Assessment of Spring Fish Communities" (Aman 2018). Briefly, Fyke nets with wings and a first chamber of 0.64 cm mesh and subsequent chambers of 0.32cm mesh were deployed at four coastal streams along the York River between early April and the first week of June in an attempt to sample upstream rainbow smelt and alewife migration. Nets were set in the thalweg of each stream, with the opening facing downstream and net wings extended across two-thirds of the channel. Nets were left to fish overnight for approximately three successive 24-hour periods, with the catch being checked daily at low tide. The fyke netting portion of the study provided estimates of smelt populations at four sites with the York River and Smelt Brook sites having th

highest two smelt catches. The abundance of smelt made these streams ideal for testing eDNA efficacy in the environmental and temporal conditions unique to these ephemeral fish.

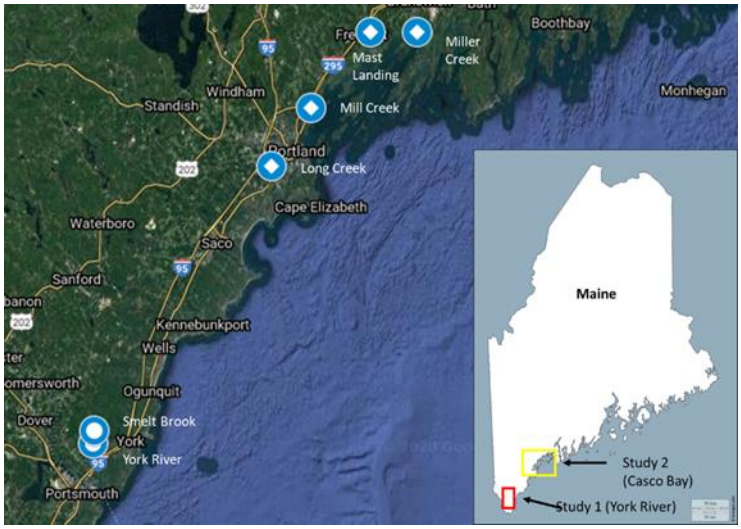

Figure 3.1: Map of surveyed streams: Sites for both studies were located in Southern Maine. Study 1 was centered around the York River, while Study 2 streams were centered around Casco Bay. (Google, n.d.)

For twelve dates in the month of April, eDNA samples were collected during the day at the York River and Smelt Brook sites. For each sampling event, eDNA sampling kits were prepared in a clean lab space to keep supplies free of contamination. Each sample kit consisted of a Ziplock bag large enough to contain two 500mL water bottles (Nestle Pure Life) for a total 1 L sample volume. Four of these kits was prepared per site, three for field samples and one as a control. We also prepared a separate bag to hold gloves, assembled all of these materials in a larger clean trash bag for each site, to keep the sampling gear free from contamination during transport. A cooler was used in taking kits to and from each site. On each day three samples were taken just upstream of and prior to fyke net setting at low tide. One sample was taken at each of river-right, river-center, and river- left of the stream. A negative control was collected at each site by opening a water bottles then closing it, to be used as a test of whether contamination

occurred prior or following collection. Once collected, the samples and the control were placed back into their labeled Zip-lock bags, separated by site in closed trash bags, and transported on ice in a cooler back to WNERR. Samples were then frozen at -20℃ in preparation for their subsequent filtration at the University of Maine.

The second study component's focus was to further refine our assessment of the power of detection of smelt via eDNA sampling using hierarchical occupancy modelling of detection rates. Sites thought to have low-mid smelt abundance were the primary targets of this study for their ability to define limits of power. High abundance sites acted as positive controls for which we would expect high rates of detection compared to the lower abundance sites. Based on Department of Marine Resources (DMR) observations from 2005-2009, we selected sites in Long Creek, Mill Creek, Mast Landing, and Miller Creek (Figure 3.1, Table 3.2). Environmental DNA was sampled near low tide on 15 dates between 3/29/2018 and 5/9/2018, equating to roughly every 2-3 days. Nine of these dates (4/16/2018 to 5/6/2018) were subsequently analyzed for this part of the study based on visual confirmation of the period when eggs were present at spawning areas. We also increased the volume per sample to 2 L (4x500 mL bottles) for each of the right, center and left channel samples along with the negative field control. Because of its very small size, the three samples collected on a given date at Miller Creek were sampled from downstream to upstream at intervals of approximately 2 meters. Again, samples were frozen at -20℃ until filtering at the University of Maine or WNERR

Table 3.2: Stream Summary: All streams observed in studies 1 and 2. Context indicates how each stream was sampled as the strength of the rainbow smelt mating run.

Study/Stream	ID	Town	Latitude	Longitude	Context
1/Smelt Brook	SMBR	York	43.1796490	-70.7349330	Fyke Netted/eDNA-Strong Run
1/York River	York	York	43.1572610	-70.7372680	Fyke Netted/eDNA-Strong Run
2/Long Creek	Long	South Portland	43.633270	-70.333263	eDNA-Weak Run
2/Mill Creek	Mill	Falmouth	43.731386	-70.225159	eDNA-Strong Run
2/Mast Landing	Mast	Freeport	43.859627	-70.0833356	eDNA-Strong Run
2/Miller Creek	Miller	Brunswick	43.8611889	-69.975642	eDNA-Weak Run

Experimental Procedure

Samples and field controls were filtered via vacuum pumping through Whatman 1.5 micron glass microfiber filters. The filters were then frozen at -20^{o}C for no more than two weeks before DNA extraction. If it was known extraction could not be accomplished in two weeks, samples were stored at -80^{o}C to prevent further degradation of DNA. The protocol for extraction followed the DNeasy Blood and Tissue Kit method of Qiagen. In the second study, there was clear evidence of a high level of PCR inhibition, potentially associated with the increased filtering volume. For that reason, we added an inhibition clean-up step to our extraction for these samples (ZYMO Research OneStepTM – PCR Inhibitor Removal Kit D6030). The eDNA filter apparatus was sanitized with 10% bleach solution and rinsed with DI water between samples. Filtering spaces were sanitized before and after use with a combination of bleach solution and UV light.

Quantitative PCRs of samples were conducted on a Bio-Rad CFX96 Real-Time System thermocycler in a 96-well PCR plate format using the thermal profile (Table 3.3). Each extracted sample and cooler blank was run with 3-4 technical replicates with assay concentration of 10µm primer and 5µm probe using the following chemistry: 10µl Taqman Environmental Master Mix 2.0 (Applied Biosystems), 5µl nuclease free water, 2µl of primer /probe/nuclease free H_2O mix, and 3µl of extracted template, for a total of 20µl with reaction concentrations of 1µM primer, 500nm probe and assay concentrations of 10µM primer, 5µM probe. A no-template control was similarly replicated on each plate, but substituted DNA-free water for the template. Positive controls in the form of a dilution series of six known concentrations of synthetic target DNA (Gblocks) were included to provide a standard curve for estimating starting copy numbers of eDNA and testing assay efficiency. An internal positive control (TaqMan™) was run in environmental samples and positive/negative control wells were included for all but three of the test plates per site, to facilitate detection of PCR inhibition.

Table 3.3: Smelt eDNA Thermocycler Settings.

Action	Time	Temperature	Cycles
Enzyme Activation	10 min	95° C	1
Denaturation	10 sec	95° C	47
Annealing	30 sec	60° C	47

Analysis

Efficiency curves were estimated by analysis of covariance of log of the synthetic gene fragments of known concentration (10, 50, 250, 1250, 6250, 31250 copies/µl) against their corresponding quantitation curve (Cq) value. As dictated by Ginzinger et al., 2002, efficiency was calculated as

$$E = -1+10^{(-1/slope)}$$

where the slope of the standard curve generated should be equal to -3.32 for 100% efficiency.

Environmental DNA concentrations per reaction were estimated from the qPCR fluorescence curves using the synthetic gene standard calibration curves. Subsequently, these reaction concentrations were volumetrically converted to copy number per liter based on extraction volumes. These numbers were compared temporally to the raw catch data of the Fyke net portion of the study.

Exclusive to the second study, hierarchical occupancy analyses were conducted in R using an eDNA occupancy package for multiscale, Bayesian models (Dorizo and Erickson 2018). This package utilizes a space-state model composed of two main equations. The first is a binary occupancy state which represents smelt DNA presence or absence on a given day (i). The second equation is dependent on the original binary occupancy state which represents smelt DNA presence or absence in a sample on a given day (j). The second equation can be applied once again for a third tier of the model (k). This tier corresponds to smelt DNA presence or absence within a qPCR replicate of a sample (Mordecai 2011).

1. $Z_i \sim$ Bernoulli (ψ_i)for i=1,2,…N
2. $\mu_{ij} | Z_i \sim$ Bernoulli (θ_{ij}) for j=1,2,… V
3. $y_{ijk} | \mu_{ij} \sim$ Bernoulli (P_{ijk}) for k= 1,2,…S

Where: Ψ= Number of days sampled, θ= Number of samples taken per day, P= Number of replicates per sample for a given day. For each tier, cumulative probability was calculated for a power analysis of the sample design. Cumulative probability was calculated as

$$x^*=1-(1-x)^n \text{ where } x= \psi, \theta, \text{ or P and } n=i, j, \text{ or k.}$$

depending on the tier of the hierarchy.

Results

Study 1 (Pre-inhibition Removal)

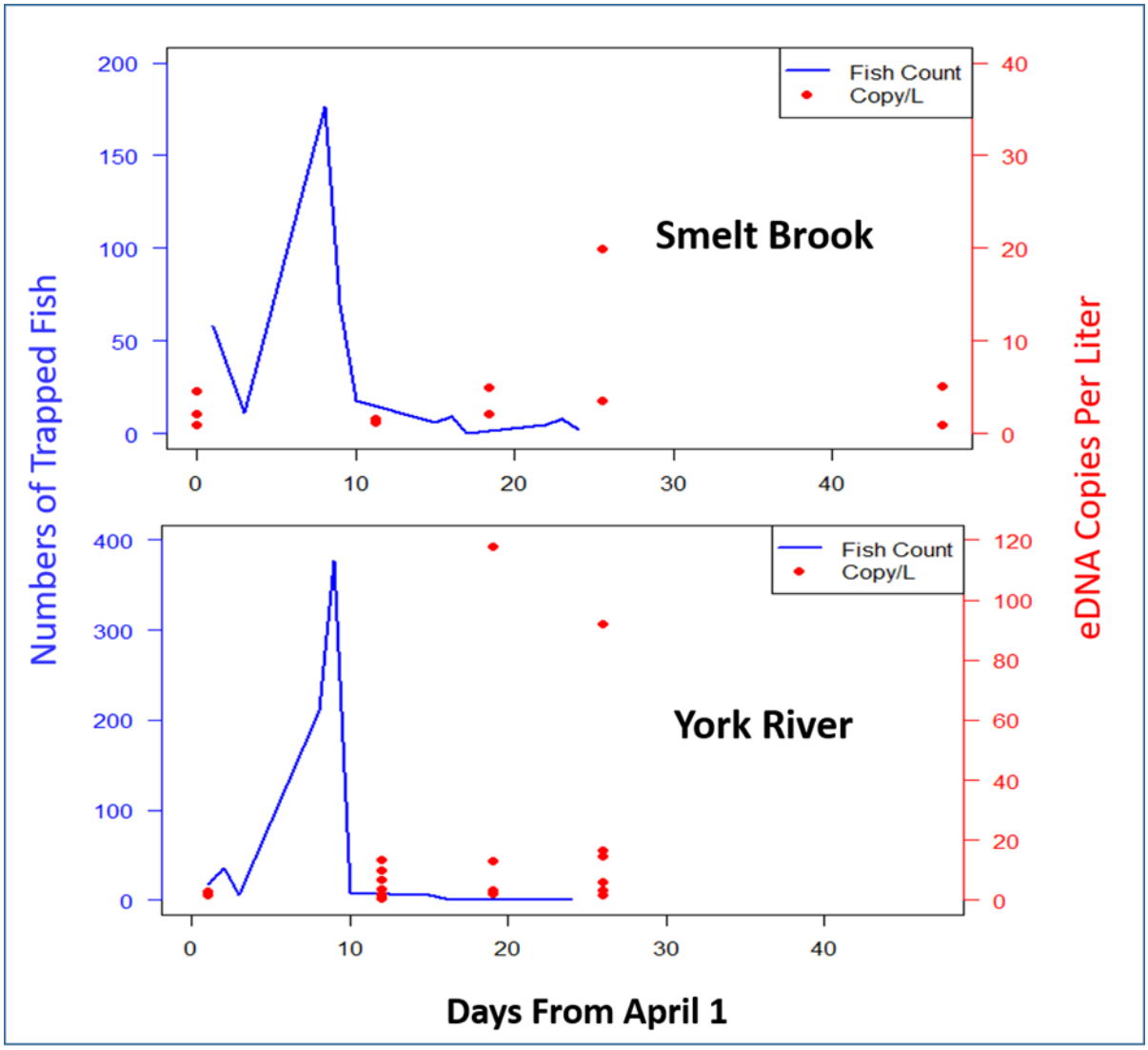

Figure 3.2: Study 1 Results: Smelt trapped plotted with eDNA concentration (copies/L) at Smelt Brook and York River in April 2017 (Aman 2018).

Both Smelt Brook and York River had a peak in smelt catch 8-9 days into the study. However, the highest eDNA results were reported anywhere from 10-18 days after peaks of Fyke

net inferred abundance (Fig 3.2). This time frame corresponds well with regional smelt egg incubation periods (Chase 2006) and informed sampling time frames for the second study.

Study 2 (Post-inhibition Removal)

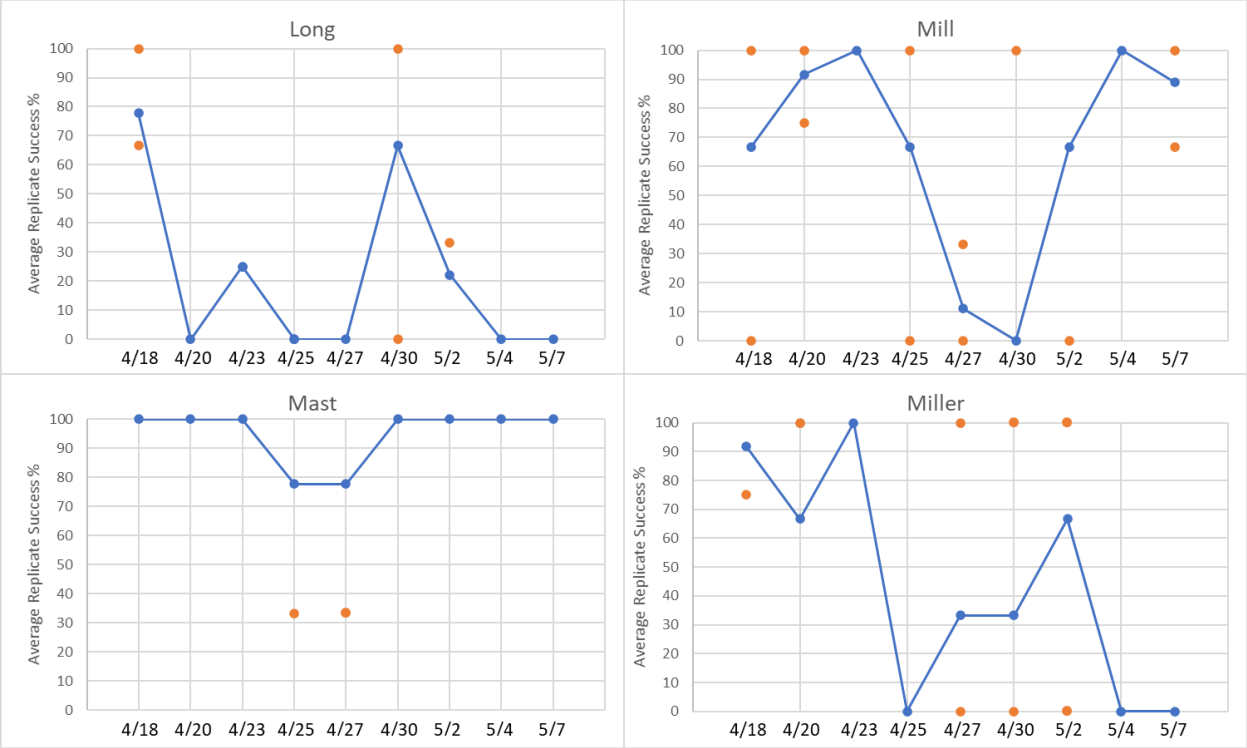

Figure 3.3: Study 2 Amplifications: Average percentage of successful amplifications(blue) out of 12 total replicates (day 4/18-4/23) and 9 replicates (day 4/25-5/7). Other values displayed (orange) are the percentage of successful amplifications for individual samples in a given date. Due to laboratory complications one sample, (three replicates) is unaccounted for at Mast Landing on 4/25.

Following inhibition clean-up, our rainbow smelt assay was successful in consistently amplifying DNA at all sites (Figure 3.3). The sites with higher known smelt abundance, Mast Landing and Mill Creek, typically had more amplifications than the other sites, though this was not always the case.

PCR Efficiency

Our smelt assay amplified smelt tissue extract as expected and did not amplify DNA of non-target species. Analysis of Covariance of Cq values against log copy number of the synthetic targets, indicated 99.5% efficiency 95% (CI of 93.0-107.0%) in the smelt assay (Fig. 3.4) and all technical replicates were positive at the lowest concentration dilution of 10 copies/L.

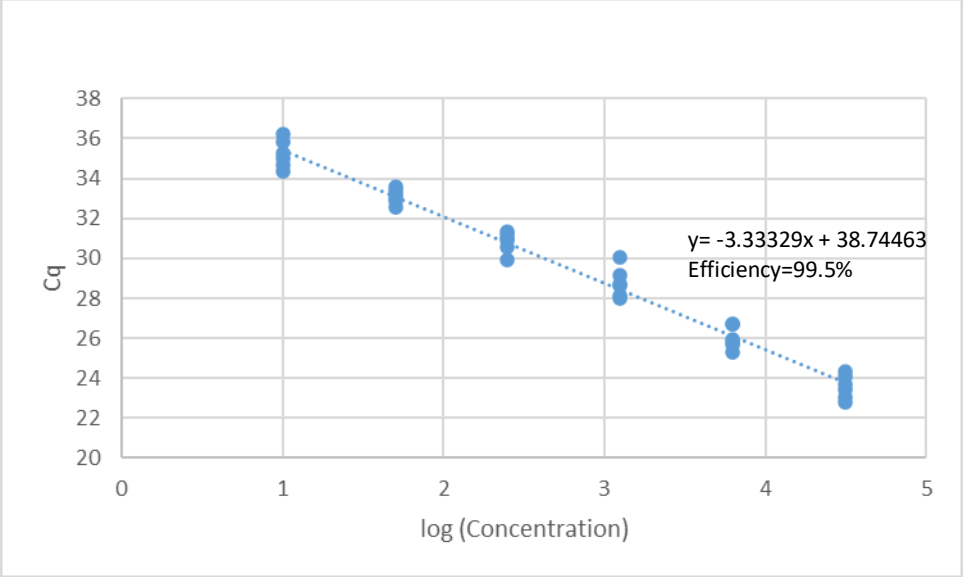

Figure 3.4: Smelt Standard Curve: The standard curve for the synthetic gene fragments of all plates. Cq value plotted against the log of synthetic gene fragments.

Occupancy Modeling

Smelt eDNA was detected at all study sites, with the greatest number of dates, samples, and positive qPCR replicates, at Mast and Mill Creeks and lower numbers of positive detections at the Long and Miller Creek sites. Estimated daily occupancy probability ranged from 0.64 (Miller) to 0.93 (Mill) (Table 3.4). Estimated per sample detection probability ranged from .68 (Long) to 0.89 (Mast) (Table 3.4). Estimated per qPCR replicate positivity probability ranged from 0.41 (Long) to 0.96 (Mast) (Table 3.4). As our study was focused on detection in low

abundance areas the lower probabilities are more impactful on our assessment of sampling design. Using the lower end estimate for each tier, we in turn estimated the number of dates, samples and qPCR replicates require for a cumulated detection probability of 95%. These recommendations are as follows: number of dates (ψ) = 3, number of samples (θ) =3, and number of qPCR replicates (p) = 6 (Fig 3.4A-C).

Table 3.4: Occupancy Parameter Estimates: The parameter estimates for each level of the occupancy model is given along with Average copy number per reaction (SCN/R) and per liter (SCN/L).

Parameter	Long	Mill	Mast	Miller
Ψ (Day)	0.7095993	0.93248	0.9259003	0.6352445
θ (Sample)	0.6810654	0.730571	0.8940187	0.6907244
p (Replicate)	0.4163081	0.903994	0.963147	0.9317895
AVG SCN/R	1.116368	2.866068	18.64544	3.535123
AVG SCN/L	9.303063	23.8839	155.3787	29.45936

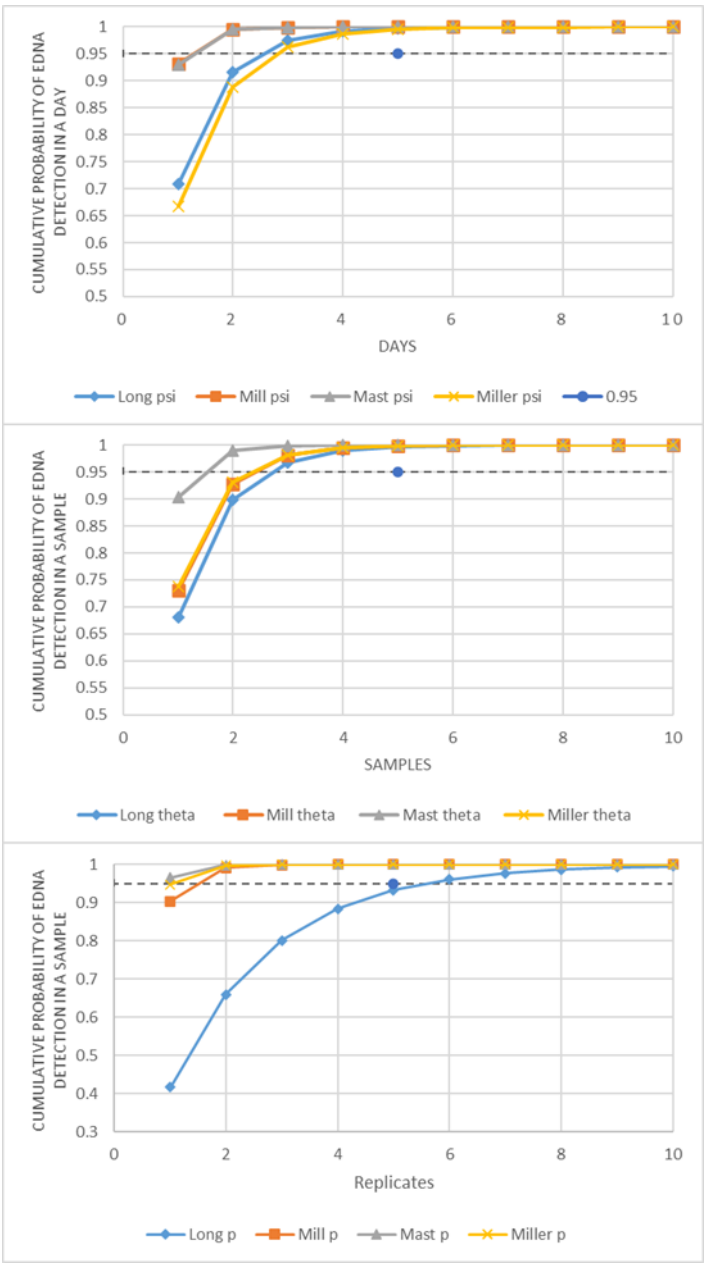

Fig 3.5A-C. Smelt Cumulative Probability Functions: Cumulative probability functions derived from occupancy model for each lake separated by tier (A=Days, B=Samples, C=Replicates). The dashed line denotes a 95% probability of detection for the above values.

Discussion

The goals of these studies were to 1. determine the viability of eDNA methods for sea-run smelt, 2. determine the duration of the detection window after active spawning, and 3. determine what sampling effort improvements could be made in order to increase future detection probabilities ($p \geq .95$). Our findings demonstrate that anadromous smelt eDNA is detectable at low concentrations in coastal streams even when samples are collected during daytime hours when adult fish are expected to have departed the system. Indeed, smelt eDNA can be detectable for weeks after the peak smelt spawning, greatly increasing the opportunity to more efficiently and safely survey for these transient stream residents over what is possible with current netting or visual methods. We further show that our actual eDNA sampling had sufficient power to detect even very low abundance smelt populations, and that this power can be improved further with modest increases in sample processing effort. These findings strongly support the role of eDNA sampling as a powerful tool for surveying anadromous rainbow smelt habitat, and we turn now to placing these findings into context of the biology of rainbow smelt, refinement of sampling design, and some added considerations for applying smelt eDNA assays more widely.

We found that rainbow smelt eDNA can be detected even after adult fish have departed streams. This is the case both on a daily spawning cycle and over the course of weeks following spawning. As previously mentioned, rainbow smelt spawn at night in small streams and typically depart those streams by morning (Chase et al., 2019). Environmental DNA samples were taken during the day, upstream of the fyke nets. As a result, it was unlikely that we detected eDNA being directly shed by upstream adults. Instead, it is likely that we detected holdover eDNA from several complementary sources that vary in importance over the spawning and post-spawning

window. Notably, eDNA did not strongly increase during or immediately following the peak spawning period of smelt as inferred from fyke netting, and was never very high overall (max copies per L) in our first study. This is consistent with most eDNA directly associated with adult fish presence and spawning activity being flushed from these short streams relatively quickly (Aman 2018; Wilcox et al., 2016). However, some eDNA was retained in these systems, which might be attributed to three different sources – eDNA from deposited eggs, eDNA from carcasses, or eDNA bound in biofilms or sediments (Merkes et al., 2014).

Carcass deposition can be an important source of eDNA in some anadromous fishes, such as semelparous Pacific salmon (Tillotson et al., 2018). Anadromous rainbow smelt are not semelparous, but some mortality can be associated with spawning even in iteroparous species (Schaeffer, 1981; Enterline et al., 2012). Dead smelt were not directly observed in the streams during survey activities, but the small size of these fish makes it possible that a few carcasses could go undetected while decomposing over a period of days to weeks. It should be noted that it is possible carcasses make up a smaller percentage of eDNA than living organisms (Yatsuyanagi et al., 2020). This may hold particularly true at low abundances in species such as crayfish (Curtis et al., 2020). Other studies (Turner et al., 2015) of eDNA production and loss have shown that eDNA can be bound by biofilms and sediments and in turn be remobilized under certain conditions. However, one would expect that detectable amounts of bound eDNA and carcass eDNA should decline over time following the peak of spawning activity, as these pools of eDNA should be gradually depleted or flushed during high flow events (Curtis et al., 2020). In contrast, we found evidence that peak eDNA concentrations actually occurred 2.5-3.5 weeks following approximate onset of spawning (inferred from fyke net captures). Although intact fish eggs are not apt to shed much eDNA, incidental death or predation on eggs could gradually release eDNA

long after spawning. Indeed, deposited eggs should become richer sources of eDNA over time as embryos develop, with the greatest eDNA released close to and during hatching. For anadromous smelt, hatching occurs around 3 weeks in our study region (Chase 2006), which coincides very closely with peak eDNA concentrations observed in study 1. Larval smelt emigrate quickly to sea, so it also makes sense that eDNA values dropped off again in our study after about 4 weeks, when hatching was likely completed.

The findings from our initial fyke net study suggest that eDNA detection of rare smelt populations might be best conducted by sampling streams 2-3 weeks following peak spawning. In practice, however, it may be difficult to target sampling with such temporal precision in areas where anadromous smelt populations are low abundance and poorly characterized. As such, it may often be necessary to distribute sampling effort across multiple dates to improve detection probabilities. Likewise, anadromous smelt eDNA was not detected in every sample or qPCR replicate in our initial study. Because of this, we sought to determine how sampling effort might best be allocated across sampling dates, samples and qPCR replicates to provide high probability of detecting rainbow smelt spawning populations using both weak and strong spawning stocks. We in turn used these probabilities to generate cumulative probability functions for a given number of dates, samples, and qPCR replicates (Fig 4A-4C).

Assuming even the most conservative (lowest) detection probabilities from our study, we found a relatively modest level of sampling effort can achieve very high predicted power of detection. For example, it is estimated a maximum 6 qPCR replicates are needed to achieve 96.04% probability of detecting smelt eDNA in a positive sample, 3 samples to achieve 96.76% probability of collecting eDNA when it is present on a given date, and 3 dates of sampling to have 96.32% probability of encountering smelt eDNA in a spawning system. This gives a

combined conditional detection power of approximately 90% in any given year. Our actual sampling effort exceeded this for number of dates (9 versus 3), matched this for number of samples, and was lower for number of qPCR replicates (3-4 versus 6), but nonetheless our odds of detecting smelt were still very good at over 77%.

Looking at stream-to-stream variation in cumulative detection probabilities suggests where effort is most needed in surveying for low abundance smelt populations and why. The number of sampling dates or samples required to detect smelt when present did not vary much among sites, with three dates or three samples per date providing >95% probability of encountering smelt eDNA. This appears consistent with the biological processes giving rise to eDNA encounter rates in space and time. Both large and small smelt populations are expected to spawn in a very synchronized fashion in a given stream even if that timing varies stream-to-stream and we showed that eDNA detection persists for weeks after spawning, so it is reasonable that a relatively low number of sampling dates would be required for most systems. Likewise, the coastal streams studied here are relatively short drainages and smelt typically do not travel very far up these systems to spawn, which likely serves to reduce sampling variability associated with greater opportunities for differences in sampling distances from spawning aggregations and hydrological variability in larger systems.

In contrast, there was a substantial difference between streams at the level of qPCR replicates. For most streams, the probability of detecting eDNA was over 90% per qPCR, indicating a need for as few as two qPCR replicates for >99% probability of detecting eDNA in a positive sample. By comparison, the qPCR detection probability for Long Creek was only 41.6%, suggesting 9 or more replicates would be required to achieve comparable power. We suggest this substantial variation in power at this qPCR replicate level likely reflects the

substantial effect that low population abundance and stream conditions can have on eDNA concentrations where it is encountered. In other words, while smelt abundance does not have much influence on how smelt eDNA is distributed in space and time in these small streams, it does influence its concentration and likelihood of detection in a given qPCR reaction. Indeed, the eDNA concentrations in positive samples for Long Creek were lower than in all other sites (Table 4). That said, detection appears to have increased dramatically with only a few fold increase in eDNA concentrations (e.g., Miller and Mill Creeks). Given the conservation goal that prompted this study is to improve documentation of declining sea-run smelt populations, survey teams may often wish to design their study around the more conservative parameters estimates from our hierarchical occupancy modelling, which are still not logistically unwieldly.

Still, there may be cases where the aforementioned conservative power design is excessive or inadequate depending on a survey's goals. For example, if the goal of stream surveys is to document whether a given stream is ever used by anadromous smelt, and streams will be surveyed in multiple years, then a lower power might be acceptable in any given year given repeated opportunities to detect that population. Likewise, the above suggested survey effort would not be appropriate if the goal of a survey is to estimate precisely when in time smelt spawn in a system in a given year. That type of study would likely benefit from sampling far more dates throughout the potential spawning season than would be required for merely documenting presence of a spawning population. Given our first study results, doing so could require a back-calculation to likely spawning date based on peak eDNA concentrations. Night eDNA sampling to detect actual spawning adults might be a more direct indicator of adult abundances in such a situation.

There are also potential ways to reduce some of the sampling intensity and analysis expenses of eDNA surveys for anadromous Rainbow Smelt. Although, when used in the proper context, eDNA surveys are often less expensive than visual, angling, netting, or electrofishing surveys (Biggs et al., 2015; Huver et al., 2015; Sigsgaard et al., 2015), some populations can be abundant enough where eDNA would likely be unnecessary. If the goal is just to document presence or absence, then eDNA use can be more limited or even avoided entirely where smelt or smelt eggs are already visually observed. At two of the sites in this study, Mill Creek and Mast Landing, eggs were observed by survey teams. We included these sites for the purpose of better understanding smelt eDNA detection, but a study only determining presence of smelt could have immediately excluded these sites from analysis of eDNA samples, saving processing costs. Likewise, if eDNA samples were processed quickly between site survey dates, a survey could save considerably on field time and sample processing by avoiding collecting or analyzing subsequent redundant samples where eDNA is already strongly detected.

Our results support an approximate relationship between eDNA detections and reported abundances, although the relationship does not appear to be linear. The two low abundance streams had less amplifications overall, but only one was estimated to require greater survey effort (more qPCR replicates) than other sites based on the hierarchical occupancy model. Although we did detect smelt eDNA in all four systems, we do recommend that future surveys include each of the types of positive controls employed in our design to be ensured that detection probabilities are comparably high even where smelt are potentially absent. Including known positive sites provided strong confirmation that our field and lab approaches were functioning as planned. Inclusion of synthetic smelt gene fragments demonstrated that our assay was functional, despite some days and samples that were negative. Finally, the inclusion of a

commercial internal positive control nested in field water helped to confirm that severe PCR inhibition was present in our samples. Almost no amplification of smelt eDNA occurred before using a commercial inhibition clean-up step in our extractions for the second experiment. We did not test for inhibition in our first study and eDNA detection rates and concentrations were substantially lower in that study than the second. It could be that difference between studies was associated with inhibition and lower sample volumes (1L vs 2L).

Negative controls, including field ("cooler") blanks, negative filtering controls, and no-template controls are of course always important in eDNA work to control for possible false positive detections. In developing our smelt assay, we also showed that it does not amplify the eDNA of other regional species and that it was negative from field water sampled in locations without smelt. We did not, however, include one type of negative control in this study that might be beneficial for some actual field surveys. In our study we only surveyed sites where anadromous rainbow smelt spawning was known or very likely and where the streams were relatively small and lacked large upstream lakes or impoundments that are sometimes inhabited by landlocked Rainbow Smelt populations in this region. Landlocked and anadromous Rainbow Smelt are the same species and differ very little in ND5 sequences. As such, surveys should be careful in applying our eDNA methods to systems where smelt eDNA might be transported from landlocked populations. If there is some uncertainty of that potential, then survey teams could take samples of eDNA well upstream of anadromous smelt spawning locations to confirm presence or absence of landlocked smelt eDNA prior to or during their sampling for anadromous populations. We also caution that the survey design we have recommended here was designed for small coastal streams. Anadromous Rainbow smelt can also migrate through, or even spawn in, much larger rivers. A subsequent study should be conducted to determine the appropriate

survey design for detecting smelt in those larger systems.

At present, approximately 131 anadromous smelt spawning locations in Maine have uncertain (Enterline 2012) status and the species has been in decline throughout the region. Regularly and reliably surveying these habitats is a daunting prospect with traditional tools given the biology of the species and challenging observation conditions. With our 3 questions answered, it is evident that eDNA can be used to detect rainbow smelt at low abundances for days to weeks following spawning events, greatly expanding the capacity for high-power surveys of smelt status We thus suggest that eDNA sampling can be a powerful and cost-effective tool for future sea-run smelt survey efforts. Moreover, smelt eDNA sampling can be conducted during the daytime and without the need to net fish, or disturb eggs, making this approach safer for both survey teams and low abundance smelt populations.

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APPENDICES

Appendix A

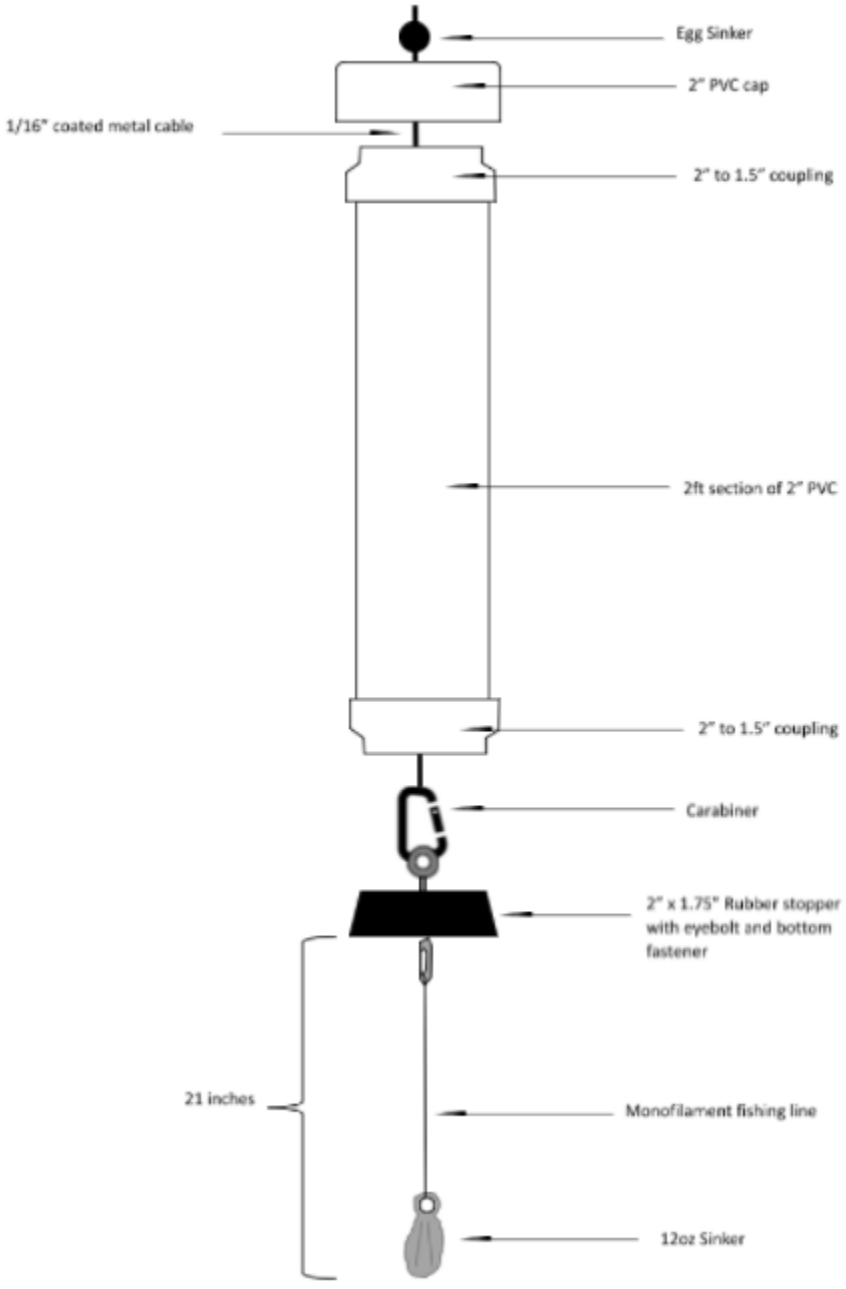

Figure A.1: At-depth Water Sampling Device. Brad Erdman

BIOGRAPHY OF THE AUTHOR

Vaughn Holmes was born in Springfield, Massachusetts in 1993. In 2015 he graduated Merrimack College with a Bachelor's degree in biology and minors in chemistry and mathematics (for some reason). In January 2018 he returned to academia in the Ecology and Environmental Sciences program at the University of Maine. Vaughn is a candidate for the Master of Science degree in Ecology and Environmental Sciences in August 2021.