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Environmental DNA (eDNA) and environmental RNA (eRNA) markers for detection of grass carp (Ctenopharyngodon idella)

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by

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ABSTRACT

Grass carp (*Ctenopharyngodon idella*) pose a threat to ecosystems within the Great Lakes. While the number of grass carp in the Great Lakes is currently low, management of this non-indigenous species will get increasingly difficult as the species establishes and becomes more abundant. Surveillance of grass carp in the Great Lakes therefore requires early, sensitive detection of the species when it is present at low abundance. The goal of this study was to determine the effectiveness of detecting low abundances of grass carp with environmental DNA (eDNA) and environmental RNA (eRNA), while limiting false positives and false negatives. In-lab experiments with aquarium tanks were used to assess eDNA and eRNA detection for low abundances of grass carp over time. This study is the first to detect eRNA from a freshwater vertebrate species in water samples, though only with a non-species-specific marker and without removal of grass carp from the system. On the other hand, species-specific eDNA markers for grass carp were detected for 32 days after removal of grass carp from experimental tanks. This study highlights the potential role of false positives and false negatives of eDNA and eRNA detection as well as the unpredictability of detection when target species abundance is very low.

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CHAPTER 1

GENERAL INTRODUCTION

Non-indigenous species (NIS) have the potential to cause tremendous harm to ecosystems and their native species. These species are introduced by humans to locations outside of their native range, and subsequently establish populations and spread (Mack et al., 2000). Before action to prevent an unwanted species from establishing or spreading can occur, the species must be detected and evaluated for its potential to cause ecological harm (Mahon et al., 2013). Many NIS are unintentionally introduced (Hebert et al., 1991), while others are purposefully moved from their native range and introduced to a new location. Intentional introductions have been documented as far back as fourth century BC (Yan et al., 2001) and have happened for various reasons, including, but not limited to: bringing crops and domestic animals to new locations (Mack, 2003), pet trade (Kraus, 2004), and biocontrol (Simberloff and Stiling, 1996; Mitchell and Kelly, 2006).

One such case of intentional introduction - for the purpose of biocontrol - pertains to stocking of grass carp (*Ctenopharyngodon idella*). Grass carp, native to eastern Asia (Cudmore and Mandrak, 2004), were first introduced in 1963 to control aquatic macrophyte growth in the southern United States. After accidental release into the environment three years later and continued stocking of fertile grass carp, the species thrived and spread (Mitchell and Kelly, 2006). Reproducing populations have since been reported in inland U.S. rivers, such as the Mississippi River (Raibley et al., 1995; Hargrave and Gido, 2004), and grass carp have been caught within the Great Lakes Basin (Wittmann et al, 2014; Cudmore et al., 2017). Wittmann et al. (2014) argued that all the

major Great Lakes, and surrounding area, could provide a physically suitable environment for the species' survival.

Not only is the climate suitable for this species, but the Great Lakes also provide aquatic vegetation in near-shore habitats that grass carp can use as a food source (Wittmann et al., 2017). While species such as largemouth bass, northern pike, and some bird species prey on juvenile carp, adults are not vulnerable to these predators (Cudmore et al., 2017). Juvenile grass carp also grow rapidly and are vulnerable to predation only for a short time (Cudmore et al., 2017). Even when considering predation and pathogens in addition to thermal tolerance and food availability, there are no known factors that would prevent survival of grass carp at any life stage in the Great Lakes (Cudmore et al., 2017). In the Great Lakes and surrounding area, dozens of grass carp individuals have been captured (Wittmann et al., 2014; Cudmore et al., 2017). Previous evidence suggested grass carp were reproducing in or near the Great Lakes as early as 2011 (Chapman et al., 2013). This was supported by microchemistry analysis of otoliths from two diploid (fertile) grass carp captured in the Great Lakes (Chapman et al., 2013). The otolith chemistry reflected an element ratio consistent with what would be expected if the fish had spent their entire lives in the Sandusky River, versus those that escaped from captivity (Chapman et al., 2013). Since then, reproduction of grass carp has been confirmed by the presence of eggs in the Sandusky River, a river that leads into Sandusky Bay, Lake Erie (Embke et al., 2016).

With the presence of reproductively viable individuals within the Great Lakes, along with potential migration from other connected waterways in Illinois (USA), grass carp could establish, if a viable population does not already exist (Wittmann et al., 2014;

Cudmore et al., 2017). If naturally reproducing populations of grass carp establish and spread within the Great Lakes, there could be severe ecological consequences. For example, grass carp can consume large amounts of nuisance vegetation (Swingle, 1957; Sills, 1970), and high abundances also reduce or eliminate desirable plant species and alter community composition (Bain, 1993; Dibble and Kovalenko, 2009). Grass carp may even selectively eliminate native plants while invasive plant species are left unharmed (McKnight and Hepp, 1995; Hanlon et al., 2000; Pipalova, 2006). Once established, spread of grass carp could exacerbate these problems. Waterfowl, for example, are attracted by aquatic vegetation and are driven away when grass carp eliminate these aquatic plants (Gasaway and Drda, 1976; Gasaway et al., 1977). Grass carp also have secondary effects on macroinvertebrates and fish. Both abundance and diversity of macroinvertebrates decrease owing to grass carp grazing vegetation (Dibble and Kovalenko, 2009). These declines in vegetation can cause increased competition for limited resources and decreased abundances of fish species such as sunfishes (*Lepomis spp.*) and juvenile largemouth bass (*Micropterus salmoides*) that depend on aquatic plants (Bettoli, et al., 1993). Some species of crappie (*Pomoxis spp.*) that are moderately dependent on aquatic plants also decrease in abundance due to grass carp overgrazing (Bettoli, et al., 1993). At least 33 fish and 18 bird species could experience severe consequences due to aquatic vegetation consumption by grass carp in the Great Lakes; 33 other fish species could experience moderate consequences (Cudmore et al., 2017; Gertzen et al., 2017).

In 2016, a binational effort was conducted by Canada and the United States to assess the potential risk of grass carp to the Great Lakes. It determined that establishment

and spread to lakes Erie, Michigan, Ontario, and Huron by 2026 is very likely, though the species had a low likelihood of spreading into Lake Superior within 50 years (Cudmore et al., 2017). While low initially, ecological consequences of grass carp are expected to worsen over time (Cudmore et al., 2017). Within 50 years, consequences of grass carp are projected to range from high for Lake Ontario to extreme for lakes Huron, Michigan, and Erie (Cudmore et al., 2017). Efforts to detect presence of this species has been ongoing (Mahon et al, 2013; USFWS, 2015), owing to concerns of potential establishment (Chapman et al., 2013; Wittmann et al., 2014), spread, and future impacts of grass carp on the Great Lakes (Dibble and Kovalenko, 2009; Cudmore et al., 2017; Gertzen et al., 2017).

Environmental DNA Detection

It is important to detect potentially harmful NIS early (Simberloff and Gibbons, 2004), as controlling species after invasion is difficult and costly as abundance increases (Pimentel et al. 2005; Pejchar and Mooney, 2009). Detecting species early and at low abundance can be difficult, however. Surveys of rare and low-abundance species frequently generate non-detection errors, as has been widely demonstrated for terrestrial species (Gardner et al., 1999; Gu and Swihart, 2004). For aquatic species hidden below the water surface, detection can be especially difficult. Traditional surveillance methods for NIS use fishing gear such as nets and electrofishing equipment (Jerde et al., 2011). These traditional methods are often unsuccessful at capturing low abundance NIS and require a tremendous and/or an impractical increase in sampling effort for adequate detection power (Magnuson et al., 1994; Dettmers et al., 1999; Hoffman et al., 2011;

Jerde et al., 2011). To detect the presence of low-abundance species - such as grass carp in the Great Lakes - detection methods that are more sensitive than traditional surveillance methods must be employed.

Detection sensitivity has been increased through eDNA surveillance. DNA present in the environment outside of an organism, termed "environmental DNA (eDNA)" (Taberlet et al., 2012), is a possible source for early detection of grass carp in the Great Lakes. Organisms such as grass carp leave DNA behind through excretion of waste and by leaving behind their remains, which can then be detected in water without directly sampling the target organism (Ficetola et al., 2008). Techniques involving detection of fish eDNA were developed by those seeking to detect American bullfrog (*Rana catesbeiana*) in Europe (Ficetola et al., 2008). Since 2009, eDNA surveillance techniques have been used for early detection of bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*) in surveys in the Chicago Area Waterway System (CAWS) (Lodge et al., 2012; Jerde et al., 2013; ACRCC, 2015). Compared to traditional surveillance methods, eDNA requires less sampling effort and is more sensitive to Asian carp presence (Wilcox et al., 2016). Traditional surveillance not only involves a great deal more effort than eDNA surveys to adequately detect rare species (Wilcox et al., 2013), but Asian carp have been known to avoid capture by conventional fishing gear (Lodge et al., 2012), especially when present at low abundance (USFWS, 2015). The high sensitivity of eDNA detection for Asian carp compared to traditional methods was demonstrated by Jerde et al. (2011). Traditional surveillance (i.e. electrofishing) was less sensitive than eDNA surveillance when comparing the same sampling locations. In one location, eDNA positively detected carp where traditional

surveillance failed to detect them (Jerde et al., 2011). The presence of bighead carp was, however, confirmed afterwards when rotenone was applied to the location (Jerde et al., 2011). Thus, eDNA surveillance can be a powerful tool for surveillance of carp species (Ficetola et al., 2008) and is more sensitive to carp presence than traditional surveillance techniques (Jerde et al., 2011; Wilcox et al., 2016).

False Negatives and False Positives

While surveillance with eDNA can be more sensitive than traditional methods, target organisms can still go undetected. This can occur even when target organisms are highly abundant where water samples are taken (Mahon et al., 2013). When eDNA surveillance fails to detect eDNA in a sample or fails to detect the target species despite the presence of target organisms, a 'false negative' or 'type II error' is committed (Darling and Mahon, 2011; Zhan and MacIsaac, 2015) (Figure 1.1). Target presence may be known, yet their DNA can still go undetected because of stochastic processes and low concentration resulting in imperfect amplification (Ficetola et al., 2008; Amberg et al., 2015; Ficetola et al., 2015). Furthermore, random sampling and preparation of samples involving eDNA of species at low abundances (i.e. newly NIS) can lead to inconsistencies in detection (Zhan et al., 2014). During the initial stages of NIS introduction, low abundance can make it difficult to effectively detect the presence of these newly invading species (MacIsaac et al., 2002; Hoffman et al., 2011). Detection thresholds can be increased by increasing sampling intensity; however, low-abundance species may still be undetected (Harvey et al., 2009).

Amberg et al. (2015) reported that current eDNA techniques have not been optimized for consistent detection of Asian carp. In one study, eDNA surveillance only detected bighead carp in 60% of water samples where the species was abundant (Jerde et al., 2011). In another study testing multiple markers, including a marker used in Quality Assurance Project Plan (QAPP) monitoring, the highest detection for all bighead carp markers was 20% despite testing in a water body known to have high abundances of bighead carp (Farrington et al., 2015). For grass carp specifically, Wittmann et al. (2014) reported that of over 500 environmental samples taken throughout western Lake Erie, Lake St. Clair and Ohio waters showed no positive detections of grass carp, despite capture of 45 grass carp individuals within the Great Lakes Basin (including Lake Erie and the Sandusky River (Ohio)). This highlights the need for more robust methods of detection before potential establishment and spread of grass carp in the Great Lakes.

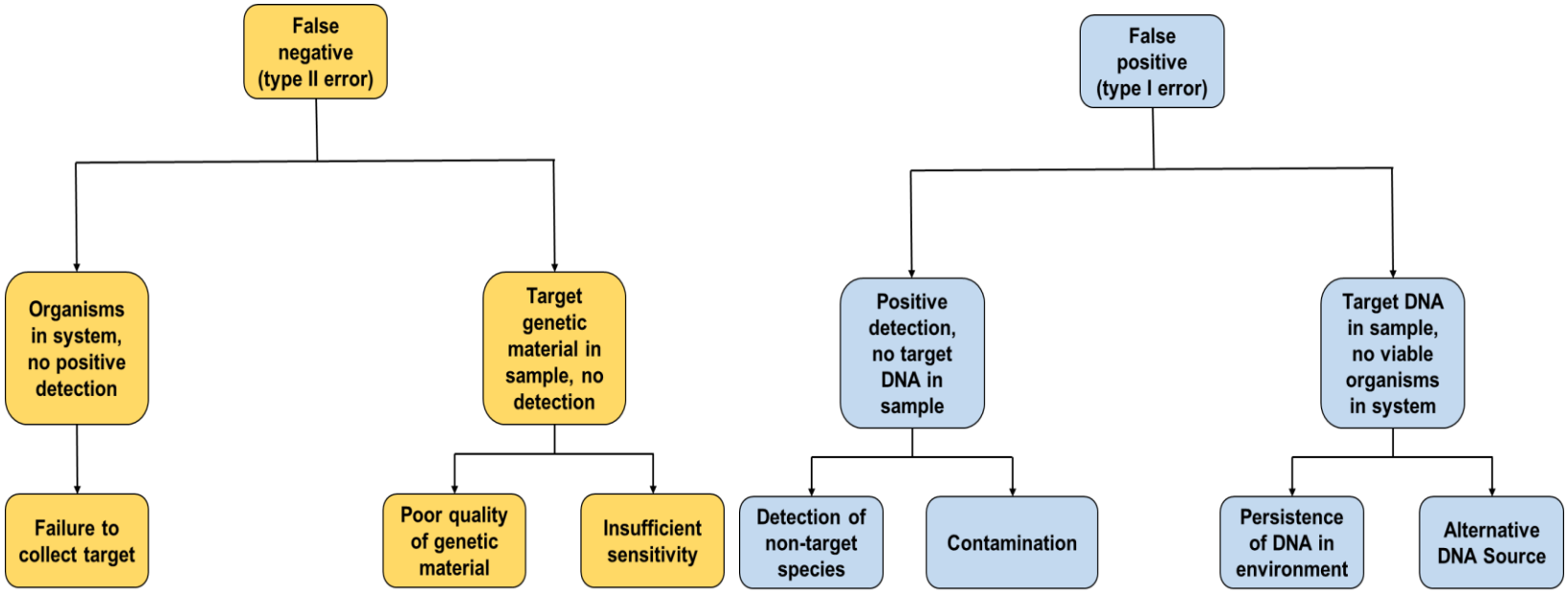
Surveillance of eDNA from NIS also suffers from false positives, where positive detection occurs despite absence of the target species or target DNA (Darling and Mahon, 2011; Zhan and MacIsaac, 2015) (Figure 1.1). Persistence of eDNA, especially in sediment, can cause false positives when sediment is disturbed during water sampling (Turner et al., 2015). Detection of eDNA that has persisted for weeks in the sediment can lead to the erroneous conclusion that the NIS was recently within the sampling area (Roussel et al., 2015; Turner et al., 2015). Predator faeces and dead organisms can also be sources of target eDNA, further complicating attempts at surveillance of NIS. A predatory bird for example could prey on fish in one location and then deposit the prey eDNA in a water body where that fish species has never been present (Darling and Mahon, 2011; Wilcox et al., 2013; Roussel et al., 2015). Resources would then be wasted

trying to find or eliminate the NIS in an area where it does not occur owing to false positives. Hence, current eDNA methods suffer from false positives that could lead to false conclusions about presence and how recently NIS occupied an area of interest. It is therefore imperative to limit false positives in eDNA surveillance.

Thesis Objectives

The primary goals of this thesis were to develop molecular genetic tools useful for early detection of grass carp in the Great Lakes and to address potential problems of false positives and false negatives so that early detection of NIS can be efficient and reliable. In Chapter 2, I tested the capabilities of multiple eDNA markers for eDNA detection to determine which would be the most sensitive for detection of grass carp. I then addressed detection errors by testing detection using low eDNA concentrations from two low-abundance treatments of grass carp. This allowed me to determine the false negative rates of each treatment and to determine the effect of low-abundance differences on detection of an NIS (i.e. grass carp). I also aimed to demonstrate changes in detection and amount of eDNA as it accumulated temporally. In Chapter 3, I tested the viability of eRNA as a tool to limit false positives. My thesis would represent the first attempt of detection of a freshwater vertebrate species using eRNA, with the objective of increasing information gained and improving true detections. I tested and compared the persistence of eDNA and eRNA to determine their potential to limit or generate false negatives or false positives in species detection. Overall, my study aimed to provide insights into the advantages and limitations of surveillance of NIS using environmental genetic material (eDNA and eRNA) for detection.

Figure 1.1. Sources of false negatives and false positives (type II and type I errors respectively) in eDNA surveillance of aquatic non-indigenous species, modified from Darling and Mahon (2011).



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CHAPTER 2

EFFECTS OF ABUNDANCE AND ACCUMULATION OF eDNA ON SPECIES DETECTION

Introduction

It can be difficult to understand how eDNA methods perform over various abundances of target species (Roussel et al., 2015). Many factors may affect the accuracy of abundance estimates. For example, filter type (Lacoursière‐Roussel et al., 2016), seasonal and temperature changes (Erickson et al., 2017), water flow (Gingera et al., 2016) and depth of sampling (Moyer et al., 2014; Klobucar et al., 2017) can all alter estimates of species abundance in aquatic systems. Furthermore, detecting a species with a small number of individuals, such as a newly introduced NIS, frequently results in false negatives (Dettmers et al., 1999). For example, in an experiment with aquarium tanks containing crayfish, 100% of samples from a tank of 100,000 individuals resulted in positive detection of eDNA (Cai et al., 2017). Detection decreased to only 55.6% for tanks containing just one individual, (Cai et al., 2017). Low abundance during the initial stages of NIS introduction can make these species especially difficult to detect (MacIsaac et al., 2002). For Asian carp, non-detection can still be an issue, even in areas where they are abundant (Jerde et al., 2011). For example, one previous Asian carp survey had a 40% non-detection rate in areas where bighead carp were highly abundant (Jerde et al., 2011). Low abundance will only decrease the detection rate of Asian carp further (Wittmann et al., 2014; Cai et al., 2017). In addition to these increases in false negatives owing to low

abundance, true positives may be filtered out of datasets during sequence processing, creating false negatives (Willerslev et al., 2014; Zhan et al., 2014; Ficetola et al., 2015).

Grass carp are still in relatively low abundance in the Great Lakes, but this species may establish a reproducing population if one does not already exist within these systems (Wittmann et al., 2014; Embke et al., 2016). After establishment, it is possible that grass carp will spread and cause severe negative consequences across the Great Lakes region, impacting waterfowl, native plant species, macroinvertebrates, and other fish species (Gasaway and Drda, 1976; Gasaway et al., 1977; Ware and Gasaway, 1978; Pipalova, 2006). Spread and increasing ecological impacts of grass carp in the Great Lakes are forecasted to occur within next decade (Cudmore et al., 2017), and will be difficult to control or eliminate as the species' abundance increases (Simberloff and Gibbons, 2004; Pimentel et al. 2005; Pejchar and Mooney, 2009). Thus, detecting grass carp at low abundances is imperative to prevent spread or to find and eliminate this species in the Great Lakes (Jerde et al., 2011, Embke et al., 2016; Cudmore et al., 2017).

Detecting grass carp requires techniques that are sensitive to extremely low abundances. Quantitative real-time polymerase chain reaction (qRT-PCR) surveillance can be faster for large sampling efforts, and more sensitive to target presence as compared to conventional PCR (cPCR) and traditional surveillance (Jerde et al., 2011; Beard et al., 2013; Wilcox et al., 2016). Moreover, qRT-PCR can be used to determine eDNA semi-quantitatively while being less costly than TaqMan (Beard et al., 2013; Farrington et al., 2015). The sensitivity and cost effectiveness of qRT-PCR makes it a powerful tool for detecting and comparing low abundances of grass carp.

To determine how different abundances of individuals affect detection of grass carp, I collected water samples from two experimental low-abundance treatments of grass carps in tanks in a controlled setting. I tested multiple primers to determine the most effective primer to use for detecting low abundances of grass carp with qRT-PCR. Since eDNA can persist for weeks in an aqueous environment (Turner et al., 2015), I expected that eDNA would accumulate over time during an experiment, and detection would increase with time that grass carp are in the tanks. I hypothesized that there would be an increase in detection and relative amount of eDNA in a treatment of multiple grass carp compared to tanks housing only single individuals. Since increasing abundance produces a non-linear increase in detection (Cai et al., 2017), and interactions between multiple grass carp will likely release more eDNA compared to a solitary fish, I hypothesized that the increase in eDNA generated from tanks housing multiple grass carp would be positive and non-linear (greater than 1:5 ratio between abundance treatments of 5 and 1 grass carp).

Methods

Experiment Design and Sampling

Experiments were conducted at the Wetland and Aquatic Research Center in Gainesville, Florida, USA, in collaboration with the USGS (United States Geological Survey) owing to restrictions on housing live grass carp in Canada. In August 2016, the USGS obtained 40 triploid (sterile) grass carp juveniles (20cm total length) from Florida Fish Farms Inc for weed control of onsite ponds. Before release into ponds, USGS animal care staff held grass carp in indoor cuboid tanks (208cmx56cmx57cm, each filled with

475L of water and two air pump inputs) which allowed me to simulate two low abundance treatments. These tanks had never before held grass carp, and inner surfaces were treated with 10% bleach to ensure no DNA contamination was present prior to experimentation. Tanks were also sampled in triplicate before introducing grass carp to ensure no contamination. To determine the differences between two low abundance treatments, I sampled water from a total of eight of tanks: three tanks held one grass carp juvenile, with one negative control tank with water only, and another three tanks had five individuals each with another negative control tank of water only. Mesh netting was fitted over top of the tanks to prevent escape. The grass carp in each tank were fed a half cup (120mL volume) of pet food pellets by USGS animal care staff each morning after I took water samples and had a mixed diet of hydrilla and fish food pellets before being put into tanks. Water samples were taken at the surface where detection of eDNA tends to be greater (Moyer et al., 2014). I used new gloves between sampling each tank to avoid cross contamination. Water samples were taken with 50mL conical tubes with 5mL of RNAlater™ preservative added to each to limit degradation. Samples from each tank were taken at 0, 1, 12, 24, 48, and 72 hours after grass carp were introduced to the tank, with three replicates samples taken for each tank at each time point (total n = 144, 72 samples for each abundance treatment). In addition to eDNA water samples, eRNA samples were also taken to compare RNA and DNA detection. However, eRNA was not detected in any sample. All 50mL samples were flash frozen using dry ice and stored at -20°C. While detection surveillance of target organisms in the field requires water samples of one litre or greater, the scale of lake or river sampling is much greater than in-tank sampling (Gingera et al., 2016). Smaller scale studies have used as little as 15mL of

water for detection of eDNA (Dejean et al., 2011). In this experiment, I took six (three eDNA and three eRNA) 50mL samples at each time point which kept the tank water volume difference between the beginning and end of the experiment negligible. Animal care workers measured the total length and biomass of each grass carp after the experiment. I recorded temperature daily at the time of sampling from in-tank temperature probes. Due to contamination concerns, dissolved oxygen and pH were measured only after fish were taken out of the tanks and experimental sampling was completed.

Filtration

I used Whatman® glass microfibre filters of 1.5µm pore size and 4.7cm diameter for filtration owing to their fast flow rate and superior yield of eDNA as compared to other filter types (Eichmiller et al., 2016; Lacoursière−Roussel et al., 2016). During filtration, the filter captures cells that are later lysed to extract eDNA. The silica-based microfibre filters also attract negatively charged DNA molecules that are tightly adsorbed to the filter surface, with greater selectivity than nitrocellulose and nylon matrices (Tan and Yiap, 2009). Benchtop and equipment surfaces were cleaned with 10% bleach before and after filtration occurred. Water samples were thawed at 4°C and then immediately filtered with a vacuum-pump system once thawed. Sample filters were then placed into 15mL tubes of RNAlater™ preservative and stored at -20°C. Sterile forceps were used to handle the new filter for each sample. Between samples, after removing the sample filter, all filtration equipment (magnetic cups, rubber stoppers, filter holders) was submerged in a 20% bleach bath for 10 minutes followed by a Milli-Q® ultrapure H_2O

bath for 10 min, then rinsed again with ultrapure water before drying in a sterile fume hood under ultraviolet light. Forceps were also sterilized between samples with 20% bleach for 10 min, ultrapure H_2O for 10 min, and then dipped in isopropanol and burned to ensure any residual bleach was evaporated. Some samples were not filtered while in Florida and were transported to Great Lakes Institute of Environmental Research (GLIER), Windsor, Ontario, Canada, on ice for later filtration. All samples remained frozen and were then stored at -20°C once again. Filtration equipment and forceps (wrapped in a Kimwipe to avoid contamination of forceps) were air dried for 20 minutes between samples instead of using a sterile fume hood with ultraviolet light and isopropanol when filtering at GLIER.

DNA Extraction

I extracted DNA from the experiment sample filters and I used grass carp fin clip tissue received from the USGS (Columbia Environmental Research Center) as positive controls. Benchtop surfaces were sterilized with a solution of 10% bleach prior to extractions. Before cutting tissue or sample filters, forceps and scissors were put into a solution of 20% bleach for 10 minutes, rinsed well with ultrapure H_2O, put into ultrapure H_2O for 10 minutes, and then air dried for 15-20 minutes covered by a Kimwipe. Fin clip tissue or sample filter halves containing eDNA were added to a 2mL screw cap tube containing 500µl of CTAB buffer and 0.5cm^3 of (1mm diameter) glass beads. I then homogenized the samples at 5,500rpm 5 times or until the sample was fully homogenized. Samples were then incubated in a 60°C water bath for one hour and transferred afterwards to new 1.7mL Eppendorf tubes. A volume of 5µl Proteinase K

solution (20mg/mL) was then added and mixed to digest samples overnight (8-12 hours) at 37°C. I then transferred sample DNA to a 96-well plate before storage at -20°C. A lab technician then completed extraction of the DNA plate using a DNA extraction robot after I incubated samples again at 60°C for one hour. Sample DNA was then suspended in TE Buffer and stored once again at -20°C. I assessed both DNA quality and estimated amount (ng/µl) using a NanoDrop spectrophotometer.

Primers and Primer Testing

Initial primer testing consisted of three species-specific primers and one non-specific primer. The three species-specific primers were based on: a mitochondrial cytochrome b (CytB) marker (Hunter et al., 2017), Cytochrome c oxidase I (COI) (Bronnenhuber and Wilson, 2013), and NADH subunit 2 (ND2) (Table 1). The non-specific primer was based on a grass carp 18S rRNA gene (Su et al., 2011) (Table 1). Serial dilutions of positive grass carp DNA were used to determine primer sensitivity using previously published annealing temperatures for each primer (Su et al., 2011; Bronnenhuber and Wilson, 2013; Mahon et al., 2013; Hunter et al., 2017). The ND2 and COI primers were not tested further after they did not amplify diluted DNA.

Grass carp are the only species within the genus *Ctenopharyngodon* and thus the risk of non-target amplification with primers designed for grass carp is reduced (Mahon et al., 2013). In 2016, I confirmed the species-specificity of the (then unpublished) CytB primer pair using the Genbank (https://www.ncbi.nlm.nih.gov/tools/primer-blast) primer-BLAST function. I also tested the primers against two other members of the Asian carp group: silver carp and black carp. I then used extracted grass carp, black carp, and silver

carp DNA to determine species-specificity of the CytB primer and 18S primer with cPCR and qRT-PCR. The non-specific 18S primer was used as to amplify any Asian carp 18S sequence. Carp DNA was extracted from fin clips stored in 70% ethanol, with grass carp fin clip DNA used as positive controls and black/silver carp DNA used as negative controls along with one non-template control. The cPCR master mix consisted of: 12.4µl ultrapure H_2O, 2.5µl of 10X Taq reaction buffer, 3.5µl of MgSO4 (20mM), 0.5µl of dNTPs, 0.5µl of forward primer, 0.5µl of reverse primer, and 0.1µl Taq DNA polymerase. One master mix was made for each primer (CytB and 18S). Each reaction consisted of 20µl of master mix with 1µl of DNA. The 21µl reaction was then run on a thermal cycler at 95°C for 5 minutes, and then 40 cycles of 95°C for 30 seconds with a 53-61°C (for CytB) or 58-65°C (for 18S) annealing stage for 1 minute and 72°C for 45 seconds, with a final elongation stage of 72°C for 5 minutes. The annealing temperature gradient was used to find the optimal annealing temperature for each primer. The annealing temperature and species-specificity was then assessed using gel-electrophoresis. Species specificity and annealing temperature were also assessed using quantitative real-time PCR. Finally, I field tested the CytB primer in August 2016. With permission from the Center for Aquatic and Invasive Plants (University of Florida, Institute of Food and Agriculture Science), an 884m^2 outdoor pond (29°43′35.87″N 82°24′59.32″W) known to have adult grass carp ($n \geq 4$) was sampled (500mL) to confirm that the primer would detect eDNA in the field.

Quantitative Real-Time PCR

To detect DNA in extracted samples, I used qRT-PCR at GLIER with PowerUp SYBR Green Master Mix (Applied Biosystems) and a Quantstudio™ 12K Flex Real-Time PCR System (Applied Biosystems). SYBR Select Master Mix was used initially, however I switched to PowerUp SYBR Green Master Mix while attempting to detect eRNA because of it's reported superior specificity and sensitivity (Thermo Fisher Scientific, 2016), plus it was less costly than SYBR Select at the time of ordering. From personal communication, other lab members reported better results with lower cycle threshold (Ct) values. Ct values are an indirect measure of initial template DNA or complementary DNA (cDNA) concentration in a qRT-PCR reaction. Binding of SYBR Green to double-stranded DNA or cDNA in the qRT-PCR reaction results in fluorescence (Nolan et al., 2006). With each PCR cycle, more copies of DNA or cDNA are synthesized (Higuchi et al., 1993; Nolan et al., 2006). SYBR Green can then bind to more double-stranded copies and emit greater fluorescence as the number of PCR cycles is increases. As the number of DNA or cDNA copies increases with each cycle, fluorescence eventually crosses a threshold that exceeds background fluorescence (Nolan et al., 2006). This threshold is automatically set by the qRT-PCR instrument (Quantstudio™ 12K Flex Real-Time PCR System (Applied Biosystems)), where the threshold intersects the exponential phase of the fluorescence curve (Life Technologies Corporation, 2012). The number of PCR cycles it takes to cross the threshold is the Ct value. The greater the initial DNA or cDNA concentration, the less PCR cycles it takes for fluorescence to surpass the threshold (Higuchi et al., 1993), which results in lower Ct values. To determine Ct values for my samples, I ran 20µl reactions using 10µl of SYBR

Green, 0.5µl of forward primer, 0.5 µl of reverse primer, 4µl of ultrapure H_2O and 5µl of template DNA in each well of a 96-well plate. Each qRT-PCR plate ran at 95°C for 10, followed by 60 cycles of 94°C for 15 seconds and 56°C (or 63°C for testing 18S) for 1 minute. Three PCR replicates were run for each sample and both an NTC (no template control) and positive grass carp fin clip control were included in triplicate for each qRT-PCR 96-well plate. Two plates where negative or positive controls failed were rerun and DNA was extracted again with new controls. All other NTC and equipment controls were negative throughout the study indicating no sample contamination. The mean Ct value of each sample triplicate was used in the analyses after correcting for PCR efficiency.

PCR efficiency

To compare Ct values among eDNA samples using Powerup SYBR Green Master Mix and eDNA samples using SYBR Select Master Mix reagent, I determined PCR efficiency using a standard curve. I generated standard curves using serial dilutions of template DNA, the CytB primer, and both SYBR Green reagents (Figures 1.2 and 2.2). PCR efficiency was calculated based on the slope of the standard curve according to the equation (Yun et al., 2006):

$$E\% = \left(10^{\left(-\frac{1}{slope}\right)} - 1\right) x100$$

Where "E%" is the PCR efficiency and "slope" is derived from the slope of the standard curve. Ct values were corrected using the PCR efficiency of the corresponding SYBR Green reagent used. The standard curves were also used to calculate the LOD (limit of detection) for the CytB primer used in this experiment. I defined the LOD as the upper 95% confidence interval using the mean Ct value of the lowest triplicate where at least

two of three replicates were positive (mean Ct +1.96(√SD)). However, Ct values beyond the limit of detection were not considered false negatives as LOD is not a limiting value, and disregarding Ct values beyond the LOD could produce false negatives (Kralik and Ricchi, 2017). Although some Ct values were higher than the estimated LOD, NTC controls account for possible contamination and primer dimer formation. If the fluorescence curve generated by the sample reaction reached the plateau phase (Life Technologies Corporation, 2012) and NTC controls were negative, I considered the Ct value to represent true detection. Thus, Ct values that were higher than the estimated LOD were not automatically excluded from analysis.

Data Analysis

For some tank samples, eDNA was undetected during qRT-PCR, which I considered to be false negatives. I calculated the false negative rate for each abundance treatment from the number of samples that resulted in no detection. I used Welch's t-test to determine if there was a significant difference in detection between the two abundance treatments using the number of false negatives from sample replicates.

To determine if using two different SYBR Green reagents would have a significant impact on qRT-PCR Ct values, I compared Ct values of three replicates of an eDNA sample using Powerup SYBR Green Master Mix and another three replicates of the same sample using SYBR Select Master Mix for qRT-PCR. I performed a one-way ANOVA using Ct values without correcting for PCR efficiency and another ANOVA after correcting for PCR efficiency to test the efficiency correction.

After removing SYBR Green as a variable, I performed repeated measures ANOVA to compare Ct values (response variable) between the two experimental abundance treatments. I analysed the significance and interactions of each variable, where "abundance" was the one versus five grass carp treatment as a categorical variable, "time" was a continuous variable representing hours after first putting grass carp into experimental tanks, and "tank" represents each individual experimental tank. A Shapiro-Wilk normality test (Shapiro and Wilk, 1965) was done to validate the assumption of normal distribution. I then performed a repeated measures ANOVA for each abundance treatment separately to determine the effect of time on eDNA amount.

Results

All four primers (Table 1) detected DNA extracted from grass carp tissue. However, grass carp were not detected with the primers ND2 (Mahon et al., 2013) and COI (Bronnenhuber and Wilson, 2013) with subsequent dilutions of DNA. The 18S (non-specific) primer amplified both grass carp and black carp DNA but failed to amplify silver carp DNA. Each grass carp sample was positive while silver and black carp samples were negative (no DNA detection), which supported the species-specificity of the CytB primer. Of 15 water samples taken from a pond for field testing, 12 were positive for grass carp with a mean Ct value of 33.46 when corrected for PCR efficiency (SEM ± 1.7). PCR efficiencies were within an acceptable range (80-120%) (Yun et al., 2006), where PCR efficiency using the CytB primer was 111.6% when using SYBR Green Select, while PCR efficiency was 90.7% when using SYBR Green Powerup (Figure 1.2 and 2). Ct mean values were corrected using the PCR efficiency of the

corresponding SYBR Green reagent. The upper limit Ct value (within a 95% confidence interval) for the CytB primer was 36.90 for SYBR Select and was 36.87 for PowerUp SYBR without PCR efficiency correction.

For some qRT-PCR reactions, fluorescence did not cross the threshold, resulting in no Ct value. Absence or low concentration of eDNA in the sample results in a fluorescence that is not distinguishable from background fluorescence (false negative). When not including control tanks in which undetected sample reactions are expected, 38% of qRT-PCR reactions resulted in non-detection (123 reactions out of 324). When taking the means across all of the sample reaction triplicates, the false negative rate was reduced to only 28% of samples. The false negative rate was higher for samples from the low (1 fish) abundance treatment (39%) than for the higher (5 fish) abundance treatment (17% non-detected samples) (Figure 3). A Welch's t-test revealed an insignificant difference ($t = 1.26$, $df = 9.54$, $p = 0.24$) between the frequency of false negatives between the two abundance treatments. Non-detection in all three qRT-PCR reactions in a triplicate were removed from data analysis because assigning a Ct value would have introduced bias. For example, assigning PCR reactions a Ct value of zero would mean threshold fluorescence was detected before any cycles of PCR occurred.

When comparing the two SYBR Green reagents, the mean Ct value of the three replicates was 37.97 (SEM ± 0.64) for PowerUp SYBR Green and for SYBR Select Master Mix the mean of the three replicates was 30.61 (SEM ± 0.22). SYBR Green had a significant impact on Ct value ($F_{2,5} = 7.22$, $p = 0.03$). Correcting Ct values for PCR efficiency limited reaction differences in Ct values, however. When Ct values were corrected for PCR efficiency of the corresponding SYBR Green reagent, the effect of

SYBR Green on Ct value was not statistically significant ($F_{1,4} = 0.308$, $p = 0.61$). Due to the insignificant effect of SYBR Green after PCR efficiency correction, I considered the effect negligible.

The repeated measures ANOVA revealed that none of the variables or interaction terms had a significant effect on Ct values (eDNA amount). Abundance ($F_{1,22} = 1.75$, $p = 0.20$), tank ($F_{4,22} = 0.81$, $p = 0.53$) and time ($F_{1,22} = 2.20$, $p = 0.15$) were all insignificant (Table 2). A Shapiro-Wilk normality test using the residuals of the ANOVA model revealed that the null hypothesis could not be rejected ($p = 0.06$), and thus the data was considered normally distributed. A repeated measures ANOVA was then performed for each abundance treatment separately, where each variable remained insignificant (Table 3).

Figure 1.2 PowerUp SYBR Green Master Mix qRT-PCR standard curve generated from serial dilutions of DNA (100 to 10 billion-fold dilution) with standard error bars. The standard curve slope was used to determine PCR efficiency using PowerUp SYBR Green and CytB primer.

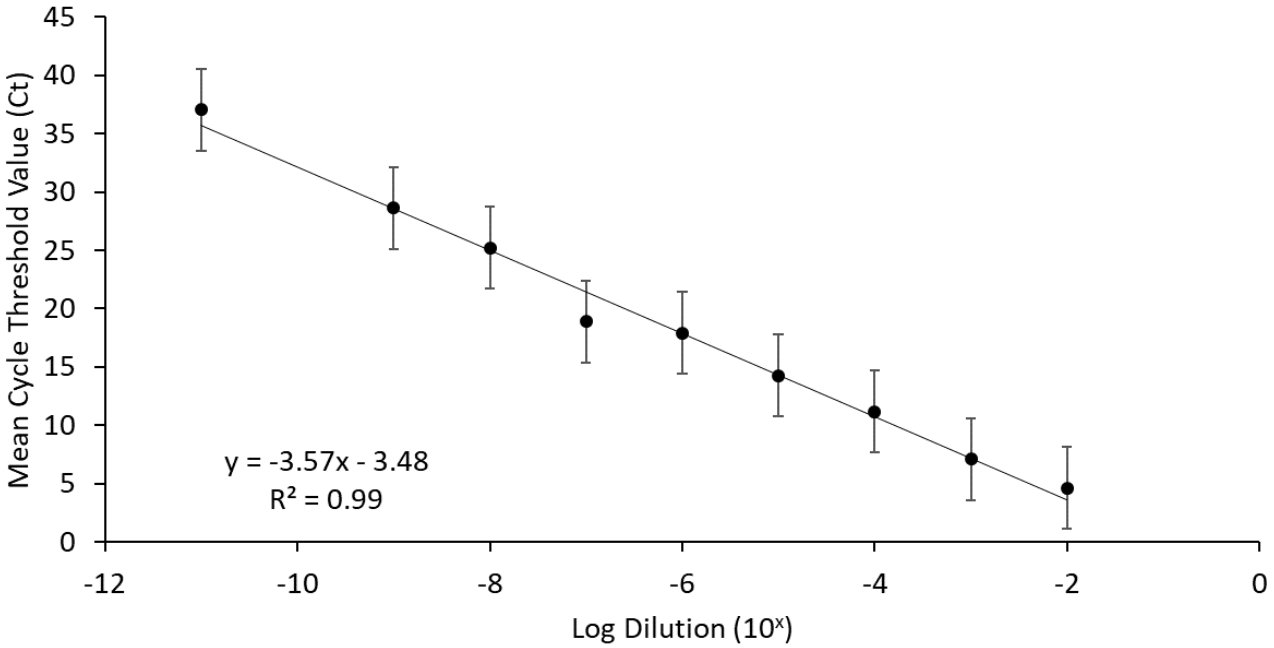

Figure 2.2. SYBR Select Master Mix qRT-PCR standard curve generated from serial dilutions of DNA with standard error bars. PCR efficiency of qRT-PCR with SYBR Select and CytB primer was calculated from the standard curve slope.

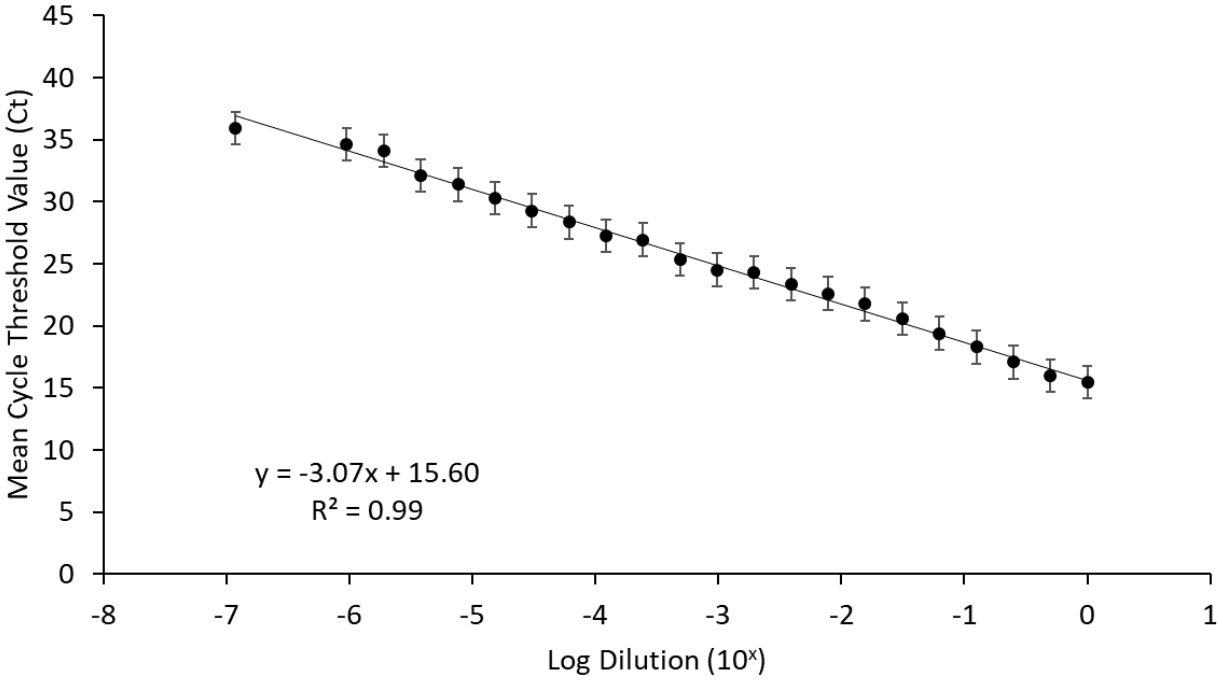

Figure 3. Frequency of undetected eDNA in sample replicates (excluding control tanks) taken at Time (hours after grass carp were placed in each tank) with standard error bars for abundances of five fish and one fish. The first samples were taken immediately after the addition of grass carp (Time "0").

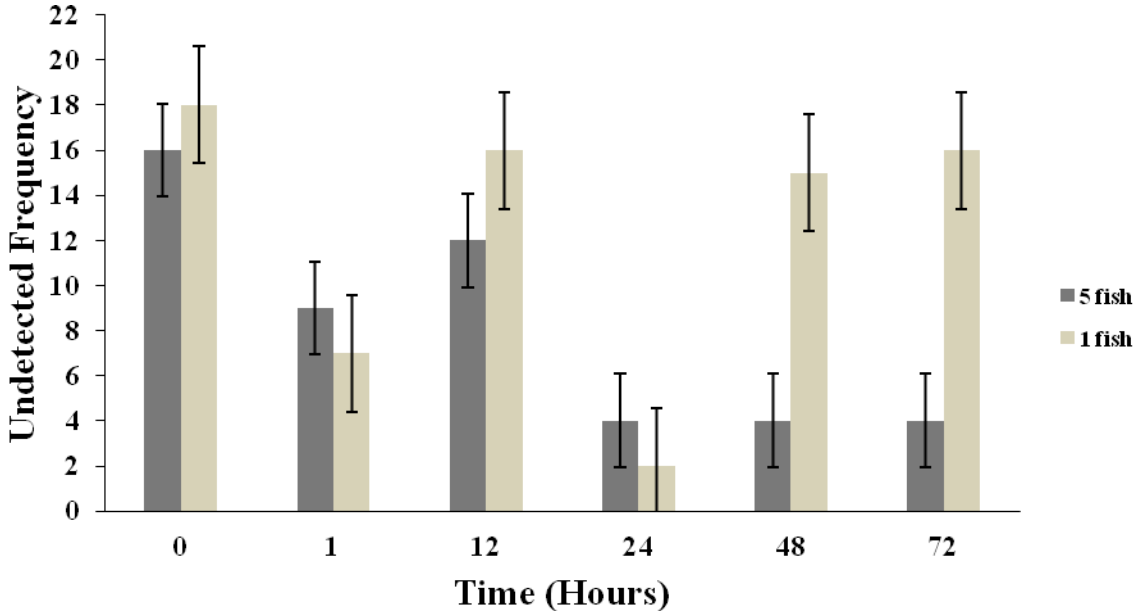

Table 1. Primer sequences for detection of grass carp, including amplicon size (measured in base pairs) for each primer pair.

Marker	Forward Primer	Reverse Primer	Amplicon Size (bp)	Citation
CytB	CAACGACGCGCTAGTCGA	TCCAAAGTTTCATCATGCAGAGA	61	Hunter et al. (2017)
COI	TGAAACCACCAGCCATCTCC	GGCGGCTAGAACTGGTAGAG	101	Bronnenhuber and Wilson (2013)
ND2	AATCAATACCTTAGCAATCATTCCA	TATTTATATCTCACTCTCCTGTAAT	157	Mahon et al. (2013)
18S	ATTTCCGACACGGAGAGG	CATGGGTTTAGGATACGCTC	90	Su et al. (2011)

Table 2. Repeated measures ANOVA model with Ct value as a response variable. Abundance is two low density abundance treatments (one versus five grass carp), tank is each separate experimental tank, time is the hours after grass carp introduction.

	DF	F Value	P
Abundance	1	1.75	0.20
Tank	4	0.81	0.53
Time	1	2.2	0.15
Residuals	22		

Table 3. Repeated measures ANOVA for each abundance treatment separately. Ct value is the response variable, tank is each separate experimental tank, time is the hours after grass carp introduction.

	DF	F Value	P
High Abundance			
Tank	2	0.01	0.99
Time	1	0.27	0.61
Residuals	10		
Low Abundance			
Tank	2	1.74	0.22
Time	1	2.51	0.14
Residuals	12		

Discussion

Grass carp are now commonly caught in the Great Lakes, and their presence provides cause for concern regarding possible negative impacts if they establish and spread (Cudmore et al., 2017). Attempts at surveillance of Asian carp - including grass carp (Wittmann et al., 2014) - using eDNA (Jerde et al., 2011) have had mixed results. While low detection rates in the Great Lakes were likely due to low abundances of these species, eDNA detection is more sensitive than traditional surveillance (Jerde et al., 2011; Wilcox et al., 2016). This makes it a potentially powerful tool in detecting both invasive and non-invasive species at low abundance, thereby partially removing the veil that tends to obscure presence and distribution of rare species (Preston, 1948). Early detection of NIS - like grass carp - requires that surveillance methods be improved substantially.

To understand how detection of eDNA changes at low species abundance, I compared two low-abundance treatments of grass carp. The mean Ct values were lower for one grass carp juvenile (Mean = 36.8, SE ± 0.79) than for five (Mean = 40.0, SE ± 1.07). This was unexpected as increasing carp abundance between treatments by 500%, even at low abundance, should have produced lower Ct values (i.e. higher eDNA amounts). The hypothesis that the higher abundance treatment would have greater amounts of eDNA was rejected.

I also hypothesized that interactions between grass carp juveniles would result in an increase in eDNA greater than the 1:5 ratio of the two treatments, as was the case for differences in crayfish abundance (Cai et al., 2017). The Ct means of the lower abundance treatment instead suggested a greater amount of eDNA compared to the higher abundance treatment (Figure 4). The results of this experiment suggest that, at least for

juvenile grass carp, interactions between group members does not produce an increase genetic material released.

One limitation of this analysis is that 28% of samples had no detection of eDNA. Since Ct values cannot be set to zero, samples without a Ct value were removed from analysis. When taking false negative rates into consideration, the higher abundance treatment had better detection of eDNA (only 17% false negatives) compared to the treatment of one grass carp (39% false negative rate). This result may partially explain why eDNA seems to be greater on average in treatments of only one grass carp. However, the higher Ct values of the higher abundance treatment cannot be ignored. False negative rates in this experiment are better than in other Asian carp surveillance studies in the Great Lakes (Jerde et al., 2011, Wittmann et al., 2014); however, those studies were conducted in the field and addressed larger sampling areas and lower eDNA concentrations as compared to my lab experiment. Other lab studies experienced false negatives in qRT-PCR replicates as well, such as a false negative rate of 44% for an abundance treatment of one crayfish in Cai et al. (2017). With a false negative rate comparable to other studies, greater specificity, and amplification of less concentrated DNA than the other three primers used in this study, the CytB primer performed well for the detection of grass carp eDNA. False negatives were reduced by 10% overall using of three PCR replicates (39% for qRT-PCR reactions versus 28% non-detection when taking the mean of PCR triplicates). Generally speaking, future eDNA studies involving low target abundance should involve at least three PCR replicates to minimize false negatives (Ficetola et al., 2015).

The time that samples were taken at after grass carp removal had an insignificant impact on Ct values and eDNA amount. While half of the experimental tanks generally increased in eDNA over time, the effect of time on eDNA amounts across experimental tanks was inconsistent (Figure 4). Although insignificant, experimental tanks “o”, “r”, and ”z” tended to decrease in Ct value, while tank “y” showed a neutral trend overall (Figure 4). Tanks “g” and “x” showed an unexpected increasing trend for Ct values over time (Figure 4). The insignificant effect of time on eDNA amount was unexpected as eDNA can persist in water for weeks (Goldberg et al., 2013; Turner et al., 2015), and other studies have demonstrated eDNA accumulating over time (Nathan et al., 2014; Thomsen et al., 2012a).

The insignificant difference between the two abundance treatments over time (Table 2) highlights the uncertainty of detection at low abundance and the difficulty in estimating abundance from eDNA. Non-uniform distribution of eDNA in aqueous environments and clumping of eDNA molecules cause variation in eDNA amounts in water samples (Wilcox et al., 2016; Lance et al., 2017), especially for small water sample volumes (Wilcox et al., 2016). Clumping of eDNA could have caused some samples to capture unexpectedly high amounts of eDNA while other samples could have missed eDNA completely. Hence sampling of eDNA, even within tanks, may have suffered from stochastic processes more than anticipated. This could explain both the false negative rates found throughout the experiment and the unexpected differences between my two abundance treatments. Further testing would require a greater difference in abundance treatments, more treatments, or more replicates of each treatment. In the field, false negatives could be reduced by sampling grass carp spawning sites. There are multiple

tributaries within the Great Lakes Basin that are predicted to be suitable for grass carp spawning (Cudmore et al., 2017), possibly up to 80 (Cudmore and Mandrak, 2004). While grass carp have spawned as early as April in China (Hargrave and Gido, 2004) and Oklahoma (Duan et al., 2009), spawning in the Great Lakes Basin is predicted to occur from June to September (Cudmore et al., 2017). Grass carp eggs have also been found within the Great Lakes Basin in June and July (Embke et al., 2016), which at least partially supports the predicted spawning period of June to September (Cudmore et al., 2017), although sampling only occurred from June to August (Embke et al., 2016). If potential spawning sites were sampled during spawning, the increased concentration of grass carp and release of gametes by fertile individuals would increase detection and would be prime locations for targeted eradication of the species.

Some samples of the control tanks tested positive for grass carp eDNA even though no fish were present. Negative controls for each PCR plate and negative equipment controls confirmed that no contamination occurred during sample processing. The occurrence of target eDNA in my control tanks is not unique to this study (Barnes et al., 2014; Lance et al., 2017) and may have resulted from aerosolization of eDNA from adjacent (0.15m) tanks with fish. At least two previous eDNA studies have suggested aerosol-containing eDNA caused contamination of nearby control tanks (Barnes et al., 2014; Lance et al., 2017). In one experiment, cross-contamination in adjacent (0.2 m) aquarium tanks could only be explained by aerosolization of target eDNA (Barnes et al., 2014). Likewise, experimental tanks in my study were adjacent to the control tank where aerosolized water droplets containing eDNA could easily contaminate the control tank. Grass carp did occasionally jump and splash as well, which could transfer water droplets

directly from one tank to the next. My study supports the view that either aerosolization of eDNA or fish splashing could have been responsible for eDNA transfer to control tanks.

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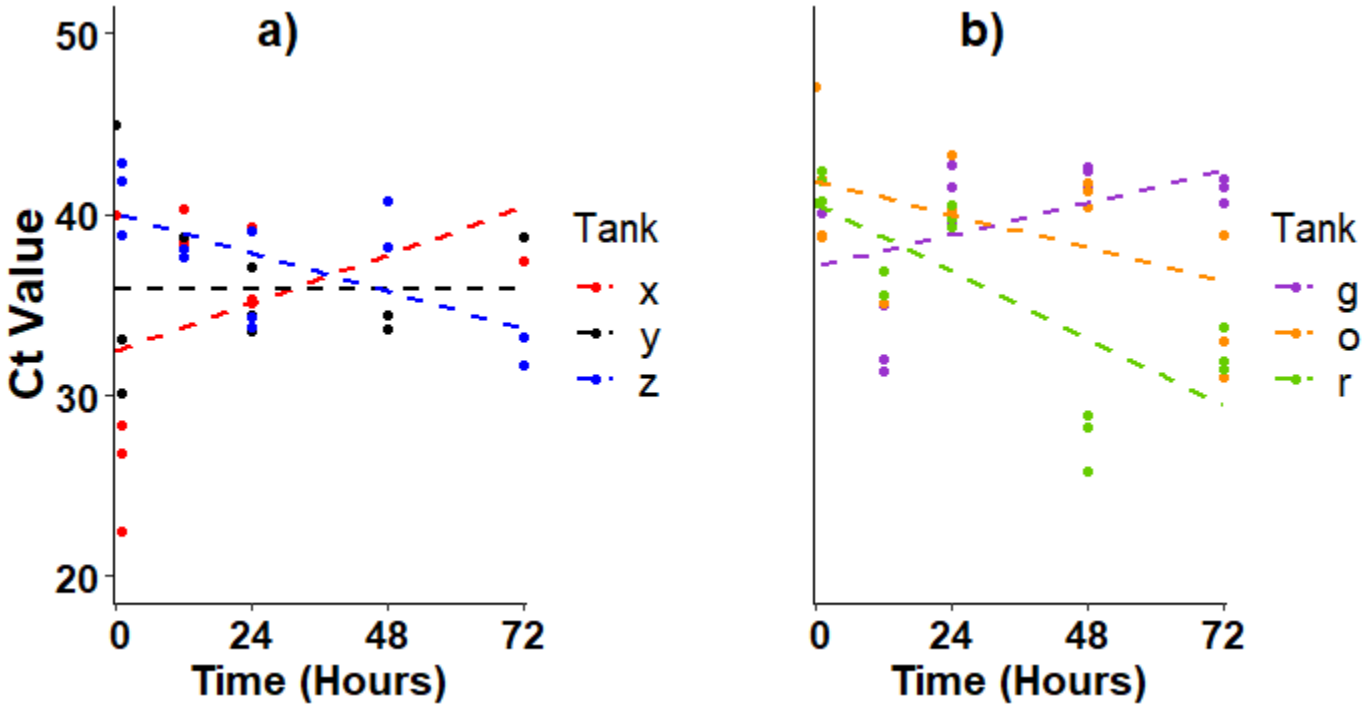
Figure 4. Ct values over time (hours) for two low abundance treatments, where a) is an abundance treatment of one grass carp individual and b) is 5 individuals. Ct values represent the mean cycle threshold value for triplicate qRT-PCR reactions and were corrected for PCR efficiency. Ct values are inversely related to eDNA concentration (i.e. lower Ct values denote higher DNA concentrations).



CHAPTER 3

PERSISTENCE OF GENETIC MATERIAL AND DETECTION OF ENVIRONMENTAL RNA

Introduction

Unlike traditional methods that involve direct capture, eDNA indirectly detects target species by the biomass they shed. Capturing DNA rather than live organisms can result in false positives (De Barba et al., 2013; Roussel et al., 2015). False positives, where a target species is detected despite being absent or dead (Darling and Mahon, 2011; Zhan and MacIsaac, 2015) can occur in a number of ways. For example, eDNA takes much longer to degrade when bound to sediment as opposed to an aqueous environment (Turner et al., 2015). When sediment is disturbed, this eDNA can be resuspended in water and cause false positives in water samples in which the target organism is no longer present (Roussel et al., 2015; Turner et al., 2015). This is especially true in near-shore sampling and in shallow ponds or lakes where wading researchers and research vessels may disturb and resuspend sediment (Turner et al., 2015). Moreover, eDNA in the environment does not always come from live organisms. Decomposed organisms release DNA that can persist in water (Darling and Mahon, 2011). In addition to live targets, eDNA can be derived from predator faeces (Wilcox et al., 2013; Roussel et al., 2015). Contamination of samples and artifacts during amplification by PCR can also elicit a false positive signal (De Barba et al., 2013; Willerslev et al., 2014). Finally,

false positives may arise when markers are not specific to one species, resulting in amplification for non-target species during PCR (Roussel et al., 2015; Zhan and MacIsaac, 2015). Sometimes these false positive errors (PCR artifacts) are removed through data processing (Zhan and MacIsaac, 2015; Scott et al., 2018). In meta-barcoding, some studies take a conservative approach by disregarding positive detections unless a sequence is confirmed in at least two independent PCRs (Willerslev et al., 2014; Ficetola et al., 2015). Due to the rarity of certain species (i.e. recent invaders) and thus low abundance of target sequences, filtering of "artifacts" also risks filtering out true positives and, in turn, creating false negatives (Scott et al., 2018). Hence, some attempts to limit false positives can actually increase the risk of false negative results, and vice versa (Zhan et al., 2014; Ficetola et al., 2015; Zhan and MacIsaac, 2015; Scott et al., 2018).

One way to address errors in eDNA methods would be to use environmental RNA (eRNA) detection in combination with eDNA. Since RNA in the environment is more rapidly degraded compared to eDNA (Lesnik and Freier, 1995; Hurt et al., 2001), eRNA would limit false positives. Sources of eDNA sample contamination, such as disturbed sediment (Turner et al., 2015) and detection of dead organisms (Darling and Mahon, 2011; Wilcox et al., 2013; Roussel et al., 2015) would be less likely to affect eRNA detection owing to the rapid degradation of RNA. Positive detections using eRNA would represent live, recently-present individuals. While RNA has been amplified from microbes (Pawlowski et al., 2014; Visco et al., 2015; Pochon et al., 2015), macroinvertebrates (Lejzerowicz et al., 2015), vertebrate tissue (Jun et al., 1997; Zhang et al., 2010), and a marine fish (*Auxis* spp.) (Pochon et al., 2017) to our knowledge there are

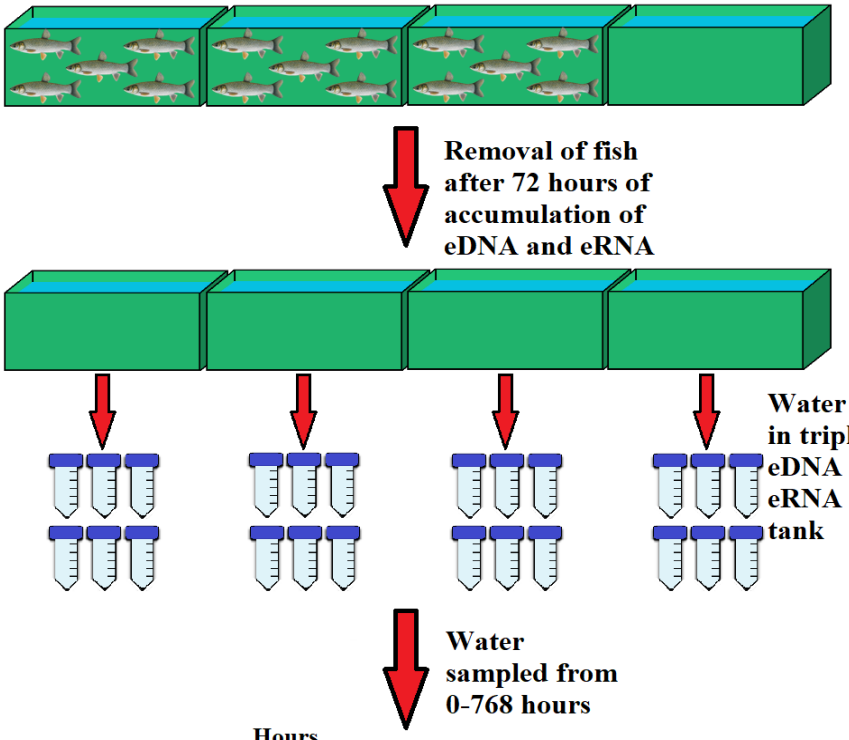
no studies that have primarily focused on detection of megafauna eRNA nor attempted to detect freshwater vertebrate species using eRNA. In addition, studies seeking to detect Asian carp genetic material have been exclusively focused on eDNA (Jerde et al., 2011; Jerde et al., 2013; Wittmann et al., 2014; Amberg et al., 2015; Farrington et al., 2015; Turner et al., 2015; USFWS, 2015). Compared to DNA, RNA is less stable and thus degrades rapidly (Lesnik and Freier, 1995; Hurt et al., 2001). Since RNA does not persist long in the environment, eRNA could be used to detect recent occupancy by the target species with greater certainty than eDNA techniques.

Conversely, the unstable nature of RNA would increase the frequency of false negatives, decreasing detection rates compared to eDNA (Lesnik and Freier, 1995; Hurt et al., 2001). Thus, using both eRNA and eDNA could provide improved insights into presence of target species and lower false positive and false negative rates than either approach alone. Thus, the selectiveness (Sharkey et al., 2004; Mora and Getts, 2007) and unstable nature of RNA (Lesnik and Freier, 1995; Hurt et al., 2001) can be exploited as a powerful tool to complement results obtained using eDNA alone.

I conducted an experiment in which grass carp were introduced into and then removed from tanks to test the persistence of eDNA and eRNA over time and to determine the viability of eRNA detection as a surveillance tool (Figure 1.3). This approach allowed me to quantify the persistence of grass carp genetic material after removing the source (i.e. grass carp). I hypothesized that grass carp would be detected for weeks using eDNA (Turner et al., 2015), while eRNA would be detected with species-specific markers only in the first few hours following grass carp removal (see Figure 1.3). This chapter of my thesis aimed to determine whether eRNA can be used as a

surveillance tool for a vertebrate species by demonstrating that it persists both long enough and in sufficient amounts for detection (i.e. reduce false negatives), but degrades rapidly enough to limit false positives.

Figure 1.3. Experiment where grass carp were held in aquarium tanks after 72 hours and then removed. Sampling of eRNA and eDNA occurred for 768 hours after removal of fish from tanks.



Hours After GC Removal	RNA Samples	DNA Samples
0	✓	✓
1	✓	✓
2	✓	✓
4	✓	
8	✓	
12	✓	
14	✓	
24	✓	
48		✓
192		✓
384		✓
504		✓
768		✓

Methods

Experimental Design

Tanks of grass carp were used to test the persistence of eDNA and eRNA in experiments at the Wetland and Aquatic Research Center (Gainesville, Florida) in collaboration with the USGS. Procurement of grass carp and their care was managed by animal care professionals at the USGS, including handling, feeding, and removal from experimental tanks. I treated experimental tanks with 10% bleach and rinsed thoroughly before drying for two days before use. Experimental tanks had never held grass carp previously. Using three indoor cylindrical aquarium tanks (215L) with five grass carp individuals (20cm) in each tank (plus one control tank with only water), grass carp were held for 72 hours to allow genetic material to accumulate through excretion of urine, faeces, mucus, etc. (SRAC, 2002; Ficetola et al., 2008). Water was sampled once in triplicate to ensure no tanks were contaminated before grass carp were added. Fish mass, total length, and standard length (in centimetres) of each individual fish was recorded. I took water samples from tanks in triplicate and then treated each sample as three PCR 'technical replicates'. When sampling, I used new nitrile gloves for each tank to take 50mL surface water samples in triplicate for both eDNA and eRNA at 0, 1, 2, and 24 hours after removal of grass carp. Additional samples were taken at 4, 8, 12, and 14 hours after grass carp removal because the persistence of eRNA was unknown but expected to be less than a day. I expected eDNA to last much longer and continued sampling at 48, 192, 384, 504 and 768 hours (32 days). Samples were frozen on dry ice after adding 5ml of RNAlater™ and were stored at -20°C.

Samples taken in August and September 2016 were filtered at the Wetland and Aquatic Research Center. Filtration equipment was submerged in 20% bleach before being transferred to a water bath for 10 minutes. Following the water bath, filtration equipment was dried in a sterile fume hood. Sample filters stored at -20°C were thawed at 4°C. After thawing, water samples were then filtered using a vacuum-pump and glass microfibre filters (VWR, 0.45um pore size, 4.7cm diameter). Sample filters were then stored in 15mL of RNAlater™ at -20°C.

Additional Sampling for eRNA

Following the experiment at the Wetland and Aquatic Research Center, further sampling was conducted in October (2017) at the Lee County Hyacinth Control District (LCHCD). To verify if eRNA detection was possible after earlier failed attempts, I took surface water samples from a 19,000L pool containing 250 grass carp juveniles. Water samples (1L) were immediately filtered by vacuum-pump using glass microfiber filters for half the samples and PES (polyethersulfone) filters for remaining samples. All filtration equipment had never been used for grass carp before and was sterilized before use (20% bleach for 10 minutes, ultrapure H_2O bath for 10 minutes, 24 hours of drying). Filtration equipment was not sterilized between samples. Sample filters were then placed into 15mL of RNAlater™ preservative following filtration. Sample tubes were transported on ice for one hour before storage at -20°C.

Primers

A primer pair based on a grass carp mitochondrial cytochrome b (CytB) marker was used for species-specific detection of both eDNA and eRNA because of the lack of introns (Hunter et al., 2017) (Table 1). Without introns within the target sequence, the CytB primer amplified cDNA synthesized from eRNA. A non-species-specific primer based on an 18S rRNA gene marker was also utilized (Su et al., 2011) (Table 1). Separate standard curves for cDNA and DNA were generated from 10-fold serial dilutions of positive grass carp using RNA extracted from grass carp liver and DNA extracted from grass carp fin tissue, respectively. This procedure was done for both CytB and 18S primers.

DNA Extraction

Surfaces were sterilized with 10% bleach before extractions. Forceps and scissors were sterilized in 20% bleach before being rinsed and placed in an ultrapure H_2O bath. Forceps and scissors were then dried completely before fin clip tissue (positive controls for DNA) and sample filter halves were put into 2mL screw cap tubes containing 500µl of CTAB buffer and 500µl of (1mm diameter) glass beads. Samples were then homogenized at 5,500rpm five times. I then incubated samples in a water bath at 60°C for one hour. Samples were added to new tubes with an addition of 5µl Proteinase K solution (20mg/mL). Sample tubes were mixed and left to digest 8-12 hours at 37°C before being stored at -20°C until ready to be processed further. After a final incubation at 60°C for one hour, DNA extraction was completed with a DNA extraction robot. DNA was stored in TE Buffer at -20°C.

RNA Extraction

Sample tubes of RNAlater™ and glass microfibre filters were thawed at 4°C before I extracted RNA from the filters. Forceps and dissecting scissors were used to take each sample filter out of a 15mL tube of RNAlater™ or to cut liver tissue (also stored in RNAlater™ at -20°C) for use as positive controls for RNA. All forceps and scissors were reused after sterilization using 20% bleach for 10 minutes, rinsing with ultrapure H_2O, and then dried for10 minutes between samples. For each sample (modified from manufacturer's TRIzol® protocol (Invitrogen)), a filter half was placed in a 2mL screw cap tube filled with 0.5cm^3 of (1.0mm diameter) glass beads and 1000µl of TRIzol®. Sample filters were homogenized for one minute four to eight times using a BeadBeater (BioSpec), then incubated at room temperature for five minutes before 150µl of chloroform isoamyl (24:1) was added. The tubes containing homogenized samples were then shaken by hand for 15 seconds before incubation at room temperature (23°C) for three minutes. After centrifugation at 11,500rpm for 15 minutes at 4°C, the aqueous top layer containing RNA was transferred into a new 1.7mL Eppendorf tube containing 1000µl of anhydrous ethanol for each sample to precipitate RNA. New sample tubes were then inverted 10 times to mix before centrifugation at 7,500rpm for three minutes at 4°C. To remove contaminants from the RNA, the pellet was washed in ethanol three times. For each ethanol wash, I removed the supernatant by pipette before adding 1000µl of 75% ethanol, followed by centrifugation at 7,500rpm for three minutes at 4°C. The supernatant was removed from each tube following centrifugation. To dry the pellet, each tube was left open and covered with a Kimwipe until almost completely dry. The pellet

containing the RNA extract was then redissolved in 20-100ul of TE Buffer for 20 minutes at room temperature before cDNA synthesis.

cDNA Synthesis

To detect RNA, cDNA was first synthesized from RNA before qRT-PCR. Synthesis of cDNA was completed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to convert RNA to cDNA. Due to difficulty getting eRNA to synthesize, sample RNA was not diluted nor standardized. DNA contamination was eliminated by a DNase I step before synthesis of cDNA from template sample RNA. For each sample, 8µl of extracted RNA was added to an RNase free PCR plate or tube. I then added 1µl of 10X RT reaction buffer, followed by 1µl of Amplification Grade DNase I (1U/1µl) to each reaction well. Mixing each reaction by pipette, the PCR plate was then spun down by centrifugation (1000rpm) for one minute before incubation for 30 minutes at 37°C. After another one minute spin down (1000rpm). 1µl of Stop Solution (50mM EDTA) was added to inactivate DNase I before synthesis of cDNA. Following incubation at 75°C for 10 minutes, denatured sample RNA was used as a template for synthesis of cDNA. To synthesize, 10µl of master mix was added to each reaction consisting of: 2µl 10X reverse transcriptase buffer, 0.8µl 25X dNTP mix (100mM), 2µl 10X reverse transcriptase primers, 1µl MultiScribe Reverse Transcriptase, 1µl RNase inhibitor, and 3.2µl Milli-Q® ultrapure H_2O. Thermocycler conditions for cDNA synthesis were 25°C for 10 minutes, 37°C for 2 hours, followed by 85°C for 5 minutes before cooling at 4°C until qRT-PCR. I then repeated the synthesis of cDNA from template RNA (without the DNase I step) three times, after initial attempts to detect cDNA from non-tissue samples,

using only one round of cDNA synthesis, failed. Repeated cDNA synthesis was done to maximize the amount of generated cDNA and increase detection. Synthesised cDNA plates that did not immediately undergo qRT-PCR were stored at -20°C.

Relative Quantification of DNA and RNA

I tested both species-specific eRNA detection with the CytB and non-species-specific eRNA detection with the 18S primers. In the same way qRT-PCR detects DNA, cDNA was detected from the fluorescence of SYBR Green (Applied Biosystems) bound to double-stranded cDNA. This produces the cycle threshold (Ct value) that is used as a relative measure of initial sample cDNA (and in turn, RNA). A reaction volume of 20µl was used where 5.0µl of template eDNA/cDNA was added to 10ul of PowerUp SYBR Green Master Mix (Applied Biosystems), along with 0.5 µl of forward primer, 0.5 µl of reverse primer, and 4µl of ultrapure H_2O. Three PCR replicates were conducted for each sample using a Quantstudio™ 12K Flex Real-Time PCR System (Applied Biosystems) at 95°C for 10 minutes, followed by 60 cycles of 94°C for 15 seconds and 56°C (or 63°C for testing 18S) for one minute. NTCs (no template controls) and positive grass carp fin clip or liver controls were included for each 96-well plate in triplicate.

Results

Experimental Results

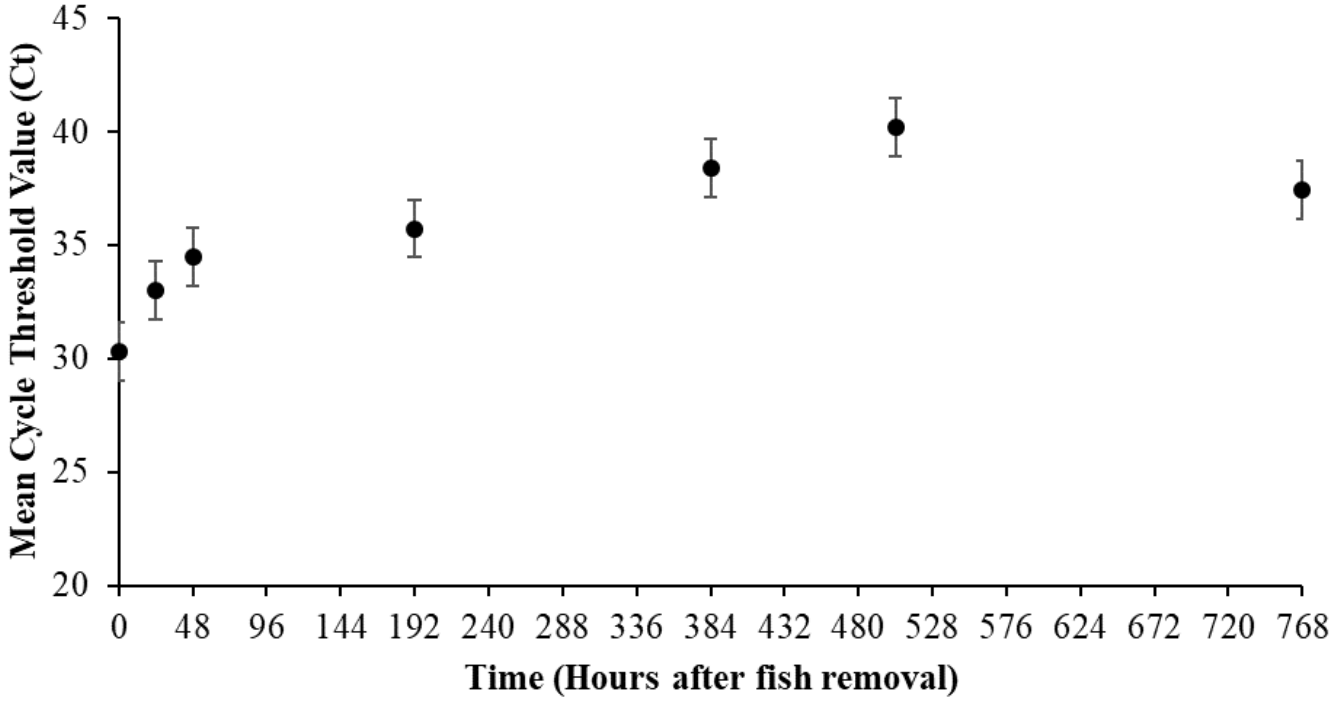
CytB primers amplified both DNA and RNA (after conversion to cDNA) from grass carp. The 18S primer amplified black carp DNA in addition to grass carp eDNA and eRNA (cDNA). In my experiment, eDNA and eRNA samples were taken after removal of grass carp from tanks. Triplicate samples taken before the addition of grass carp and from the control tank containing no fish produced no Ct value from qRT-PCR. Despite removing grass carp one month earlier, eDNA was detected from tank samples for all days sampled during the 32 day experiment (Figure 2.3). Controls (in triplicate) were negative for each sample plate, suggesting that no contamination occurred

While eDNA was detected over one month, eRNA could not be detected at all in this experiment. This included samples taken right after removal of grass carp and at one hour post-removal (i.e. samples expected to have the most eRNA). I also used RNA samples taken at hour 1, 48, and 72 from an experiment where grass carp stayed in tanks during sampling. Of 135 qRT-PCR reactions using cDNA, no eRNA could be detected from samples despite positive detection of cDNA synthesized from grass carp liver RNA.

Since eRNA was not detected during the experiment, I performed additional sampling to improve the probability of detection. I successfully synthesized and subsequently amplified cDNA from eRNA using water samples from the pool of 250 grass carp juveniles. For each PCR sample plate, negative controls were negative and positive controls were positively detected. The species-specific CytB primer was successfully used with grass carp liver RNA but failed to detect eRNA in these water samples. The non-specific 18S primer did, however, detect eRNA in both glass

microfiber and PES filters. Of 13 non-diluted PCR reactions (and 15 diluted reactions) from these samples, only six reactions produced a Ct value. Only one of these sample reactions (Ct value of 31.82) was below a Ct value of 48. Values >48 are highly suspect and are likely PCR artifacts (Zhan and MacIsaac, 2015). While only one glass microfibre sample produced a reasonable Ct value, this sample was also diluted, which decreased PCR inhibition, although other diluted samples produced no reasonable Ct values (Takahara et al., 2015). Of 13 PCR reactions with cDNA synthesized from PES filter eRNA, eight produced Ct values. Four of these reactions had Ct values (mean=30.50).

Figure 2.3. Changes in mean cycle threshold value for three experimental tanks after removal of grass carp. Sample eDNA amounts are inversely related to Ct value. At 768 hours, eDNA could no longer be detected from one of the three tanks, which could have skewed the mean at that time point.



Discussion

Detection of recent grass carp presence and validation of eDNA positives could inform management decision making, considering the species is generally considered a nuisance NIS. On the other hand, insufficient data on location and spread of potential invaders can be detrimental. Such was the case in 2002, when the U.S. Army Corps of Engineers constructed a barrier to stop the spread of the non-indigenous round goby (*Neogobius melanostomus*) from the Great Lakes to the Mississippi River without knowing that the species had already moved past the barrier (USFWS, 2015). Without knowledge of how recently a target species was in an area, attempted prevention or management of a NIS can appear rather blind.

The persistence of environmental genetic material determines how long detections can occur after source removal. The less persistent the genetic material is, the more recently the target was in the area sampled. In one marine fish study, eDNA was below detection in less than a day (Thomsen et al., 2012b). However, numerous freshwater studies report that eDNA can be detected for weeks, depending on temperature and other factors (Dejean et al., 2011; Thomsen et al., 2012a; Goldberg et al., 2013). In my experiment, eDNA was detected for at least 32 days (the maximum time sampled) following grass carp removal. A general rise in Ct value (decreasing eDNA amount) was observed in Figure 2.3. However, the mean at 768 hours after grass carp removal was less than the mean at 504 hours. This was because at hour 504, one of the experimental tanks had a mean Ct value of 45.06, and at hour 768, no eDNA could be detected from this tank. Owing to this missing value, the overall mean Ct value at hour 768 is likely skewed. The results of this experiment suggest that detectable sequences of grass carp DNA

persisted for at least 32 days (the maximum amount of time sampled), with no contamination of negative controls. This is longer than previous studies of the persistence of carp eDNA in an aqueous environment, but much shorter than that reported by Turner et al. (2015) where bighead carp eDNA was detected for 132 days in sediment. In one experiment of similarly sized (20cm) sturgeon (*Acipenser baerii*), eDNA was able to be detected for 14 days (the maximum number of experimental sampling days) from just 15mL water samples each day (Dejean et al., 2011). In Barnes et al. (2014), two sample replicates detected common carp (*Cyprinus carpio*) eDNA a week after removal and one replicate after two weeks. Beyond carp, eDNA from mudsnails (*Potamopyrgus antipodarum*) (Goldberg et al., 2013) persisted between 21 and 44 days after removal while bullfrog (*Lithobates catesbeianus*) tadpole eDNA could be detected up to 58 days (Strickler et al., 2015). The persistence of DNA clearly varies between eDNA studies. While 32 days of persistence is longer than other carp studies (Dejean et al., 2011; Barnes et al., 2014), it is not inconsistent with other freshwater animal studies (Goldberg et al., 2013; Strickler et al., 2015).

The persistence of eDNA in the Great Lakes is likely to differ from that in lab experiments. In my experiment, indoor aquarium tanks were not exposed to the same amount of ultraviolet light that would be found in nature and the tanks had a relatively high pH (8.35-8.72). These factors could contribute to the long persistence (32 days) of eDNA in my experiment (Strickler et al., 2015; Lance et al., 2017). Compared to the lake environment, the experimental tanks would contain less humic substances that could decrease detection of eDNA (Takahara et al., 2015). My experiment thus showed the maximum amount of time eDNA could persist after grass carp removal, with eDNA

persistence in the Great Lakes likely lower than 32 days. Lower lake temperatures in colder months could decrease the rate of eDNA degradation compared to experimental tanks (23.0-24.9°C) (Barnes et al., 2014; Strickler et al., 2015; Lance et al., 2017).

This is the first study to amplify vertebrate eRNA from freshwater sampling. Both the species-specific CytB primer and 18S (non-specific) primer detected RNA extracted from grass carp liver tissue. While many attempts were made to amplify species-specific eRNA markers, only the non-specific 18S primer amplified eRNA, and only from a pool containing a large number of fishes (250 grass carp). This is likely because 18S sequences are highly expressed (Bas et al., 2004), and thus 18S eRNA sequences are more likely to be captured in greater amounts during sampling than cytochrome b sequences. 18S rRNA genes are required for cell function (Butte et al., 2001), rendering development of species-specific 18S primers problematic when genes are conserved between species. To amplify species-specific eRNA, highly expressed species-specific markers need to be developed.

Due to the ubiquity of RNase enzymes in the environment, eRNA seems to degrade too rapidly for adequate detection with the methods employed in this study. For detection of eRNA in the field, it would need to persist long enough to be collected and in sufficient amounts for cDNA synthesis. Furthermore, different cell types have different amounts of total RNA (Thermo Fisher Scientific, 2008) and transcription rates (Martinez and Walhout, 2009). I was able to obtain positive detection from liver cells, which had high amounts of RNA, but these cells are less likely to be shed or excreted into the environment than epithelial cells (which I found to yield insufficient RNA for conversion to cDNA), and intestinal cells. Cells present in my water samples clearly had insufficient

RNA amounts for detection. To synthesize quality cDNA, 20-2000ng of RNA was required with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Cell type and rapid degradation of eRNA could have led to insufficient amounts of template RNA for cDNA conversion, even with the repeated cDNA synthesis used in my study. While eRNA was amplified with non-specific primers from a pool of 250 grass carp, eRNA detection may not be practical for surveillance of low abundance species due to low eRNA concentrations. To use eRNA practically, more sensitive detection methods must be developed.

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CHAPTER 4

CONCLUSIONS

Surveillance of NIS with eDNA is a sensitive method of detection. However, eDNA surveillance is still troubled with false negatives, especially at very low abundances. In Chapter 2, I confirmed that the CytB primer developed from a grass carp mitochondrial cytochrome b marker (Hunter et al., 2017) was species-specific to grass carp. This marker positively detected the species both in-lab and during field-testing without detecting silver or bighead carp DNA. Although my samples came directly from aquarium tanks with grass carp, there were still some false negatives. Compared to surveillance of Asian carp in previous field studies (Jerde et al., 2011; Wittmann et al., 2014), the CytB primer had a lower false negative rate of 38% for qRT-PCR replicates. Detection increased by using the mean values of each qRT-PCR sample triplicate, where false negatives decreased by 10% compared to evaluating each replicate individually. The false negative rate of five grass carp (17%) was lower than one individual (39%), which is consistent with the false negative rate of one crayfish (Cai et al., 2017). Due to low non-detection rates in this experiment, the cytochrome b marker was effective at detecting grass carp. The effectiveness of the CytB primer for surveillance in the field will need to be tested further, although 80% detection from a pond of grass carp is promising. This experiment also showed that false negatives in detection can occur even when grass carp are very near, minimized by increasing the number of samples and PCR replicates (Ficetola et al., 2015; Xia et al., unpublished).

False positives are also problematic for eDNA surveillance. In my Chapter 3 experiment, eDNA in an aqueous medium was detected for 32 days after removal of

grass carp from tanks. Furthermore, the eDNA amounts between samples from day 8 (Ct value mean of 37.8, SEM ± 1.25) and day 32 (Ct value mean of 37.4, SEM ± 0.49) were similar enough to suggest that degradation between 8 and 32 days was of no consequence to detection. In sediment, eDNA can last even longer (Turner et al., 2015). While eDNA in the field is unlikely to persist as long as in my lab experiments, this experiment showed that eDNA does have the potential to last for weeks in an aqueous environment. The long persistence of eDNA could lead to erroneous conclusions in eDNA surveillance and cause false positives. When determining where NIS occur, persistence of eDNA for 32 days would be very problematic as one could have potentially detected a fish that had been absent from the system for weeks with no indication of when the fish was last in the area. When monitoring invasion fronts of newly introduced or spreading NIS, weeks of eDNA persistence would mean that the target species could have been within the sampling area when sampling occurred, or the target could have been moving away from the area for weeks. Water flow carrying eDNA away from its source location would further confound efforts to detect and locate NIS. The long persistence of eDNA in this study and variable rate of degradation found in other studies (Dejean et al., 2011; Thomsen et al., 2012a; Goldberg et al., 2013; Strickler et al., 2015) indicate that false positives are of major concern for eDNA surveillance. While eDNA surveillance can be more sensitive than traditional surveillance methods (Jerde et al., 2011; Wittmann et al., 2014), locating live NIS with eDNA alone could prove difficult, especially for mobile target species.

To limit false positives in eDNA surveillance, I assessed the viability of eRNA for detection. Less persistent RNA would only be detected soon after being and shed from

the target species. Detection of eRNA would be a better measure of actual target distribution at the time of sampling. In Chapter 3, I successfully detected eRNA from a vertebrate species (i.e. grass carp) from water samples. Detection was only successful using a primer designed from a non-species-specific 18S rRNA gene marker however, with no detection from the species-specific CytB primer. Furthermore, only samples that were filtered immediately from a pool of 250 grass carp were positive for eRNA. While eRNA was able to be detected, it is clear that it is impractical to detect species-specific eRNA for low abundance target species with the current methods presented here. Although detection of eRNA would give more accurate temporal and spatial information than eDNA surveillance, detecting eRNA is difficult and prone to false negatives.

Early detection of grass carp is imperative to preventing establishment and spread (Mack et al., 2000; Simberloff and Gibbons, 2004; Mahon et al., 2013; Wittmann et al., 2014). If undetected and unmanaged, grass carp could have destructive impacts in the Great Lakes (Gasaway and Drda, 1976; Mahon et al., 2013; Cudmore et al., 2017). While eDNA is a powerful tool for surveillance of grass carp, false negatives and positives are problematic (Darling and Mahon, 2011). I showed that false negatives in eDNA detection could occur even when I sampled tanks of grass carp, consistent with other studies (Jerde et al., 2011; Wittmann et al., 2014; Cai et al., 2017). Although a higher abundance of carp was detected more often in my experiment, the effect of abundance, biomass, and time on eDNA amount was unclear. I also showed that eDNA can last a very long time (32 days), which could contribute to false positives. Species-specific detection using eRNA could limit false positives; however, false negatives were much more prevalent for eRNA. While I successfully demonstrated that eRNA can be detected, more sensitive methods

must be developed to use eRNA for species-specific detection of low-abundance species. Clearly, eDNA and eRNA from low-abundance sources must be studied further to limit false negatives and positives in early surveillance of grass carp and other NIS.

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