COMPARING MOLECULAR METHODS TO ESTIMATE FISH STOMACH CONTENTS AND GASTRIC EVACUATION RATES: IMPLICATIONS FOR MEASURING THE IMPACTS OF PREDATION ON CENTRAL VALLEY CHINOOK SALMON

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ABSTRACT

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Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) migrating through the Sacramento – San Joaquin Delta (the Delta) in central California have higher mortality rates than salmonids migrating through other west coast estuaries. Some hypotheses for high mortality rates include entrainment into water export facilities, physical alterations of the river system, and predation. Of these factors, predation is considered the least understood but may have the largest impact. Predation from large populations of non-native piscivorous fishes in the Delta is believed to effect abundances of Central Valley Chinook salmon, however sufficient data supporting this hypothesis is scarce. In this study, I investigate the possibility of using three molecular methods (qPCR, metabarcoding, SNP) to improve fish diet analyses by measuring the gastric evacuation of Chinook salmon in the stomachs of two common Delta predators, largemouth bass (*Micropterus salmoides*) and channel catfish (*Ictalurus punctatus*). Before molecular analysis can occur using field samples, it is important to conduct laboratory feed trials to determine how factors affect gastric evacuation and the detectability of DNA within

predator digestion systems. My experiment was conducted to determine gastric evacuation of Chinook DNA and visual decay of organic tissue within the digestive tracts of two common piscivores in the Delta at different temperatures (15.5˚C and 18.5˚C) and feed ratios (predator weight: prey weight). Results from metabarcoding and qPCR analysis indicate greater detection ability and longer durations of digestion at higher feed ratios and decreased temperature. Temperature and ratio effect was species dependent with a larger effect on evacuation within channel catfish compared to largemouth bass. Channel catfish also had slower digestion rates, indicating lower metabolism and a longer duration of DNA detection. Lastly, visual degradation of prey tissue followed a highly correlated decay pattern to metabarcoding read count and qPCR copy number over time. Gastric evacuation results from this study can provide information for future models on the effect of species, time, temperature, and feed ratio on digestion rate needed to predict population level predation occurring on native salmonids.

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INTRODUCTION

The Sacramento – San Joaquin Rivers within central California historically nurtured abundant, self-sustaining populations of Chinook salmon (*Oncorhynchus tshawytscha*), a valuable resource for the fishing community and local economy. Juvenile Chinook utilize the river system as a rearing habitat and migration corridor to the Pacific Ocean. Current populations of Central Valley salmon are in decline with some studies suggesting more than a 75% loss in recent decades and the listing of two local races under the Endangered Species Act (Yoshiyama et al. 2000; Lindley et al. 2004). Estimated survival rates of juvenile salmonids have been low for at least two decades, averaging less than 5% and decreasing (Buchanan et al. 2018). Factors attributed to the population decrease include entrainment and flow alterations, habitat degradation, physical barriers, and predation (Baker and Morhardt 2001; Brandes and McLain 2001; Wim 2008; Grossman 2016; Sabal et al. 2016). Predation, one of the least studied factors, could have one of the highest impacts on mortality of juvenile populations (Lindley and Mohr 2003; Grossman 2016; Grossman et al. 2013). Specifically, predation by invasive fishes may be a leading factor, but current information on the percent of mortality due to predation is scarce (Grossman 2016).

One of the most popular methods for studying trophic interactions between predators and prey are diet studies, but it can be difficult to quantitatively assess the results from these studies. Results from studies investigating the effects of invasive fish predation as a factor for salmonid population decline suggest that predation could be important (Michel

et al. 2018), but these data are limited by the constraints of visual gut content analysis (Loboschefsky et al. 2012; Demetras et al. 2016; Grossman 2016). The primary protocol for analyzing diets is to visually assess stomach and intestinal content, but rapid digestion rates may introduce ambiguity into results (Schooley et al. 2008). Gut content becomes an indistinguishable mixture containing digested material and unknown species usually within a matter of hours (Legler et al. 2010; Figure 1). Not only are some fish within stomach contents labeled as "unidentifiable" (Grossman 2016), but empty stomachs are usually excluded in quantitative predator analyses even though recent prey may have been consumed (Rindorf and Lewy 2004). Results from Nobriga and Feyrer (2007) suggest predators in the Delta are opportunistic feeders, with diets comprised of numerous taxa including both fish and invertebrates. The combination of multiple taxa present in a sample and differences in prey tissue altering digestion rate can lead to misidentification, and in some cases, lack of data (Thomas et al. 2014).

Figure 1:Contents from a stomach lavage depicting the difficulty of visual identification. Performed on a striped bass caught in the Georgianna Slough during a predation study.

One approach that has been used to improve upon these weaknesses of visual diet analyses are molecular methods that can identify diet contents throughout the entire digestive tract. For example, quantitative polymerase chain reactions (qPCR) and metabarcoding methods can identify the presence of a species in a mixed DNA sample long after visual identification of gut contents becomes ineffective (Brandl et al. 2016; Michel et al. 2018). Whereas soft tissues, which includes early juvenile fish (Schooley et al. 2008; Legler et al. 2010; Brandl et al. 2015; Brandl et al. 2016), can become

unidentifiable within 30-60 minutes, the ability to detect Chinook DNA in striped bass stomachs using molecular methods was estimated to be 66.2 h (Brandl et al. 2016). Molecular methods have been successful in determining stomach content of predators in areas of the Delta where predation is high due to the proliferation of invasive species and degradation of native habitat (Nobriga and Feyrer 2007; Perry et al. 2010; Grossman 2016; Michel et al. 2018).

Areas where predators congregate near physical structure or where there are changes in flow patterns are often referred as "predatory hot spots" due to the large amount of prey consumed (Grossman et al. 2013). For example, lower flow rates result in lengthened duration of juvenile salmon migration (Cavallo et al. 2012), increasing the interval spent in areas with high populations of predators (Feyrer and Healey 2002; Nobriga and Feyrer 2007). Many of these predators are invasive to the Delta and are known to prey on juvenile Chinook salmon (Nobriga and Feyrer 2007; Grossman 2016).

The San Francisco Bay along with the Sacramento – San Joaquin River Delta is considered one of the most invaded aquatic ecosystems in the world, with a total of 234 invasive species, of which 30 are fish (Cohen and Carlton 1998). Feyrer and Healey (2002) stated that 99% of their overall catch throughout the Delta consisted of invasive fish species, likely due to degradation and alterations of natural habitats. Invasive predators, such as the striped bass (*Morone saxatilis*), largemouth bass (*Micropterus salmoides*), and channel catfish (*Ictalurus punctatus)* dominate areas of the Delta where native fish reside, particularly in disturbed areas known to be important habitat for

juvenile salmonids during their migration to the ocean (Brown and Michniuk 2007; Michel et al. 2018). Of these three predatory species, channel catfish were found to have the highest frequency of Chinook in their diets (Michel et al. 2018), and largemouth bass are thought to have the highest per-capita impact on native fishes near shore (Nobriga and Feyrer 2007). Largemouth bass was also the most abundant predator species captured from a 2007 study in the Delta (Nobriga and Feyrer 2007). Because salmonids are not abundant, they generally do not encompass a large proportion of predator diets (Michel et al. 2018), but even a relatively small fraction can result in substantial decreases in salmon populations due to the large abundances of the different predator populations. Indeed, telemetry studies indicate low juvenile survival from route-specific migration through the multiple river corridors (Perry et al. 2010; Cavallo et al. 2012; Buchanan et al. 2018; Michel et al. 2020). Although the source of this mortality is not clear, the proximate cause is likely predation.

I selected channel catfish and largemouth bass as the subjects of my study due to their overall importance to the Delta ecosystem and their potential impact to the Central Valley Chinook population. Specifically, these species were selected because: 1) the large population abundances and wide geographical distribution of each species imply these predators can influence salmonid survival along multiple outmigration routes and in different regions of the Delta, 2) these two species have unique life histories and feeding strategies, therefore they are expected to have different trophic interactions with juvenile Chinook salmon, 3) these species were expected to have different physiology and

digestive systems, thus making an interesting comparison of evacuation rates.

The different habitats occupied by these species suggests that they should have vastly different feeding strategies. Channel catfish were at one point only found in the Gulf region and throughout the Mississippi Valley towards Canada, but have now spread across the United States, including throughout the Delta River systems (Wellborn 1990). Although preferring clear water, channel catfish do well in muddy water and can thrive in brackish systems due to taste buds covering the entirety of their body, resulting in an effective food detection strategy (Wellborn 1990). In comparison, largemouth bass feed primarily by sight and cannot reproduce in brackish water systems, thus preferring clearer waters (Moyle 2002; Davis and Lock 2007). However, largemouth start feeding on fish when they reach merely two inches in length, leading to a predominantly piscivorous life history (Davis and Lock 2007). In combination, these two species exist in many areas of the Delta, providing little refuge for juvenile salmon (Brown and Michniuk 2007).

A method which can be used to study the effect of predation on prey populations in complex ecosystems (e.g., the Sacramento – San Joaquin River Delta) is to use gastric evacuation rates modeled with population abundances (Bromley 1994). However, before these methods can be implemented, information regarding gastric evacuation is needed. More specifically, the gastric evacuation of DNA within predator digestive tracts and the factors that affect the evacuation. Information from these experiments can provide managers with necessary information on digestion rates that have previously led to uncertainties in mortality rates from predation on juvenile salmon by invasive species.

Previous studies (Grossman 2016; Michel et al. 2018; Moran et al. 2016) have indicated the need for standardized feeding trials assessing the deterioration rate of DNA within predator digestive tracts to reduce bias of prey estimation and determine the effect of environmental variables on species-specific gastric evacuation rates. In this study, I conducted laboratory feed trials on well-known predators of juvenile Chinook to determine their gastric evacuation rates. The Delta was chosen as my study site due to the large number of non-native piscivorous species (Brown and Michniuk 2007) and the high rate of predation on native juvenile salmonids, including listed Central Valley Chinook salmon (Nobriga and Feyrer 2007).

Gastric evacuation rates and bioenergetics models have been used in combination with gut fullness to predict consumption levels (i.e predation) (Hansson et al. 1996). Synonymous to gut fullness in standard visual protocols, molecular methods use a measurement that estimates the amount of DNA remaining in the digestive tract. Immediate predation levels could then be transformed from estimates of DNA abundance, into daily consumption rates dependent on gastric evacuation rates (Bromley 1994; Hansson et al. 1996; Santos and Jobling 1992). Daily consumption rates from field samples and gastric evacuation rates from laboratory trials can then be used to predict ecosystem level predation following models by Hansson et al. (1996). However, many physiological factors can affect the outcome of gastric evacuation rates including surface area-volume ratio, species specific digestion rates, temperature, feed ratio, and life stages of prey (Legler et al. 2010; Baerwald et al. 2012; Brandl et al. 2016). When attempting to

incorporate gastric evacuation analysis on populations, it is vital to include prey type, temperature, predator size, and meal size or ration (Bromley 1994). A large predator-to-prey meal ratio is assumed to increase the duration of detection of DNA. Laboratory feed trials are required to examine these factors between species and standardize evacuation rates based on these variables (Brandl et al. 2015).

The results from these laboratory feed trials can be used to inform subsequent studies designed to estimate the total consumption of juvenile salmon by populations of predatory species. Molecular field sampling of predatory stomachs would yield immediate consumption rates and provide an estimate of gut fullness. Evacuation rates calculated from laboratory trials generally correlate with the metabolism of the fish (Armstrong 1986). From this information, the amount of prey that a piscivorous fish needs to consume to maintain size, or grow at an average rate, can be calculated using bioenergetics models. Previously, field studies that used molecular methods to determine whether predators consumed Chinook salmon smolts assumed that a fish with a stomach that contained Chinook DNA had only consumed one smolt, when multiple smolts could be in the digestive tract (Grossman 2016). To correct this potential under estimation of the number of smolts that were consumed, novel methods in determining the ‘number of contributors’ of a sample using individual DNA markers (allelic estimation) is available (Sethi et al. 2019). A study completed in a laboratory setting was able to estimate the number of individuals contributing to a DNA mixture by counting individual allelic markers (Sethi et al. 2019). The presence/absence of allele genotyped samples were

applied to a likelihood equation and successfully produced a 5-fold increase in the estimation of predation rates by largemouth bass on yellow perch (Sethi et al. 2019). By using allelic estimation to estimate predation within the Delta, comparable conclusions about predation rates could be determined.

Assessing gastric evacuation rates, with multiple influencing variables, using the molecular methods developed by Brandl et al. (2016) and Sethi et al. (2019) can give management officials the resources to help understand the proportion of predation taking place on juvenile Chinook by different invasive species. Throughout this study, we aimed to address the following questions:

1) How long is juvenile Chinook DNA detectable within predator stomachs using unique molecular methods?
2) How does temperature, predator-prey feed ratio, and species affect the gastric evacuation of juvenile Chinook DNA?

My hypothesis for DNA detection was 66.2 hours based on the previous study by Brandl et al. (2016). I also hypothesized that increased temperature and larger predator-prey ratio size would increase the evacuation of DNA. Hypotheses for this experiment came from previous studies on evacuation and digestion of fish. Largemouth bass fed higher proportions of emerald lake shiners had longer stomach evacuation times than those fed smaller proportions (Beamish 1972). Brett and Higgs (1970) witnessed a decrease in evacuation from over 100 hours down to 18 hours as temperature increased 20 degrees C. Lastly, I hypothesized that largemouth bass would have an increased rate of digestion

compared to channel catfish based on physiological life history traits. Channel catfish digestive systems are elongated compared to largemouth bass due to the differences in diet items including algae, aquatic plants, and seeds (Wellborn 1990). The elongated digestive system of channel catfish could contain DNA remnants of prey items for a longer duration than the shorter system of largemouth bass. These difference in gut tissue evacuation detailed in the above hypotheses may result in systematic biases in predation models developed in the Delta.

METHODS

Feeding

During the summers of 2020 and 2021, I conducted gastric evacuation experiments on hatchery and wild largemouth bass (LMB, n=382) and hatchery channel catfish (CCF, n=220) to understand the effects of temperature, species, and predator-prey ratio on gastric evacuation of juvenile Chinook DNA. Adult hatchery LMB (fork length 205-552 mm) and CCF (fork length 257-545) were transported to the University of California – Davis Center for Aquatic Biology and Aquaculture (UC – Davis CABA). Hatchery LMB and CCF were obtained from The Fishery Inc. in Galt, CA, where LMB were reared in 10-foot flow through tanks set to a temperature of approximately 18.8˚C and CCF were reared in aquaculture ponds with a water temperature of approximately 19.2˚C. Wild LMB (fork length 223-552 mm) were captured via USGS electroshocking vessel on May 10th, 2021 near Frank's Tract within the Delta region and transported to UC – Davis CABA where they were placed in 10-foot flow through holding tanks. Prior to the initiation of the experiment, predators were acclimated for approximately two weeks in 10 ft treatment tanks set to the experimental treatment temperatures of 15.5˚C and 18.5˚C without feeding. Acclimation for this length of time was deemed necessary to 1) ensure previously digested material be eliminated and, 2) reduce overall stress and allow digestion rates to return to normal levels (Brandl et al 2015). The experimental treatment temperatures were selected based on the range of temperatures found within the Delta system during the spring, which have an effect on fish metabolism and digestion rate

because they are exothermic (Brett and Groves 1979).

The feed for the experiments were juvenile fall run Chinook smolts (80-120mm) that were received from a discontinued California Department of Fish and Wildlife (CDFW) project and transported to CABA prior to the first feed trial. Smolts were reared in 4-foot flow through tanks set to 12˚ C and fed a diet of fish feed pellets through automated fish feeders. Smolts in ration sizes of either one or three individuals were sacrificed, weighed for wet weight (g), and fin clipped for DNA identification. These ration sizes were representative of the typical amount of prey found within piscivore's stomach in previous field studies (Nobriga and Feyrer 2007). During hatchery LMB trials, smolts required separation into smaller, third-sized pieces due to the smaller stomach size of hatchery LMB, which were not accustomed to piscivorous prey. Smolts were cut near the posterior portion of the gill operculum and the anterior portion was used for feeding. The smolts were not cut for the wild LMB and hatchery CCF gastric evacuation trials.

After the two-week acclimation period, individual predators were anesthetized, and force fed the appropriate ration. Prior to being force fed the smolts, all predators were anesthetized in a buffered neutral solution of MS – 222, tagged for future identification, fork lengths were measured to the nearest mm, and fin clips were collected for DNA identification. Smolt rations were force fed to anesthetized LMB and CCF, ingestion time was recorded, and predators were promptly returned to their respective temperature treatment tanks for recovery. Reflexes from the trachea provided a simple feeding procedure. After opening the predator's jaw, a smolt was placed deeply in the gape using

a set of decontaminated tweezers. Pressure on the trachea caused a reflex action to occur where the predator consumed the smolt with ease. Handling time for single ration samples averaged approximately 45 seconds while handling time for triple ration samples averaged approximately 65 seconds. During the first 30 minutes of recovery, predators were observed to ensure no regurgitation occurred. If regurgitation was observed, the predator sample was removed from the treatment and a new individual was sampled from a batch of spare predators. If unknown regurgitation occurred beyond the 30-minute observation period, a note was made on the data sheet indicating a potential regurgitation from select tank and stomachs were inspected upon dissection. If regurgitation was suspected, a new individual from the spare predators was sampled.

At regular intervals post-ingestion, a subset of five predators from each treatment were euthanized and the digestive tracts were removed. A sterile surgical scalpel was used to make an incision from the predator's anal cavity along the ventral side of their body, anterior to the gill plate. A second sterile scalpel was then used to make incisions internally until the entire digestive tract could be removed. The entire digestive tract consisted of the trachea, the pyloric cecum, stomach, pyloric sphincter, intestinal tract, and anal cavity resulting in one sample. The dissections began 6 hours post ingestion (t=6) and continued every 12 hours until t=96 hours. After that, a final sample occurred at t=120 hours (5 days), resulting in a total of 10 sample intervals and an expected 50 predator samples per temperature per ration. The dissected digestive tracts were injected with a 3ml solution of 100% non-denatured EtOH to halt any further enzymatic digestion

and were then stored in 200 ml conical vials. The conical vials were filled with 100% non-denatured EtOH ensuring that the sample to liquid ratio was approximately 2:3. Sample vials were refreshed with 100% non-denatured EtOH 24 hours post dissection and wrapped in parafilm to prevent evaporation. Samples were then stored at room temperature until DNA extraction began.

A duration of 5 days was selected to determine the decline of DNA detection ability and estimate digestion rate of predators based on previously observed DNA detection time (Brandl et al. 2016). Initial treatment numbers included 5 fish beyond those necessary for the experiment to replace fish that were unusable due to regurgitation or incidental mortality. Due to available tank space and timing of trials, the experiment consisted of 2 trials of 55 fish per month over 2020 and 2021 with replicate tanks. Four cages were placed within tanks to separate feeding groups and for organization at time of dissection. Cages filled the holding tanks to near max capacity, reducing the flow-through holding tanks by less than $2m^2$.

After all gastric evacuation experiments and dissections were complete, digestive tract samples were shipped NOAA's Auke Bay Laboratory for DNA analysis. Unexpected deaths, regurgitation, and shipping damages resulted in a total of 587 predator samples being analyzed (215 CCF and 372 LMB).

Three molecular detection methods (qPCR, metabarcoding, and a single-nucleotide polymorphism (SNP) microhaplotype panel) were used to investigate the presence and enumeration of salmon DNA. Quantitative polymerase chain reaction (qPCR), also

referred to as real-time PCR, is the process of enzymatic amplification and detection of specific DNA sequences. The typical definition for amplification efficiency is the number of amplification cycles required to register a certain amount of DNA, calculated from standards with known concentrations. Once amplification efficiency for a sample is determined, the number of DNA molecules within the sample can be calculated relative to the total number of molecules within the initial sample (Kubista et al. 2006).

The qPCR process is covered in detail by Kubista et al. (2006), but I will briefly outline the major steps of the analysis. The first step of qPCR amplification is melting, annealing, and elongation of sample DNA onto a DNA template, or previously known sequence assay. The process of melting, annealing, and elongation is referred to as a cycle and typically takes between 20-40 cycles before amplification of 125 pre-selected target loci can be detected. Detection is determined once the target DNA reaches a fluorescent signal threshold. Target DNA is fluorescently labeled, typically with cyanine-based dyes, and the amount of fluorescence released by the target DNA corresponds to the amount of initial DNA present. Fluorescent signals create a response curve for each sample, transformed into a standard curve equation, which is assumed to be correlated to the number of molecules within the initial sample. The selection process for the above-background fluorescence threshold level is mostly arbitrary (Kubista et al. 2006), meaning cycle threshold and amplification efficiency alone do not equate to quantity of molecules. Standard curves, comparisons to standards (controls), and amplification calculations are required to establish a basis of cycle threshold.

Metabarcoding is also a complex process, which is described in detail in Deiner et al. (2017) and Liu et al. (2020), but here I describe the primary concepts and methods. Herbet et al. (2003) first defined the use of barcoding using mitochondrial DNA (typically cytochrome *c* oxidase subunit 1, COI) as a way to identify species across the entire animal kingdom. Barcoding refers to the sequencing and identification of a single species using diagnostic markers (COI for example), while metabarcoding refers to the identification of multiple species within a mixed sample. Metabarcoding uses a high-throughput sequencing (HTS) platform to analyze millions of sequences at a time (Deiner et al. 2017). Examples of high-throughput platforms include Illumina and Ion Torrent. As with qPCR, a previously sequenced assay must be provided as a reference for the unknown sample. An unknown mixed sample undergoes a first-round PCR amplification and primer-template sequence alignment (similar to qPCR above). The unknown sample then goes through a second round of PCR amplification where all paired sequences are given HTS primer tags so that sequencing reads can be sorted as original, unknown samples after HTS (Liu et al. 2020). Following the two rounds of PCR, tagged samples are pooled into operational taxonomic units (OTU's) and read into a reference database to be taxonomically assigned.

The third, and final, molecular method used within this study was a single-nucleotide polymorphism (SNP) microhaplotype panel. SNP refers to the variation within a sequence at a single location within species (Kwok 2001). This can also be referred to as allelic expression within a gene, where alternative forms of DNA sequence produce

variations. One advantage of using SNP panels is the ability to identify unique individuals, whereas metabarcoding and qPCR analysis can only detect presence or absence of a species and can estimate an abundance of DNA molecules present within a sample. However, metabarcoding and qPCR cannot be used to estimate how many individuals are present within the sample. In contrast, SNP methods following Baetscher et al. (2018) are able to estimate haplotype frequency within a mixed sample. Using the assumed haplotype frequency, number of contributors (NOC, unique individuals) within the mixed sample can be estimated following a likelihood-based model by Sethi et al. (2019) and Andres et al. (2021). This model takes into consideration the likelihood of diploid individuals masking the number of distinct alleles within the mixture. For example, a mixture with 2 heterozygous and 1 homozygous individuals could have a range of 2 – 5 unique alleles. This would be translated into a range of 2 – 5 unique individuals within the mixture when the true value is 3. The likelihood-based model attempts to incorporate this masking effect by determining the number of haplotypes, their frequencies, and the relative distribution of haplotypes within a mock mixture (or previously known assay).

Within this study, qPCR and metabarcoding were not able to directly quantify the number of individuals in a diet, but fluorescence (qPCR) and number of reads (metabarcoding) may be correlated to the number of individuals present from an initial unknown sample (Kubista et al. 2006; Liu et al. 2020). The SNP panel was used to directly quantify the number of individuals in the diet sample using the methods of Baetscher et al. (2018) and

Sethi et al. (2019). Metabarcoding and qPCR analyses were directly compared between their two respective quantity estimates and the factors that may affect them (Figure 2).

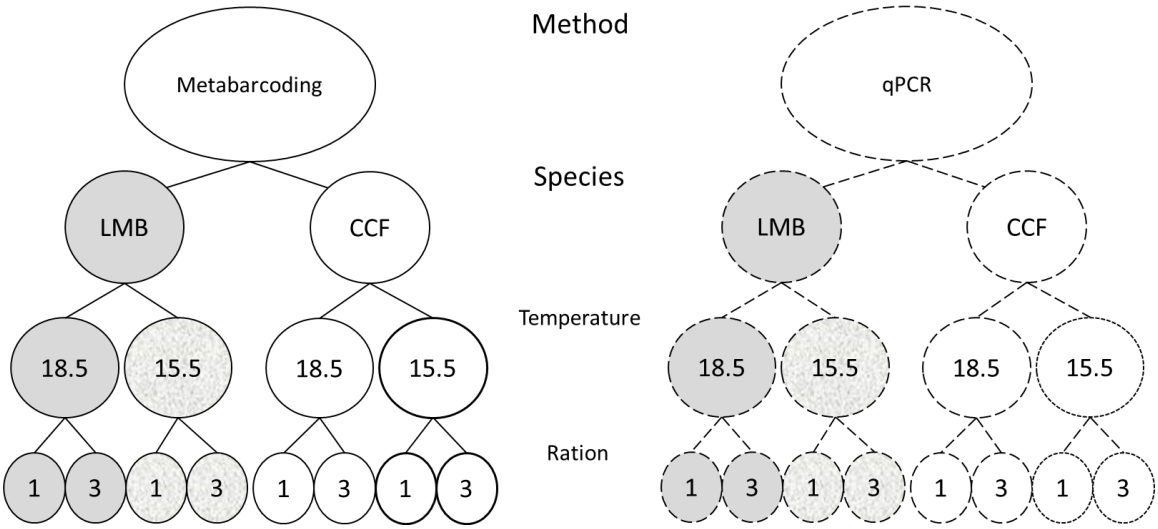

Figure 2: *Bubble flow chart depicting the differences between molecular method analyses and factors within trials.*

Comparing these three methods through gastric evacuation can provide a template for future studies on how sensitive each detection method is at different stages throughout digestion. Managers and researchers can use this information to understand how recently prey items were ingested depending on which molecular method is used. A recent study by Brandl et al. (2021) found low rates of Chinook salmon comprising the diet of striped bass but believe predation on soft-bodied prey (such as juveniles) may be overlooked. Biotic factors may not be the only factor affecting detection sensitivity in this case. The choice of molecular method may also influence rate of detection. Each method used within this study provides unique outputs that may be needed for different studies (Figure

3). Providing a baseline for detection sensitivity for three unique methods on soft-bodied prey could provide an explanation for the loss of detection on juvenile fishes.

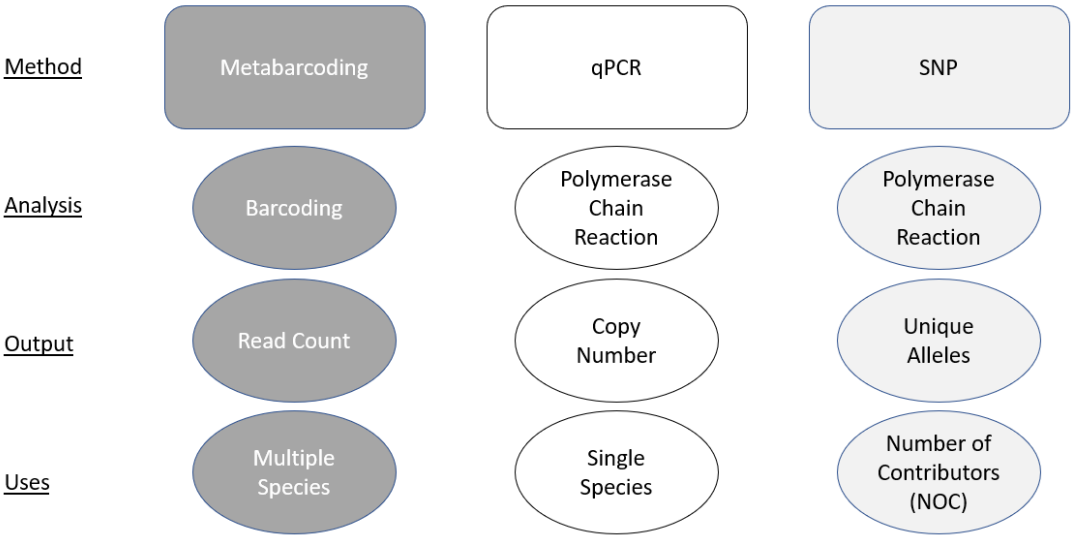

Figure 3: Bubble flow chart depicting the differences between the three molecular methods used: metabarcoding, qPCR, and SNP microhaplotype panel.

IACUC

Laboratory experiments and predator captures were conducted under the institutional animal care and use committee (IACUC) protocol number 2021F5A and Scientific Collection Permit (SCP) number S-200520003-20081-001. Approval date for 2021F5 was 04/27/2021 and an expiration of 04/27/2022.

Digestive Tract Content Dissections & Visual Analysis

Using aseptic technique, the stomach contents were dissected from the stomach and

added back into the jar of ethanol. The stomach was then washed in the jar of ethanol to remove any remaining stomach contents and discarded (tissue samples from the bass were previously collected). Following this, the intestine was isolated from the cecum and surrounding tissue, and any intestinal contents were also added to the jar of ethanol. The remaining intestinal and other bass tissue was discarded, leaving only the stomach and intestinal contents.

To prepare 1.5 mL tubes for extraction, a 1 mL pipette was used to pipette as much stool as possible into the tube. In cases where a high amount of stool was present, any clear ethanol toward the top was pipetted out of the 1.5 mL tube and this process was repeated. To sample chunks of partially digested tissue, a small piece was taken from each visible chunk to ensure thorough sampling. These were frozen until the time of extraction.

A visual assessment was performed with each dissection, noting whether stool was present or absent and whether it was a low, medium, or high amount relative to other samples. The same was done for any undigested tissue. Stool was defined as relatively homogenous and generally dark in color, while undigested tissue was lighter in color and in chunks of variable size and shape. Visual analysis of prey tissue and stool during laboratory dissections led to two categorical scores assigned values of 0 – 3. The first categorical score was given to undigested tissue within the stomach. Undigested tissue that could be discerned as either fish or non-fish was labeled as “tissue.” A score of 3 represented a large amount of tissue while a score of 0 represented no amount of tissue present. The second categorical score was given to undiscernible tissue. Homogenized

tissue that could not be discerned as fish or non-fish, and was either in the stomach or beyond, was labeled as “stool.” A score of 3 represented a large amount of stool while a score of 0 represented no amount of stool. A total visual score was calculated based on the sum of both “tissue” and “stool” categories, giving total visual score a range from 0 – 6. During analysis, equal weights were given to both “tissue” and “stool” remnants as there is little known information on the different DNA abundance produced between stool amounts and tissue amounts.

Between samples, all utensils and surfaces were wiped down with a dry paper towel to remove the bulk of the ethanol, followed by a 5% bleach solution, followed by 70% ethanol, then dried thoroughly.

DNA Extractions

Fin clip extractions were performed using two methods; some samples were extracted using a Qiagen DNEasy Blood and Tissue kit following the manufacturer’s instructions, while others were extracted using a 10% chelex slurry (1% v/v Triton-X 100, 1% v/v Tween 20, 10% w/v Chelex 100) and heated on a thermal cycler at 95°C for 10 minutes.

Stomach and intestinal contents in 1.5 mL tubes were centrifuged 3 minutes at 5000 G and any available ethanol was pipetted off the top. Samples were then allowed to sit in the fume hood to evaporate any remaining ethanol prior to DNA extraction. Stomach and intestinal contents were extracted using a Macherey Nagel Nucleospin 96 DNA Stool kit modified by replacing bead-induced lysis with enzymatic lysis, using a per-sample volume of 25 ul proteinase-k and with 850 ul lysis buffer ST1. Samples were incubated

overnight at 56°C, and the manufacturer's instructions were followed using the centrifuge protocol for subsequent DNA extraction. One negative control was included on each extraction plate for all sample types.

Response Variables

Read count has been defined as the number of matching sequencing reads assigned to a DNA sample during taxonomic assignment through barcoding or metabarcoding (Deagle et al. 2019). Read count is then typically translated into two types of results: 1) occurrence (e.g., presence and absence), and 2) relative read abundance (RRA). RRA can then been used to estimate the relative abundance or frequency of prey items in a diet mixture (Ford et al. 2016; Thomas et al. 2017). For the purpose of this study, read count represented relative abundance of Chinook DNA remaining within prey digestive tracts through metabarcoding molecular analysis (i.e., lower read counts corresponded to smaller portions of DNA remaining). Comparatively, qPCR analysis used quantity of DNA copies per volume of solution (ng/ul) for each sample as a means of DNA measurement. The following equation was used to convert qPCR quantity to copy number:

$$copy\ \# = \frac{x * 6.0221E^{23}mol/mole}{N * 660g/mol * 1E^{9}ng/g}$$

where x is quantity in ng/ul and N is length of sample in base pairs (15,000 for our case). Similar to metabarcoding, copy number represented relative abundance of Chinook DNA remaining. A lower copy number corresponded to a smaller portion of DNA remaining

within the sample.

Analysis

All analyses were completed using RStudio (RStudio Team 2021), R programming language, and relevant data packages included under CRAN. A hurdle model was fit to determine the number of reads from metabarcoding or copy number from qPCR with parameters: time since ingestion (time), temperature of tanks (temp), predator-to-prey size ratio (ratio), and species effect (species).

Time post-ingestion was calculated by back dating dissection time from ingestion time, giving an exact duration of time spent in digestive tract. Time post-ingestion is expected to decrease read count due to excretion of prey items and degradation of DNA material through digestion, reducing extraction ability (Moran et al 2016). Comparisons between visual tissue and stool amounts with metabarcoding results utilized time post-ingestion as a categorical factor with each hour (6, 12, 24, … etc.) representing a unique factor.

Treatment tanks were designated as a binary factor between two average temperatures: 15.5˚C and 18.5˚C. A higher tank temperature is expected to increase the metabolic rate of fish and subsequently increase evacuation (Brett and Groves 1979). In contrast, cooler temperatures are expected to lengthen the evacuation of DNA, potentially prolonging detection.

The size of the meal relative to the size of the predator may increase detection ability due to longer evacuation times for larger prey items (Beamish 1972). Predator-to-prey ratio

was transformed to a continuous factor from the binary ration of 1 or 3 smolts fed. Individual smolts were wet weighed to nearest 0.1 g to give prey mass for the predator-to-prey ratio. Predators were not wet weighed but measured to nearest fork length (nearest 1 mm). Weight-at-length regressions previously formed in the literature were used to calculate individual predator weights (Keenen et al. 2011; Henson 1991). Traditional weight-at-length regression for largemouth bass

$$w(l) = al^b$$

was used where weight in grams (w) at length in mm (l) is equal to growth parameter *a* times length raised to growth parameter *b*. Parameters *a* and *b* determined through linear-least squares regression (Henson 1991). An alternative model for channel catfish

$$w(l) = (l/L)^b$$

was used to determine length at weight where L is equal to the standard length of a catfish at 1 kg (Keenen et al. 2011). I used this alternative model because channel catfish are known to have a lean, longer form at higher weights compared to largemouth bass, which have an oval, rounder growth pattern at higher weights. Unique weight-at-length regressions were used for each species as the condition of each species widely vary. From there, predator mass was divided by their respectively fed prey mass to obtain proportional prey size relative to predator, or predator-to-prey mass ratio (PPMR). PPMR has been used frequently in food web and community sized predation studies to understand metabolic constraints of predators (Woodward and Warren 2007). An inverse

relationship was hypothesized for PPMR, meaning a large ratio would display a negative effect on digestion rate, decreasing gastric evacuation time. Alternatively, a small ratio would display a positive effect on gastric evacuation, lengthening digestion duration. To illustrate by example, imagine two predators of the same size. One is fed a small prey while the other is fed a large prey. The predator fed a large prey would have a smaller ratio and therefore, a slower rate of digestion. The predator fed a smaller prey would have a larger ratio and therefore, a quicker rate of digestion.

Both qPCR copy number and metabarcoding read counts needed to exceed a minimum threshold to be considered valid, which resulted in an overabundance of zeros in the data, thus I analyzed these data with a zero-inflated model (i.e., a hurdle model). The hurdle model was a combination of two models where one accounted for the zero-inflated portion ("zi", zero's introduced into the data), and the second accounted for the conditional, linear portion of the data. The standard equation for this model is

$$E[y|x] = \frac{1 - f_1(0|x)}{1 - f_2(0|x)} u_2(x)$$

where y is our response variable, x are the covariates included in the model, and f is equal to the distributions (Welsh et al. 1996). I used a binomial distribution to model the zeros and a Gamma distribution to model the positive values. The package "glmmTMB" (Brooks 2021) was used within R to fit the hurdle model. Raw residual diagnostics are affected by the abundance of zero's within the data, thus simulated diagnostics using the "DHARMa" package (Hartig 2019) were used.

Model Selection

All combinations of factors of interest were used during model selection because I had no *a priori* reasons to exclude any candidate models. Using evacuation rates of prey DNA within predators to supplement current modeling efforts is a novel approach and required full examination of covariate effects, thus the model selection results should be considered exploratory. Time post-ingestion was expected to have the largest effect on evacuation and therefore was included in each model during the model selection process. Time post-ingestion is expected to reduce DNA extraction ability due to the degradation and digestion of prey tissue (Moran et al. 2016). Temperature, feed ratio, and species were each expected to have an effect on evacuation as well (Beamish 1972; Brett and Groves 1979). These three variables (species, ratio, and temperature) were included into models as single variables and as an interaction term paired with time post-ingestion (Table 1). The interactions between time and each of the other covariates was include to determine if there was an effect of any covariate on the evacuation rate, which is defined as a change in the amount of material in the digestive tract over time. In addition, an interaction term was included between species and both feed ratio and temperature. Channel catfish are known to have elongated intestinal tracts compared to bass, therefore larger meals may remain for a longer duration, while smaller meals may pass through rapidly. Likewise, because largemouth bass and channel catfish have different habitat preferences, it is likely they will have different temperature responses. Interaction terms were only included if the variable was included into the model as a main effect (Table 1).

Table 1: Hurdle model selection table. The 'x' denotes if the variable was included in the model. Headers with two variables separated by a colon indicate an interaction term. Species factor labeled as 'Sp'.

ID	Time	Sp	Temp	Ratio	Sp:Temp	Sp:Ratio	Time: Sp	Time: Temp	Time: Ratio
1	x	x	x	x	x	x	x	x	x
2	x	x	x	x	x	x	x	x	
3	x	x	x	x	x	x	x		x
4	x	x	x	x	x	x	x		
5	x	x	x	x	x	x		x	x
6	x	x	x	x	x	x		x	
7	x	x	x	x	x	x			x
8	x	x	x	x	x	x			
9	x	x	x	x	x		x	x	x
10	x	x	x	x	x		x	x	
11	x	x	x	x	x		x		x
12	x	x	x	x	x		x		
13	x	x	x	x	x			x	x
14	x	x	x	x	x			x	
15	x	x	x	x	x				x
16	x	x	x	x		x	x	x	x
17	x	x	x	x		x	x	x	
18	x	x	x	x		x	x		x
19	x	x	x	x		x	x		
20	x	x	x	x		x		x	x
21	x	x	x	x		x		x	
22	x	x	x	x		x			x
23	x	x	x	x		x			
24	x	x	x	x			x	x	x
25	x	x	x	x			x	x	
26	x	x	x	x			x		x
27	x	x	x	x			x		
28	x	x	x	x				x	x
29	x	x	x	x				x	
30	x	x	x	x					x
31	x	x	x	x					
32	x	x	x		x		x	x	

ID	Time	Sp	Temp	Ratio	Sp:Temp	Sp:Ratio	Time: Sp	Time: Temp	Time: Ratio
33	x	x	x		x		x		
34	x	x	x		x			x	
35	x	x	x				x	x	
36	x	x	x				x		
37	x	x	x					x	
38	x	x	x						
39	x	x		x		x	x		x
40	x	x		x		x	x		
41	x	x		x		x			x
42	x	x		x		x			
43	x	x		x			x		x
44	x	x		x			x		
45	x	x		x					x
46	x	x		x					
47	x	x					x		
48	x	x							
49	x		x	x				x	x
50	x		x	x				x	
51	x		x	x					x
52	x		x	x					
53	x		x					x	
54	x		x						
55	x			x					x
56	x			x					
57	x								
58	x	x	x	x	x				
59	x	x	x		x				

The hurdle model used during analysis required further model selection due to the second equation explaining the zero-inflation within the data. Similar to the initial equation explaining non-zero values, time post-ingestion, species, ratio, and temperature were the main variables expected to influence evacuation. The same process, and models, as the

non-zero portion was used to model and select the equation for the zero-inflated portion. There was no previous information indicating alternative effects towards the zero-inflated portion, therefore identical models were fit along with the conditional portion.

Initial hurdle models were created based on previous literature and background information on which covariates can affect evacuation. Models were analyzed using the function glmmTMB (Brooks 2021) and compared using the function 'aictab()' within the "stats" package and "AICcmodavg" package, respectively (Rstudio Team 2021; Mazerolle 2020). Psuedo r-squared values were estimated using the function 'r2_zeroinflated' within the "performance" package (Lüdecke et al. 2021). Residual diagnostics were simulated an analyzed for error and a pattern of heteroscedasticity using the "DHARMa" package (Hartig 2019). Post-hoc Tukey tests for difference in means and estimated marginal means (EEM) were assessed using the "emmeans" package (Lenth et al. 2022).

RESULTS

Metabarcoding

A total of 588 specimen samples between channel catfish and largemouth bass were sent for analysis and completed. Metabarcoding analysis returned 536 non-zero sample results, or 91%. 9% of the returned samples from metabarcoding did not return a positive Chinook (*Onchorhynchus tshawytscha*) DNA presence. The frequency and detectability of read count from metabarcoding analysis decreased as time post-ingestion increased (Figure 4). Figure 4 below also shows how the number of non-zero results decreased as time post-ingestion increased.

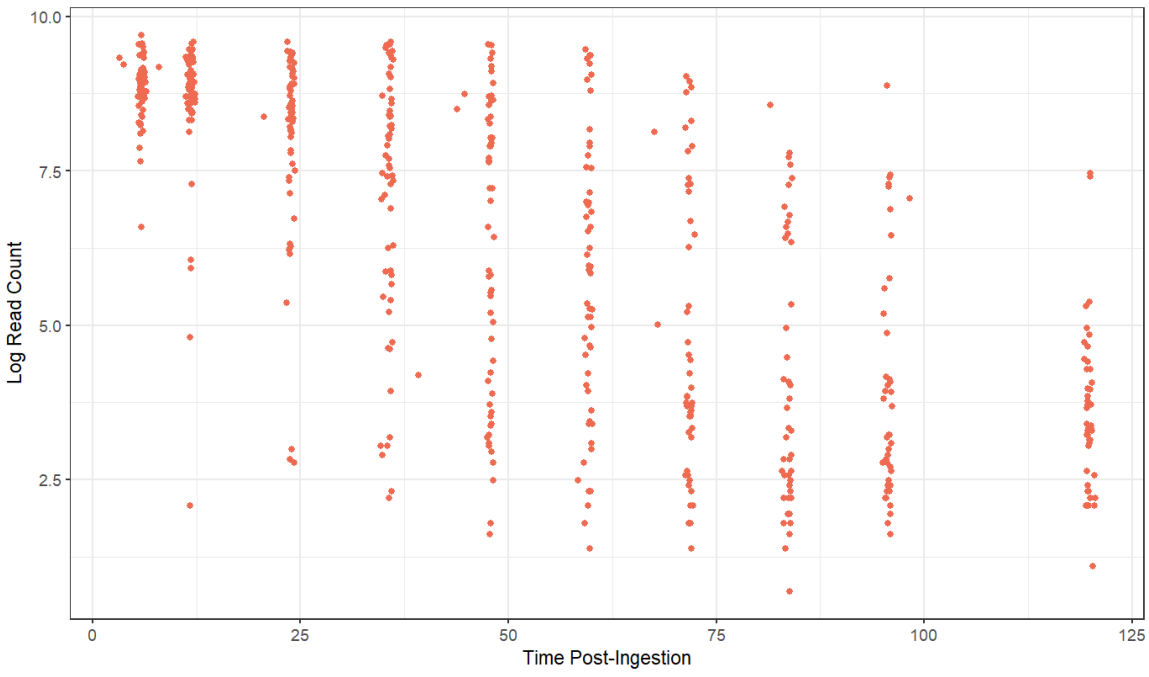

Figure 4: Non-zero metabarcoding sample results displaying log read count quantity over time after ingestion (hours).

A total of 59 hurdle models were assessed and selected based on dAICc values (Table 3).

The model of best fit using metabarcoding analysis had a logarithmic evacuation equation with coefficients listed in Table 2 and a dispersion estimate of 1.81 from the Gamma distribution. The correlative measure of fit (R^2) for this model was equal to 0.45, including zero-inflated residuals. Diagnostic plots for this model found some patterns in the residual plots that may indicate the model is missing some covariates or interactions. However, I proceeded with this model as it was the most parsimonious from our original candidate model set (Appendix 1).

Table 2: Beta coefficients and standard error values for most model of best fit under metabarcoding analysis. 'ZI' refers to the zero-inflated portion of the model.

Variable	*Estimate*	*SE*	*ZI Estimate*	*ZI SE*
Intercept	8.35	0.14	-3.28	0.52
Time	-1.08	0.13	0.50	0.37
Species	-1.08	0.18	0.26	0.65
Temperature	-0.97	0.19	-0.23	0.70
Ratio	-0.36	0.06	0.31	0.14
Time:Species	-0.23	0.13	0.77	0.40
Time:Temperature	-0.32	0.13	-0.10	0.37
Species:Temperature	0.55	0.24	1.01	0.80

Channel catfish and largemouth bass both showed exponential decay patterns in gastric evacuation of juvenile Chinook DNA through time (Figure 4). Read count had a continuous rate of decline that began rapidly post-ingestion but slowed as values approached 120 hours post-ingestion. An interaction was seen between time and species and time and temperature indicating both a species and temperature effect on the rate of digestion. Largemouth bass had a faster rate of digestion than channel catfish. Higher temperatures also resulted in a faster rate of digestion. Conversely, predator-prey ratio did not have an interaction with time, indicating no effect on digestion rate. Although predator-prey ratio did not affect rate of digestion, it was included as a main effect variable within the model displaying an effect on DNA detection (read counts). The negative coefficient indicates a negative result on read count of smolt DNA. Previously stated, this is an inverse relationship, meaning as predator-prey ratio increases, measured DNA becomes less. Or in other words, as prey intake size decreases, detection quantity of DNA decreases. Lastly, temperature and species had a positive interaction, indicating that the difference between the two temperature treatments was different between the two species. Largemouth bass had more similar amounts of DNA between the two temperature treatments than channel catfish (Figure 5).

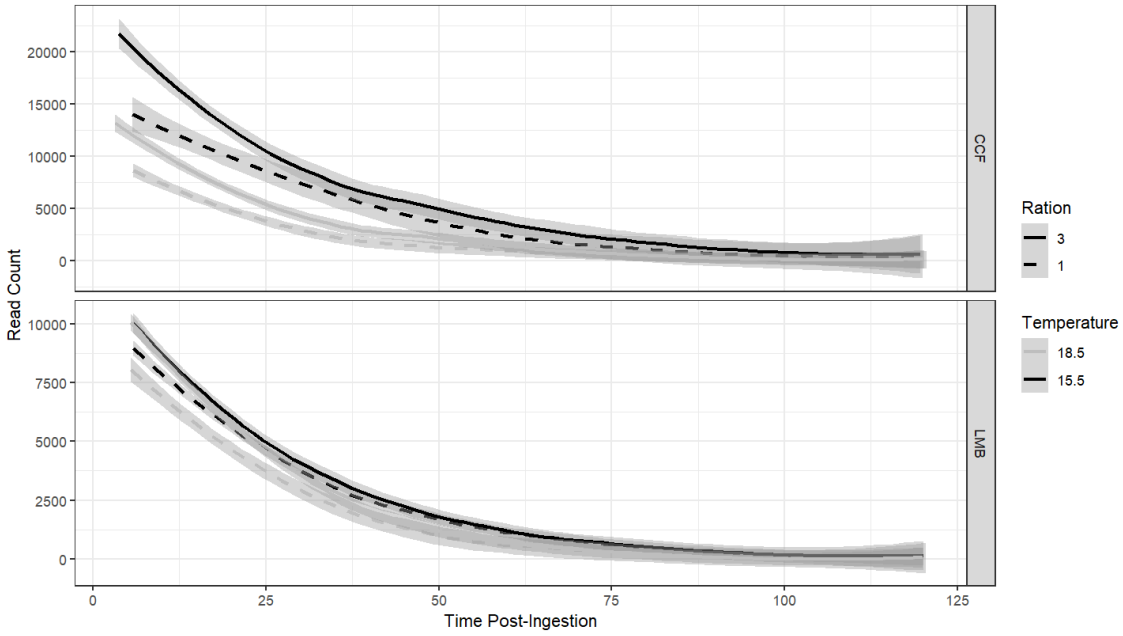

Figure 5: Gastric evacuation of juvenile Chinook DNA read counts within channel catfish and largemouth bass digestive tracts under metabarcoding analysis. (Note the differing y-scales for CCF and LMB).

One important aspect to note in Figure 5 is the difference between y-axis scales, or amount of metabarcoding reads determined during analysis. Channel catfish read count was nearly double over largemouth bass until approximately 85 hours. Largemouth bass also had less of an effect from both temperature and predator-prey ratio than channel catfish. Although higher temperatures increased evacuation in largemouth bass, it was not to the same degree as was seen in channel catfish (Figure 5). Similarly, predator-prey ratio had only a marginal effect on detection of DNA within largemouth bass when comparing effects to channel catfish.

The model with the next best fit had a dAICc value of 2.11 and did not include an interaction between the time and temperature (Table 3). The top model candidates all had

dAICc values <5 (Table 3).

Table 3: Metabarcoding hurdle model selection table showing top 10 models based on AICc. Model numbers correspond to Table 1.

Model	*Parameters*	*AIC*	*dAIC*
10	17	9276.07	0
12	15	9278.18	2.11
25	15	9278.52	2.45
9	19	9279.23	3.16
2	19	9279.64	3.56
14	13	9279.91	3.84
58	13	9279.91	3.84
6	18	9280.7	4.64
27	13	9280.77	4.7
29	13	9280.78	4.71

Channel catfish had increased estimated marginal means (EEM) of DNA on average throughout the digestion period compared to largemouth bass (Figure 6). Through EMM post-hoc analysis, channel catfish were predicted to have a higher detection quantity for a longer duration when compared to largemouth bass (Figure 6). This analysis continued to predict higher quantities of smolt DNA detected within channel catfish as was seen in the metabarcoding hurdle model (Figure 6). Significant detection differences began to occur

at approximately 36 hours for both species, with a large change in detection occurring at 120 hours (Figure 6).

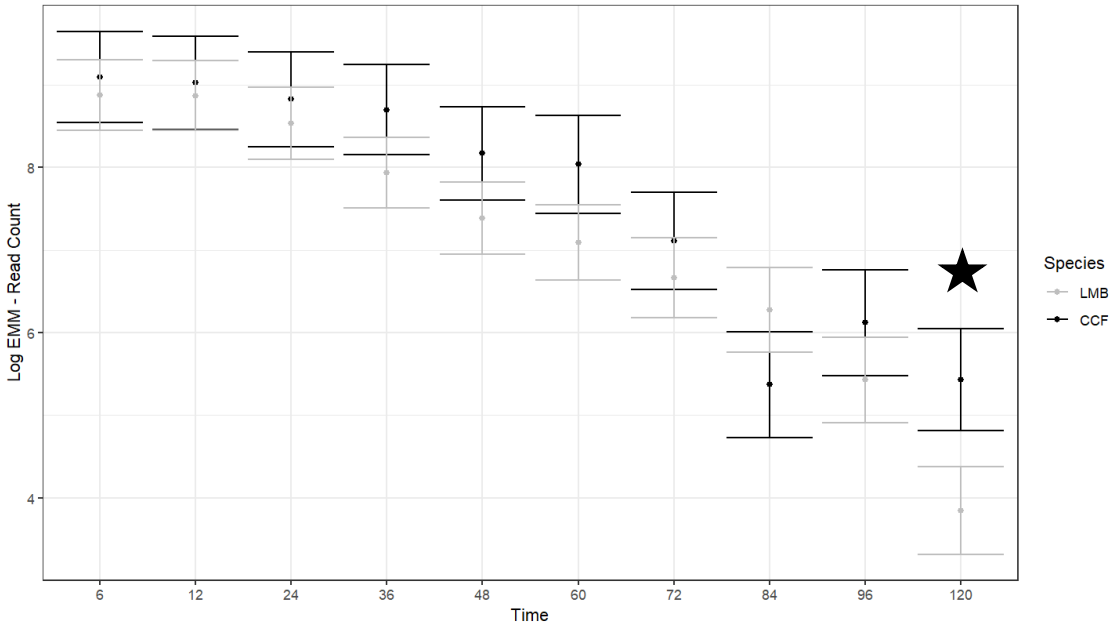

Figure 6: Estimated marginal means (EEM) plot contrasting species:time interaction. Lettering indicates significant differences between estimated marginal means within groups.

Estimated marginal mean post-hoc analysis determined lower temperature retained higher quantities of DNA for a longer duration, slowing down evacuation rate (Figure 7). A large decrease in DNA quantity occurs at 48 hours between the two treatment temperatures and is continuously seen through the digestion time (Figure 7).

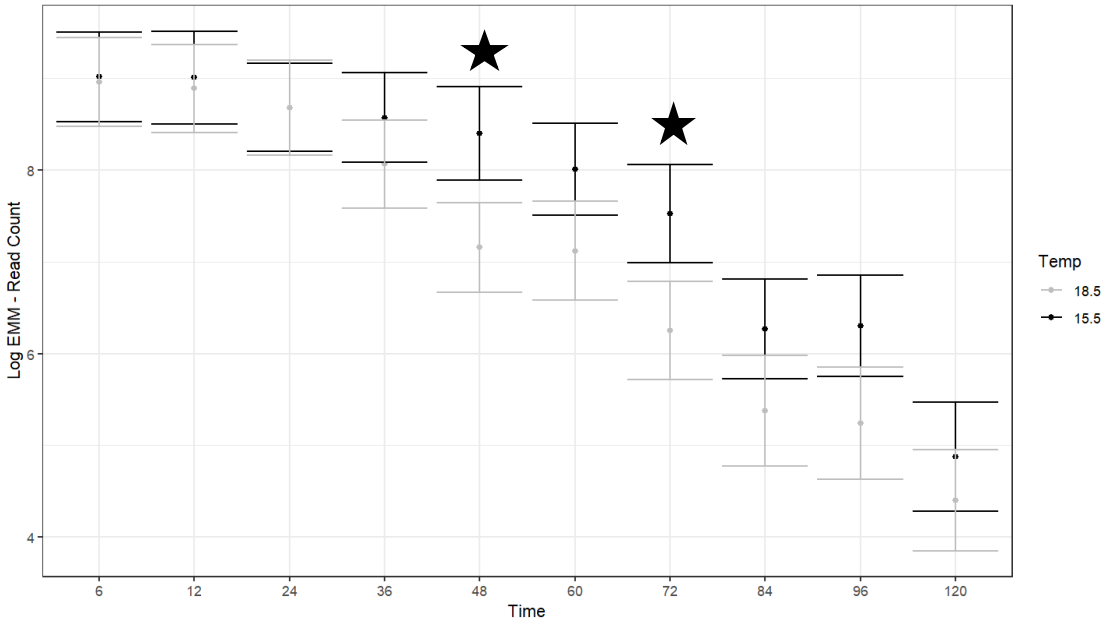

Figure 7: Estimated marginal means (EEM) plot contrasting temperature:time interaction. Lettering indicates significant differences between estimated marginal means within groups.

Decay of read count quantity was observed beginning between 6- and 12-hours post-ingestion (Table 4). This decay continued throughout the entire 120-hour digestion period with 90% decay observed at approximately 72 hours (Table 4). On average, largemouth bass displayed larger percent loss in read count compared to channel catfish (Table 4).

Table 4: Percent decay of simulated read count estimates between time post-ingestion groups using metabarcoding analysis. Calculated from initial sampled dissection (hour 6).

Species	***CCF***				***LMB***			
Temperature	*18.5*		*15.5*		*18.5*		*15.5*	
Ration	*1*	*3*	*1*	*3*	*1*	*3*	*1*	*3*
Hour 6	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
12	19.46	32.74	17.24	34.65	18.98	15.97	11.81	25.95
24	61.94	62.06	37.68	51.97	50.13	55.64	48.32	49.35
36	74.45	73.04	54.56	65.57	74.58	74.78	65.93	68.82
48	84.38	82.91	73.12	75.35	85.22	85.01	78.78	80.19
60	86.45	88.94	84.53	84.47	91.02	92.90	86.06	88.55
72	91.73	94.07	89.31	88.62	95.29	95.64	87.34	91.89
84	97.03	96.63	91.83	93.11	97.49	97.83	94.73	94.10
96	96.87	98.00	95.34	95.00	98.72	98.73	96.42	97.03
120	98.96	99.11	97.43	97.67	99.68	99.69	99.02	98.99

qPCR

A total of 59 hurdle models were assessed and selected based on dAICc values for samples analyzed using qPCR techniques (Table 1). 39% of the 588 samples analyzed with qPCR were below the quantification threshold required to return a postive DNA quantification. These samples were all given a value of zero for copy number (calculated

from quantification). The frequency and sensitivity of copy number from qPCR analysis decreased as time post-ingestion increased (Figure 8). Figure 8 below also shows how the number of non-zero results for copy number decreased as time post-ingestion increased.

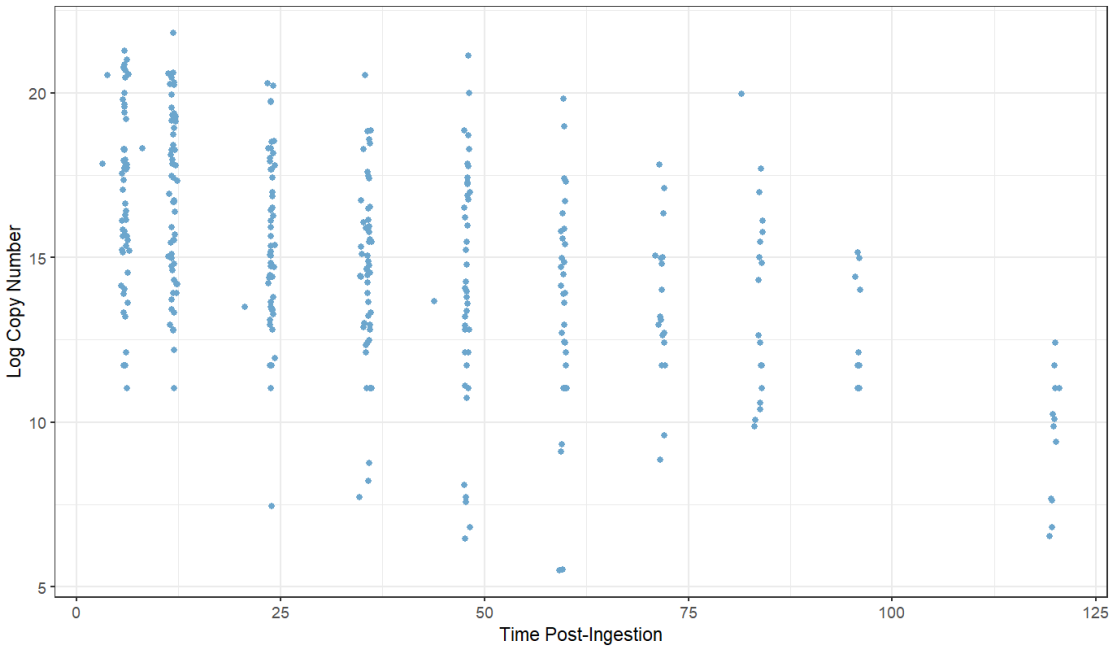

Figure 8: Non-zero qPCR sample results and time after ingestion (hours).

The best fit hurdle model after model selection included all four main effect variables (time, species, ratio, and temperature) and interaction terms species:ratio and time:ratio (Table 5). The model of best fit using qPCR analysis had a logarithmic evacuation of DNA quantity with coefficients from Table 6 and a dispersion estimate of 3.51 from the Gamma distribution. The correlative measure of fit (R^2) for this model was equal to 0.39. Examination of the diagnostic plots indicated that the model did not violate any assumptions (Appendix 2).

Table 5: Beta coefficients and standard error values for model of best fit under qPCR analysis. 'ZI' refers to the zero-inflated portion of the model.

Variable	*Estimate*	*SE*	*ZI Estimate*	*ZI SE*
Intercept	17.86	0.22	-0.25	0.21
Time	-1.87	0.13	1.59	0.13
Species	-2.01	0.23	-0.85	0.24
Temperature	-0.42	0.21	0.52	0.21
Ratio	-0.90	0.17	0.19	0.13
Species:Ratio	-0.75	0.29	0.30	0.28
Time:Ratio	-0.58	0.16	0.04	0.14

Results from qPCR molecular anlaysis showed similar decay patterns to metabarcoding molecular analysis, but had an alternative model of best fit with unique interactions. qPCR results showed effects between gastric evacuation and the interactions between species:ratio and time:ratio where metabarcoding results had effects from the interactions between time:species and time:temperature. Predator-prey ratio was found to be species dependent, which was not seen in metabarcoding analysis. Largemouth bass at higher predator-prey ratios (or smaller prey intake) had decreased DNA concentrations comparative to channel catfish (Table 5). In other words, predator-prey ratio effected largemouth bass to a greater, negative degree than for channel catfish. Predator-prey ratio was also the only variable that effected the rate of evacuation for qPCR anlaysis (Table 5, Figure 9). At higher prey intake, or smaller predator-prey ratios, evacuation rate of prey

DNA was slower than at larger predator-prey ratios (Table 5, Figure 9). Finally, similar to metabarcoding, increased temperature decreased detection quantity (Table 5, Figure 9)

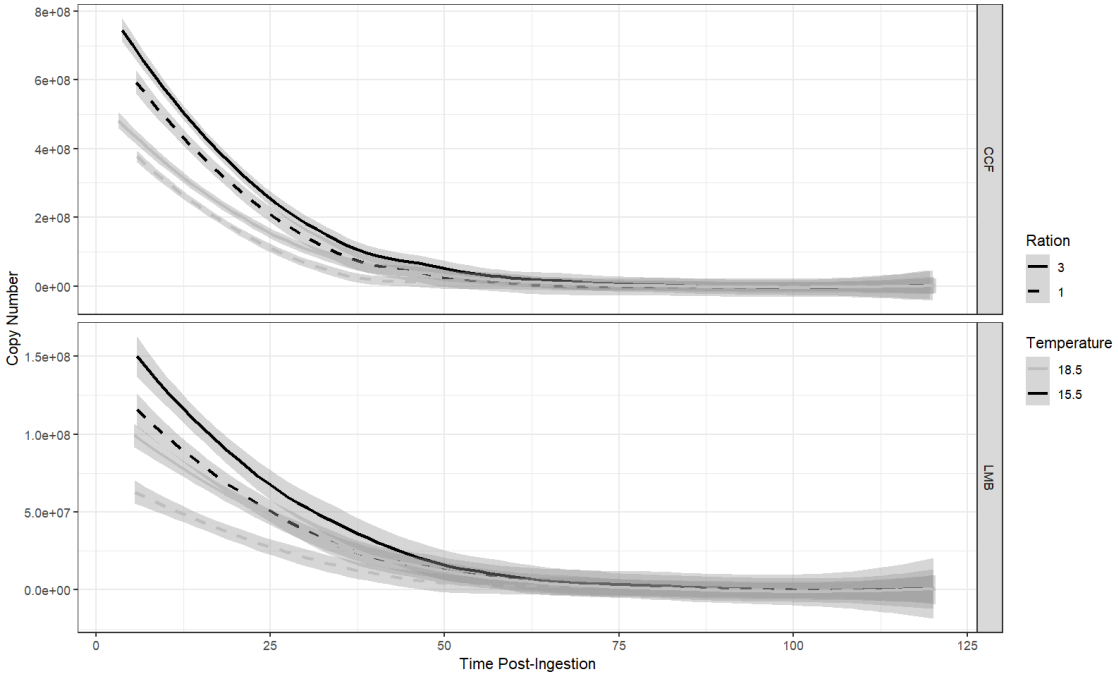

Figure 9: Gastric evacuation of juvenile Chinook DNA copy number within channel catfish and largemouth bass tracts under qPCR analysis. (note the differing y-scales for LMB and CCF).

The model with the next best fit included an interaction between time:temperature and had a dAICc value of 0.41 (Table 6). Top model candidates tested had dAICc values <5 (Table 6).

Table 6: qPCR molecular analysis hurdle model selection table showing top 10 models. Model numbers correspond to Table 1.

Model	*Parameters*	*AICc*	*dAICc*
22	15	12886.37	0
20	17	12886.78	0.41
18	17	12887.65	1.28
28	15	12887.81	1.44
16	19	12888.63	2.26
30	13	12889.79	3.42
7	17	12890.31	3.94
24	17	12890.57	4.2
5	19	12890.84	4.47
3	19	12891.17	4.8

Higher prey intake (lower predator-prey ratio) averaged increased estimated marginal means (EEM) of DNA from qPCR analysis throughout the digestion period compared to smaller prey intake levels (Figure 10). Through EMM post-hoc analysis, higher prey amounts were predicted to have a higher detection quantity for a longer duration when compared to lower prey amounts (Figure 10). The rate at which DNA was digested in predators differed significantly depending on the amount of prey fed (Figure 10). Smaller

prey amounts resulted in a more rapid digestion rate, or a steeper slope, while larger prey amounts resulting in a slower digestion rate (Figure 10). Significant detection differences began to occur at 84 hours with a large drop in detection quantity at a single ration compared to triple ration feeding (Figure 10). Although the first significant different was observed at 84 hours, large difference between the two rations can start to be seen at 60 hours post ingestion.

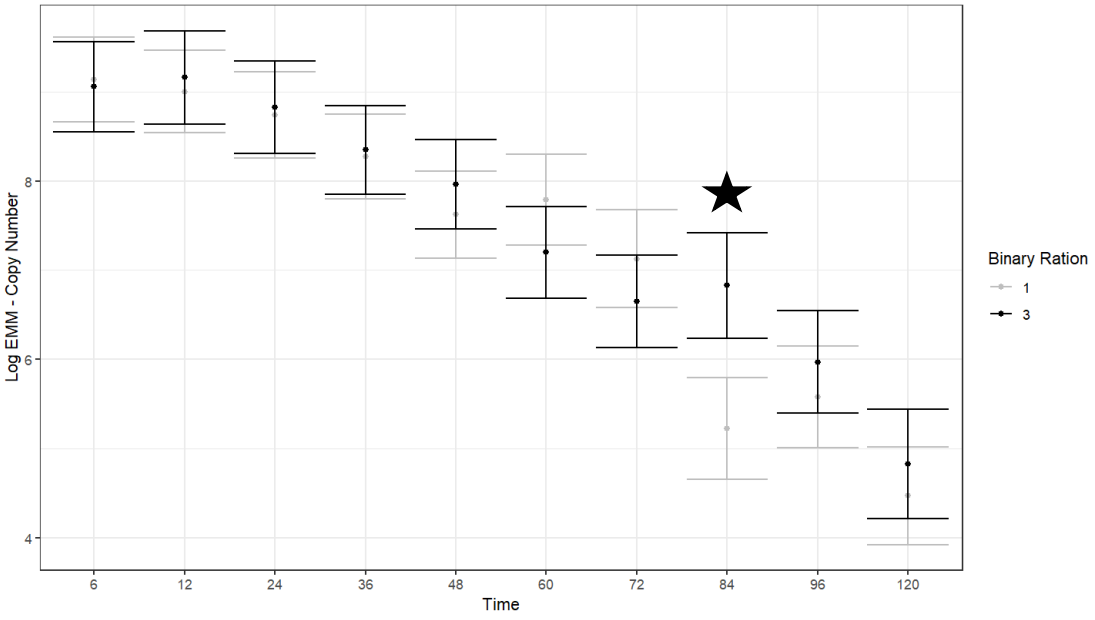

Figure 10: Estimated marginal means (EEM) plot contrasting time:ratio interaction. . Lettering indicates significant differences between estimated marginal means within groups.

Decay of copy number quantity was observed beginning between 6- and 12-hours post-ingestion (Table 7). This decay continued throughout the entire 120-hour digestion period with 90% decay observed at approximately 48 hours (Table 7). On average, channel

catfish and largemouth bass observed similar percent loss of copy number abundance after 36 hours (Table 7).

Table 7: Percent decay of simulated copy number estimates between time post-ingestion groups using qPCR analysis. Calculated from initial sampled dissection (hour 6).

Species	***CCF***				***LMB***			
Temperature	*18.5*		*15.5*		*18.5*		*15.5*	
Ration	*1*	*3*	*1*	*3*	*1*	*3*	*1*	*3*
Hour 6	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
12	31.90	28.86	33.49	35.53	5.41	3.33	-2.25	35.11
24	78.47	70.43	67.58	66.51	46.51	54.11	56.50	51.27
36	91.01	83.38	84.12	82.99	81.78	72.73	74.92	76.81
48	96.99	92.10	94.57	91.22	89.14	82.10	85.19	87.60
60	96.85	96.11	98.53	96.58	93.06	94.76	91.49	95.29
72	99.16	98.95	99.30	98.27	96.84	96.41	93.47	96.18
84	99.94	99.69	99.83	99.43	98.59	98.93	98.20	96.74
96	99.90	99.91	99.97	99.78	99.41	99.20	98.64	99.12
120	100.00	99.98	99.99	99.98	99.93	99.89	99.88	99.84

Visual

Total visual score (tissue score + stool score) had a minimum value of 0 and a maximum value of 6. Mean visual score across all hours for channel catfish was equal to 1.84 and mean visual score for largemouth bass was equal to 2.81. Mean visual score for both

species was generally higher across digestion time in the lower temperature treatment tank than compared to the higher temperature treatment tank (Figure 11). Data for both mean read count of metabarcoding and mean visual score showed highly correlated decreasing trends over digestion time with an R^2 value of 0.89 (Figure 11). The correlation between qPCR copy number and mean visual score showed was not as highly correlated, with an R^2 value of 0.58 (Figure 11). A mean visual score greater than 3 corresponded to metabarcoding read counts greater than 15,000. Once mean visual score dropped below 1.5, read counts were reduced to approximately 5,000 and below.

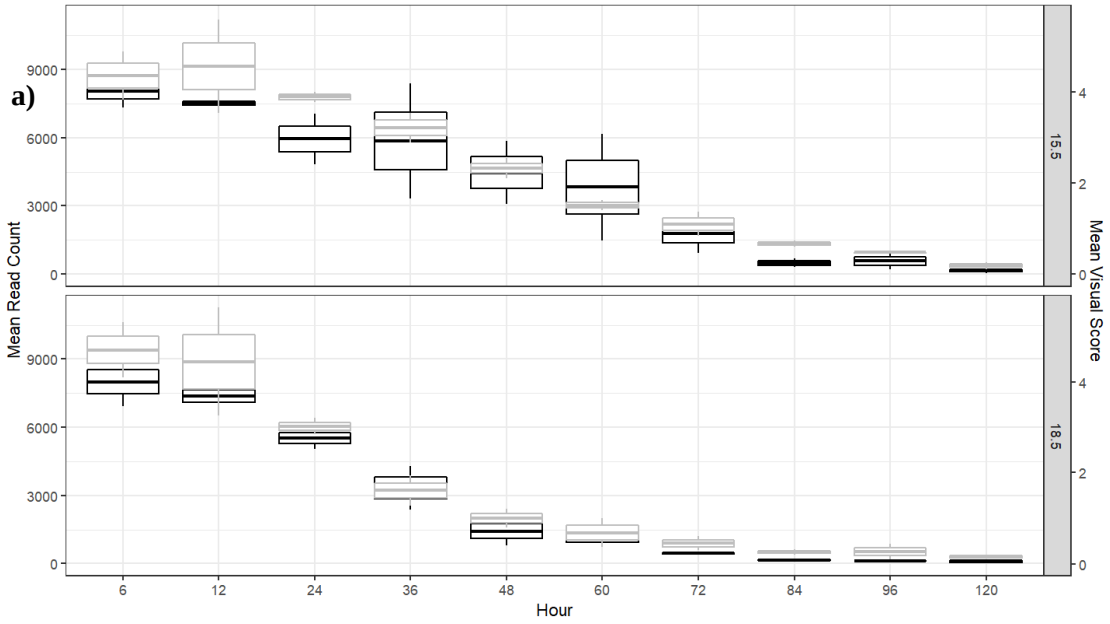

Figure 11: Mean read count from metabarcoding (a, black) and mean copy number from qPCR (b, black) plotted against mean visual score (grey).

Number of Contributors (NOC)

Feeding rations within trials involved either 1 smolt or 3 smolt rations. Determination of

the number of contributors (NOC) or individuals across digestion time was possible with slight over estimation of error (Figure 13). Single ration samples were typically overestimated by a single individual, assuming approximately 2 NOC within most treatments. Triple ration samples overestimated NOC at earlier digestion times and became more accurate as time post-ingestion increased (Figure 12). Channel catfish had on average a larger difference than largemouth when estimating number of individuals (Figure 12). Temperature affected NOC estimation with warmer tank treatment having less overestimation of individuals (Figure 12).

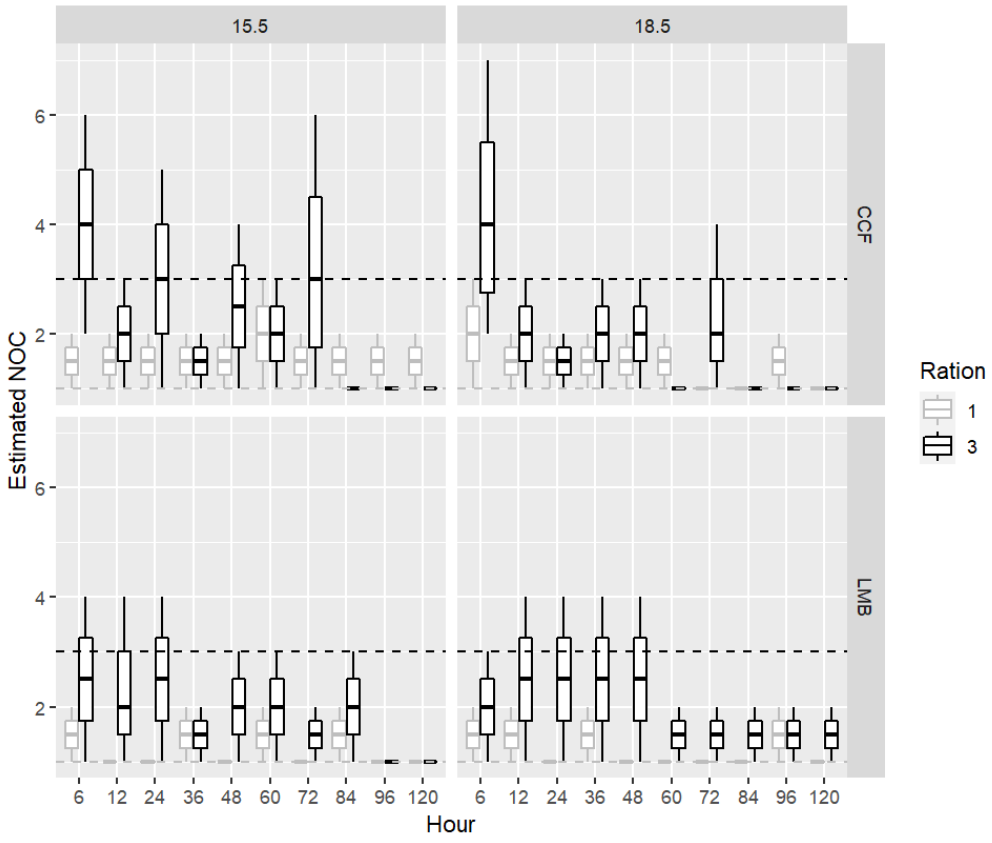

Figure 12: Estimated number of contributors (NOC) for species and temperature at different rations with true rations shown by dashed line.

The current algorithm developed by Sethi et al. (2019) makes the *a priori* assumption that there is at least 1 NOC in the sample. For this reason, single smolt samples were considered ‘control’ and ‘estimated NOC’ was calculated as triple individual estimated NOC minus single individual estimated NOC plus one (triple individual – ‘control’ + 1). ‘Estimated NOC’ averaged greater than 1 until approximatley 72 hours implying accurate estimation of greater than 1 individual for a duration of 72 hours (Figure 13). NOC ‘error’ decreased to zero as time-post ingestion increased, resulting in a pattern of decay similar to the degradation of DNA (Figure 13). High temperature and channel catfish species had the least consistent estimation of NOC compared to lower temperatures and largemouth bass species (Figure 13).

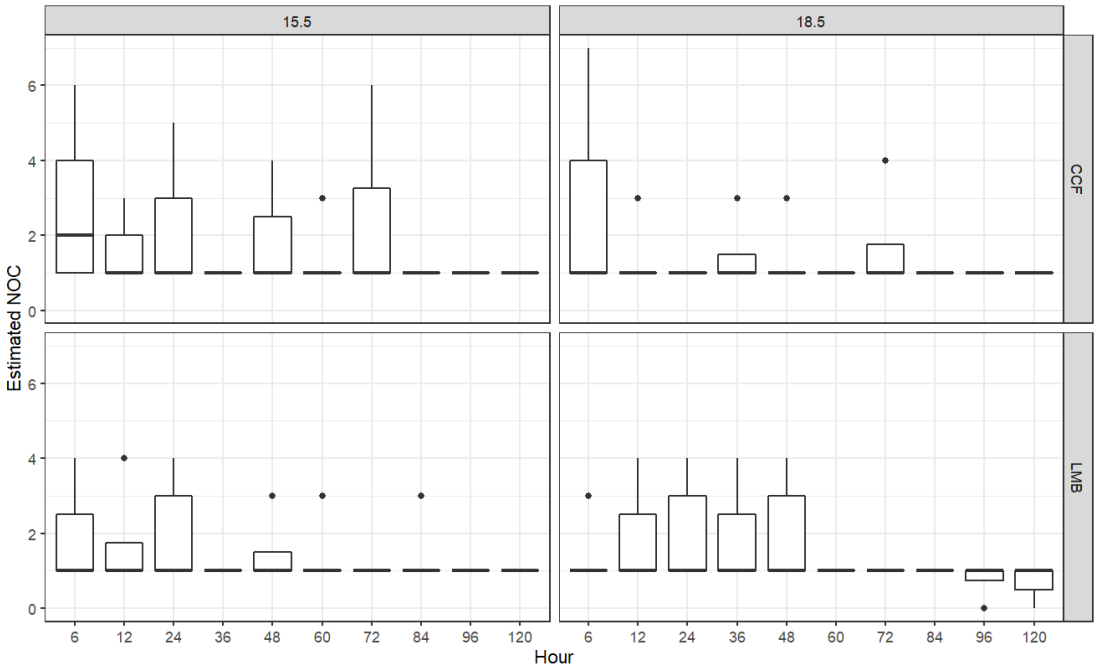

Figure 13: Estimated number of contributors (NOC) standardized against single ration feeding.

DISCUSSION

Largemouth bass and channel catfish populate many areas of the Delta where native salmonids reside and are known to be dominant predators (Brown and Michniuk 2007; Michel et al. 2018; Nobriga and Feyrer 2007). Predation by these invasive fishes may be a leading factor in juvenile Chinook decline within the Delta, but current information on the amount of predation occurring has been scarce. A review by Grossman (2016) determined most predators were occasional consumers of prey, but much of the modeling efforts are limited by a lack of resolution (i.e., fish are labeled as unidentified) due to standard protocols of prey identification. Standard visual analysis protocols paired with rapid deterioration rates of prey items has led to uncertainty in previous predation studies resulting in unidentified fish species (Thomas et al. 2014; Demetras et al. 2016; Grossman 2016). Alternative molecular methods have been able to detect prey DNA for a longer duration and at a higher sensitivity than visual identification (Brandl et al. 2015, 2016). Recent advances have also indicated there is potential to use molecular methods to estimate the number of individuals in a DNA mixture (Andreas et al. 2021; Sethi et al. 2019). Taking advantage of newly formed methods can lead to prolonged detection times, increasing the detection ability of prey items, and can ultimately lead to more accurate estimates of predation rates.

Inspection of stomach contents from this study indicated juvenile salmon would need to be captured within 6 hours of ingestion to be accurately identified at the species level using visual assessment, similar to the findings of Schooley et al (2008). Contrary to

visual assessment, molecular methods such as metabarcoding and qPCR were able to identify juvenile Chinook tissue up to 120 hours post-ingestion within largemouth bass and channel catfish digestion tracts. Results from this study also indicate that evacuation rates differed based on temperature and predator species, and detection of smolt DNA differed based on temperature, species, and predator-prey ratio. These factors were shown to influence the digestion rate and, thus, the interpretation of how much prey is consumed during diet analysis. Numerous studies have used methods described above to estimate predation rates on juvenile Chinook but were subject to bias due to assumptions on the effect of temperature and smolt size on evacuation times (Michel et al 2018; Brandl et al 2015; Grossman 2016). As recommended by Bromley (1994), laboratory feed trials similar to this study can increase the accuracy of these estimates for management purposes by standardizing covariate effects on DNA evacuation. Species, predator-to-prey ratio, and temperature all had significant effects on the decay and detection of Chinook DNA and should be incorporated in future studies attempting to model predation rates.

Currently, predation studies using conventional visual methods generally disregard empty stomachs and unidentified material, potentially underestimating predation activity (Grossman 2016). Results from this study were able to positively identify juvenile Chinook after 120 hours using molecular methods with little amounts of visual “stool” observed in the digestive tract. Disregarding stool and unidentifiable tissue within predator stomachs will lead to significant decreases in predation estimates and I

recommend that molecular methods are used to identify these prey items. Using an arbitrary scale of visual tissue and stool amount, I found a significant correlation with metabarcoding read count. This can be used as a guide towards expected DNA detection ability, predicting a timeframe of predation history, and estimating DNA quantity, or read count. Estimation of prey items using molecular methods has previously resulted in higher accuracy and longer periods of detection time (Brandl et al. 2015). Similar results were observed from this study through metabarcoding and qPCR analysis and highlight the effects that time since ingestion, predator-prey ratio size, and temperature have on the rate of decay for DNA. Chinook smolts within the digestive tracts of both channel catfish and largemouth bass were detectable using molecular methods for at a minimum of approximately 60 hours. When compared to the current standard protocol of visual detection, which can last only a matter of hours (Legler et al. 2010) and low taxonomic resolution (Grossman 2016), metabarcoding and qPCR has a much longer time frame of detection.

Temperature

Higher treatment tank temperature reduced the duration that DNA was detected within predator digestive tracts for both the metabarcoding and qPCR methods. Metabarcoding analysis determined that temperature was a significant factor altering the rate at which DNA degrades within the digestion tract. Seasonal water temperatures within the Delta system fluctuate but tend to average around 18.0˚C based on water gauges (Michel et al 2018). Given that the average water temperature of the Delta may rise with climate

change effects, my results suggest that gastric evacuation of predators would also increase as temperatures increase. Recent predictions under two Representative Concentration Pathways (RCP) adopted by the Intergovernmental Panel on Climate Change (IPCC) suggest an average temperature rises between 2 – 3˚C in most areas of the Delta (He 2022). Recommendations from both Michel et. al (2018) and Grossman (2016) include accounting for potential temperature effects within models assessing predation on a large spatial scale, which coincides with the results from this study. Failure to account for temperature effect on both visual and molecular protocols could result in large over or underestimation of predation rates within seasonality changes of temperature alone. Pairing this with the potential predicted effects of climate change calls for an increased need in the explanation of temperature on digestion.

Previous studies within the Delta by Kjelson and Brandes (1989) and Nobriga et al. (2020) have confirmed increased predation on juvenile Chinook as temperatures increase and approach 20˚C. Hypotheses for the increase in predation include an increase in predator risk, predator migration due to temperatures, and reduced swimming capacity from juvenile Chinook (Nobriga et al. 2021; Michel et al. 2020; Lehman et al. 2017), although further research of predator dynamics is needed at higher temperatures. Within the same study by Nobriga et al. (2021), using data from Michel et al. (2018, 2020), bioenergetics models were built to estimate prey consumption by largemouth bass and striped bass. Temperature effect on digestion rate was not included, however, and results from this laboratory trial have highlighted the importance of temperature to predator

digestion rates. Impacts seen within my study show an interaction between both species and temperature, altering the rate of digestion. Including temperature related digestion coefficients within bioenergetics models could yield alternative results as to which species are consuming a greater amount of prey within the Delta.

Ratio

Predator-prey feed ratio was determined to be a significant factor in the detection of Chinook DNA within predator stomachs which coincides with previously known information on piscivores (Beamish 1972). Predators fed three smolts (i.e., those that were fed a higher mass of prey) retained DNA copies for a longer period of time initially, slowing down evacuation. This was seen to a greater degree in channel catfish compared with largemouth bass.

Implications from my results may explain the underestimation of predation due to the rapid deterioration and evacuation of prey items at higher ratios. Prey tissue is often in low abundances during predator stomach dissection, or gastric lavage, and is rarely identifiable to the species (Nobriga and Feyrer 2007; Jarret et al 2019). Many molecular studies have also found infrequent occurrences of prey items within predators (Grossman 2016). This would indicate these predators are feeding opportunistically and will have rapid digestion rates due to the low levels prey mass. Although many dissected stomachs from field studies are recorded as empty (Grossman 2016), collection of fish could be between feed times while gut content is located in the lower tract of the digestion system. This would also lead to underestimation or negative bias in predation rates of population

models. A similar trend can be seen between predatory species. If a species has a more rapid evacuation of gut content, it may create difficulty in detecting prey presence immediately after ingestion. This was observed in largemouth bass where evacuation of Chinook DNA occurred at a more rapid pace at both temperatures and ratio compared to channel catfish.

Species

The differences in evacuation of DNA between species could result in considerable differences in the way that stomach content data are used to estimate predation rates. Both Grossman (2016) and Michel et al. (2018) found high frequencies of Chinook DNA within stomach samples of channel catfish, a predator not previously thought as a high consumer of juvenile Chinook. Although detection frequency was high, the unique nature of catfish digestive systems can allow for lengthened DNA detection periods and altering patterns in prey decay. Channel catfish had slower evacuation times compared to largemouth bass, implying that channel catfish had slower metabolisms and, as a result, may have different consumption rates than largemouth bass (Armstrong 1986, Millidine et al. 2009). This could indicate a longer detection period of prey within lower metabolizing species. Thus, prey species may be detected within the digestive tracts of channel catfish at higher rates than other species (e.g., largemouth bass) even if they were feeding less than a predator with a faster metabolism. Correcting for the species effect would lead to a more accurate representation of the predation directly from channel catfish, as well as other predators with faster digestion or higher metabolisms.

Similar to channel catfish, a more rapid evacuation of tissue and DNA from largemouth bass can lead to misrepresentation of predation rates. Largemouth bass had lower DNA detection than channel catfish across all predator-prey ratios and temperatures. This would imply field crews need to capture predatory largemouth bass more quickly after prey were consumed to accurately identify prey. Regardless of the metabolism of the predator, clarifying species specific predation rates on juvenile Chinook is crucial to the management of the species as mismanagement could result in a negative effect on the population. Additionally, the high metabolism of largemouth bass, and species with similar digestive tracts, will need to be accounted for during community sized predation estimates. If similar amounts of DNA are detected within different types of predators (e.g., channel catfish and largemouth bass), a scaling equation or parameter should be included to account for the speed at which the prey will be excreted. This parameter is necessary to control for the differential consumption rates of different predators. As an example, consider a field study investigating the effect multiple predator species have on juvenile Chinook survival. If the study sampled the digestive tracts from 1000 largemouth bass and channel catfish and found one smolt in the stomach of each species, it would typically be assumed there was equal predation rates for each species. However, if each species metabolizes the smolts at different rates, then this would not be the case. As seen in this study, channel catfish have slower gut evacuation, which is correlated with lower metabolic rates (Armstrong 1986). Therefore, according to mass balance bioenergetics models (e.g. the Wisconsin model; Deslauriers et al. 2017), they require less consumption to meet their energetic needs. Thus, channel catfish will consume fewer

salmon smolts over time than largemouth bass even though the snapshot of their predation rates were ‘equal.’ To accurately assess the predation rate for each different species in this hypothetical situation, the evacuation of DNA and prey items would need to be accounted for. In order for this to occur, further laboratory digestion studies on notable predators, such as striped bass, would need to occur to establish temperature parameters.

Conclusions

It has been shown within this study that molecular methods can detect prey for longer periods of time than the standard visual protocol and show similar duration of detectability to previous studies (Brandl et al. 2015). It can be difficult to identify prey items to species using visual analysis methods within 6 hours, whereas this was possible for an average of 60 hours using molecular methods within this study. Unlike previous studies, multiple molecular methods were conducted on predator samples. Metabarcoding, qPCR, and an SNP microhaplotype panel were all conducted to determine evacuation of DNA within the two predators. Metabarcoding had the highest threshold success rate, with 91% of samples detecting Chinook DNA. On the other hand, 39% of the qPCR samples did not reach the threshold for detection. Advantages for metabarcoding based on this study, and previous studies listed by Grossman (2016), include sensitivity of detection and multiple species detection. Since predators rarely consume a single prey species, metabarcoding is a valuable method that could be used to create a food web model of predation within the Delta. Alternatively, if managers or

researchers are only interested in detecting a single species, both qPCR and metabarcoding are viable methods. Each method showed similar trends through this study, although metabarcoding had a higher frequency of detection. Based on results from this study, it is suggested that metabarcoding analysis be used for studies on fish prey if species and quantity are at interest.

Protocols for analyzing prey tissue with molecular methods such as metabarcoding is to take a sample of the tissue located in digestive tracts. This equates to a subsample of a sample (or a molecular sample of prey tissue from an entire stomach sample of tissue). This has the potential to alter molecular quantities and should be assumed variable between laboratories. Within this study, equal sample portions for molecular analysis were obtained from unequal stomach content sizes. For example, a 6-hour dissection sample may have contained 5 grams of stomach content, whereas an 84-hour dissection sample may have contained 2 grams of stomach content. The procedure for each sample was the same with stomach contents filling 1.5 mL tubes. This results in a subsample of the original stomach sample and could result in unequal representations of DNA quantity. Future studies are recommended to investigate the differences in this sampling technique.

Although the quantity of DNA molecules can only be estimated through qPCR and metabarcoding, the specific number of individuals can be estimated through SNP allelic estimation. My results implied that detection of NOC was viable for equal durations in channel catfish compared to largemouth bass. This was alternatively observed in qPCR and metabarcoding methods and was likely due to the difference in digestion tracts.

Current methods using NOC have widely varying biases and are recommended to be evaluated more thoroughly within laboratory settings before utilizing in the field for model parameterization. One advantage of using NOC over qPCR or metabarcoding includes knowing the exact number of individuals within a predator diet sample. qPCR and metabarcoding can provide estimates of DNA quantity which correlates to tissue amount, not solely number of individuals. If a predator diet sample has a large quantity of DNA, it may consist of either one or many individuals. NOC estimation would be the preferred method in understanding this situation and can alter the survival estimates of juvenile Chinook during outmigration. With further advancement and accuracy of NOC estimation, I would eventually recommend this method as the standard protocol for future diet studies. Without the use of NOC, it is recommended that future studies incorporate prey mass and DNA quantity correlations when conducting molecular analyses on prey tissue.

A final investigation within this study was to compare the cost of molecular analysis to the cost of visual analysis. For this, there are a few things to consider including the cost of labor for field collection, cost of labor for dissections, cost of labor for visual analysis, and molecular analysis cost, or bioinformatics operation. Simplified, the cost for field collection and the cost for labor of dissections would be very similar for each method. Field sampling is required for each method to obtain samples and stomach dissections are required for each method. The difference in cost then equates to who conducts the analysis and where the analysis will occur. If an agency is able to conduct their own

analysis within a local lab, costs would be reduced significantly and would be similar to the cost of visual analysis. The length of time to visual identify contents within a single sample is similar to the length of time it takes to analyze a single sample using molecular methods. However, if an agency requires to outsource molecular analysis, the cost may increase by approximately $10-50 per sample. Costs can be reduced by dissecting and processing samples prior to shipping or may increase by shipping whole stomach samples to a lab require future dissection. Overall, labor costs between visual and molecular methods are similar while bioinformatic processing has the potential to increase the cost of molecular analysis. In-house molecular analysis can decrease the cost significantly, while outsourcing will likely raise the cost.

Conclusions from this study coincide with gastric evacuation models from Bochdansky and Deibel (2001) where initial gut fullness is proportional to evacuation length and the model shape of decay is exponential when prey items are retained in the lower gut for prolonged periods. Chinook DNA evacuation follow the exponential decay model within both largemouth bass and channel catfish. DNA was detected in the lower intestine tract for at least 120 hours. With the continuous advancement and reduced cost of molecular analysis, future studies are recommended to begin using molecular analysis on prey tissue samples or entire stomach samples of predators within the Delta. Along with this, it is recommended that field studies consider the physiological processes behind their species of interest to estimate gastric evacuation lengths and rates. Expanding the knowledge base on gastric evacuation of salmonid DNA can supplement current models on predation

activity occurring. Using predator species, temperature, and feed ratio as parameters in future predation models can reduce prevalent uncertainties pertaining to digestion rate. Managers can then develop bioenergetics models using these parameters to get a clear understanding of individual level and population level predation, leading to effective management decisions.

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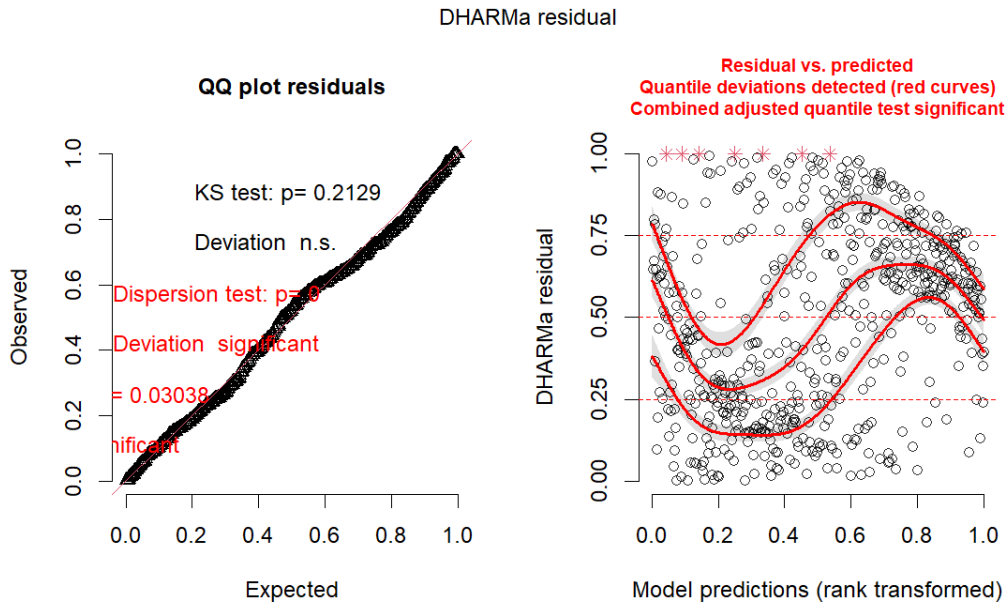
APPENDICES

Appendix A: DHARMa simulated residual diagnostics for metabarcoding analysis model of best fit



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Appendix B: DHARMa simulated residual diagnostics for qPCR analysis model of best fit.