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RESOURCE ARTICLE

Prey ration, temperature, and predator species influence digestion rates of prey DNA inferred from qPCR and metabarcoding

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Abstract

Diet analysis is a vital tool for understanding trophic interactions and is frequently used to inform conservation and management. Molecular approaches can identify diet items that are impossible to distinguish using more traditional visual-based methods. Yet, our understanding of how different variables, such as predator species or prey ration size, influence molecular diet analysis is still incomplete. Here, we conducted a large feeding trial to assess the impact that ration size, predator species, and temperature had on digestion rates estimated with visual identification, qPCR, and metabarcoding. Our trial was conducted by feeding two rations of Chinook salmon (*Oncorhynchus tshawytscha*) to two piscivorous fish species (largemouth bass [*Micropterus salmoides*] and channel catfish [*Ictalurus punctatus*]) held at two different temperatures (15.5 and 18.5°C) and sacrificed at regular intervals up to 120 h from the time of ingestion to quantify the prey contents remaining in the digestive tract. We found that ration size, temperature, and predator species all influenced digestion rate, with some indication that ration size had the largest influence. DNA-based analyses were able to identify salmon smolt prey in predator gut samples for much longer than visual analysis (~12 h for visual analysis vs. ~72 h for molecular analyses). Our study provides evidence that modelling the persistence of prey DNA in predator guts for molecular diet analyses may be feasible using a small set of controlling variables for many fish systems.

KEYWORDS
diet analysis, evacuation rate, fish, metabarcoding, qPCR, trophic ecology

1 | INTRODUCTION

One of the most common methods for studying trophic interactions between predators and prey are diet studies (Nielsen et al., 2018). Diet studies facilitate understanding of the dynamics of aquatic ecosystems and are frequently used in fisheries science (Amundsen & Sánchez-Hernández, 2019; Hyslop, 1980). Examples of how diet studies have been incorporated into fisheries management include: estimates of predator–prey interactions to inform management of groundfish on the west coast of the United States (U.S.; Bizzarro et al., 2017; Livingston et al., 2017), integration into stock assessment models for multiple commercially important species (Gaichas et al., 2010), and the development of ecosystem-based models (Szoboszlai et al., 2015). Historically, these trophic interaction studies have relied on the visual identification of diet items (Buckland et al., 2017; Hynes, 1950; Hyslop, 1980). While visual gut content assessment is relatively simple to conduct, identification of diet items can require substantial taxonomic training and it is often challenging to identify some prey that can quickly lose identifying morphological characteristics within a predator's digestive tract (Buckland et al., 2017; Cottrell et al., 1996). Similarly, smaller prey items, and those with softer tissue, can quickly become difficult to identify due to rapid digestion rates (Buckland et al., 2017; Schooley et al., 2008). Ultimately, gut contents in many organisms often become an indistinguishable mixture of digested material within a matter of hours (Berens & Murie, 2008; Legler et al., 2010). Thus, the limitations of the visual diet assessment approach have led researchers to explore alternative methods for understanding feeding habits and trophic interactions.

Molecular genetic tools can address some limitations associated with visual diet analysis, because DNA can be amplified even when diet items are substantially degraded (Nielsen et al., 2018). Molecular diet analysis was first developed in the early 2000s (Farrell et al., 2000; Symondson, 2002) and began with amplification of small species-diagnostic regions of mitochondrial DNA followed by cloning and sequencing of cloned products (Blankenship & Yayanos, 2005; Carreon-Martinez et al., 2011; Deagle et al., 2005). More recently, quantitative PCR (qPCR) has been used to accurately detect, and quantify, specific prey items within diet samples (Brandl et al., 2015; Zhang et al., 2007). Alternatively, studies aiming to identify all of the prey items in a sample employ metabarcoding (Nielsen et al., 2018). Analysis of diet samples taken from wild little blue penguins (*Eudyptula minor*) suggested that results from qPCR and metabarcoding can be comparable (Murray et al., 2011), but metabarcoding accuracy can be compromised in natural systems due to a variety of biological and analytical factors such as differences in digestibility among prey types and filtering parameters (Deagle et al., 2013).

One major limitation of both visual and molecular approaches for diet analysis is a lack of information on how different environmental and biological factors influence the detectability of diet items. For example, factors such as predator species, prey species, size of meal, and temperature could all potentially impact the rates of chemical digestion and physical movement of material through the digestive tract. Furthermore, interactions between these factors could result in complicated relationships affecting what influences the detectability of prey DNA in gut content samples. Only a few controlled diet experiments have been conducted in fish species to investigate how specific factors influence molecular diet analyses. For example, Brandl et al. (2016) focused on qPCR detectability half-lives of different prey species consumed by a single fish predator, but they did not explore the influence of other factors, such as temperature. Carreon-Martinez et al. (2011) investigated differences in prey detectability between visual and DNA-based fish diet analyses in a laboratory setting across controlled temperatures. While these studies represent valuable insights into factors that can influence molecular diet analyses, a more comprehensive investigation including multiple molecular techniques and exploring how interactions between biological and environmental factors affect prey item detectability would provide valuable information for interpreting molecular diet analyses and integrating results into species conservation and management.

The ability to improve the precision of diet analysis could be invaluable, especially in ecosystems where piscivores could impact species that are listed under the U.S. Endangered Species Act (ESA). For example, the Sacramento-San Joaquin Delta (hereafter referred to as the Delta) in the state of California (USA) contains multiple ecotypes of Chinook salmon (*Oncorhynchus tshawytscha*) whose abundances have been declining for decades, and some are now considered threatened or endangered under the ESA. One of the hypotheses for this decline has been increased mortality due to predation by non- native predators as these juvenile salmon migrate through the Delta (Grossman, 2016). Two major non-native predators of these juvenile salmon are largemouth bass (*Micropterus salmoides*) and channel catfish (*Ictalurus punctatus*), which are abundant and widespread throughout the Delta (Grossman, 2016; Michel et al., 2018; Nobriga & Feyrer, 2007; Weinersmith et al., 2019). Unfortunately, accurate quantification of the potential impact of these predators on Chinook salmon, and other native fishes, has been extremely limited due to a lack of sufficiently high-quality diet data and many prey items listed as 'unidentified fishes' due to an absence of visually distinguishable features after digestion (Grossman, 2016).

The primary motivation for our study was to determine if molecular methods could be used in place of visual analyses to better study the impacts of non-native predators on Chinook salmon in the Delta. To do that, it is necessary to understand what factors could influence how long a prey item will be detected within a predator's digestive tract. Diet analysis data can then be used in bioenergetics models to quantify the number of prey consumed by various predator species (Deslauriers et al., 2017). To address the question of what factors influence predator digestion rates, we conducted an experiment that included two ration sizes, two temperatures, and two predator species. We then analysed samples from these predator's digestive tracts using two molecular methods: qPCR and metabarcoding. Our specific aims were to: (1) understand how the interactions between temperature, meal size, and predator species

affect digestion rates, and (2) compare, and contrast, molecular and visual-based diet assessment approaches. Our results provide important insights on factors influencing molecular and visual diet analyses that can be leveraged to better interpret findings from past and future studies.

2 | METHODS

2.1 | Overview

We dissected digestive tracts from largemouth bass and channel catfish that were sacrificed at regular intervals post-ingestion of a meal to determine how different covariates affect digestion rates. We define digestion rates as the reduction in prey item DNA detected in predator gut contents through time due to the processes of degradation, digestion, and evacuation. We used two different molecular methods to quantify the amount of DNA within the digestive tracts at each time interval and then used generalized linear models to quantify the effects of multiple covariates (i.e., species, ration size, and temperature) on digestion rates.

2.2 | Controlled experiment

We conducted experiments on wild-caught and hatchery-reared largemouth bass (n fish = 382) and hatchery-reared channel catfish (n fish = 220) to investigate how temperature, predator species, and ration size influence digestion rates (Table 1). Hatchery largemouth bass (n = 202) and channel catfish (n = 220) were obtained from a private aquaculture facility in Galt, CA (The Fishery Inc.). The wild largemouth bass (n = 225) were captured via electroshocking in the Delta. All fish were transported to the University of California, Davis Center for Aquatic Biology and Aquaculture (UC Davis CABA) for gastric evacuation experiments. Predator fish were transported at 20°C, then held at 18.5°C for 3 days to initiate acclimation and reduce the stress from transport. After the initial 3-day acclimation period, fish were placed in their respective 3 m flow through temperature treatment tanks (15.5 and 18.5°C) and cages for acclimation. The treatment temperatures were selected based on the range of temperatures observed in the Delta during the outmigration period of fall and late-fall Chinook (March – June), which are the most abundant ecotypes. We held 55 predators within each treatment tank, for a total of 110 predators per trial. Acclimation for 2 weeks was deemed necessary to (1) ensure previously digested material was eliminated and, (2) reduce overall stress and allow digestion rates to return to normal levels (Brandl et al., 2016). Ration sizes of one and three smolts were chosen based on the typical amount of prey found within a piscivore's stomach (Nobriga & Feyrer, 2007). Within each treatment tank, fish were placed into 4 cages to organize the predators based on time of ingestion throughout the feeding trials. We started the trials with five predators for each combination of temperature and ration (e.g., 15°C and 1 smolt), thus, ideally we would have a total of 200 samples throughout 10 dissection intervals (see below). Each tank contained 5 spare predators in the case of mortality or regurgitation.

Prey items for the diet study were fall run Chinook salmon smolts received from a state run hatchery in Clements, California (Mokelumne River Hatchery). Smolts were reared in 1.2 m flow through tanks set to 12°C and fed fish pellets through automated feeders. Smolts were sacrificed by a single strike to the top of the

TABLE 1 Summary of predator (CCF = channel catfish *Ictalurus punctatus*), LMB = largemouth bass (*Micropterus salmoides*) lengths and prey (Chinook salmon *Oncorhynchus tshawytscha* smolts) weights within laboratory feeding trial treatments to determine the effect of temperature and ration on predator digestion rates.

Species	Temperature	Ration	Sample size (n)	Mean predator FL (mm)	Predator FL std. dev.	Average prey mass (g)	Prey mass std. dev.
LMB – Hatchery	18.5	1	50	320.54	16.80	5.13	1.10
		3	51	336.00	19.68	8.98	1.12
	15.5	1	51	321.98	20.36	5.10	1.02
		3	50	341.52	22.20	9.07	1.09
LMB – Wild	18.5	1	50	356.62	67.59	6.26	1.04
		3	40	303.55	70.88	5.63	1.50
	15.5	1	50	333.74	46.73	6.54	1.26
		3	40	300.75	75.63	5.11	1.34
CCF	18.5	1	55	326.95	47.35	4.09	0.95
		3	55	339.95	56.20	8.27	1.40
	15.5	1	55	329.05	63.53	3.96	0.78
		3	55	350.27	62.38	8.46	1.38

Note: Largemouth bass specimens were collected both from a hatchery and in the wild; channel catfish were sourced exclusively from a hatchery.
Abbreviations: FL, Fork Length, Std. Dev., Standard deviation.

skull, wet weighed (g), fin clipped for DNA identification, and force fed to predators. For the feeding trial with hatchery-reared largemouth bass, smolts were divided into smaller portions due to smaller stomach sizes of the hatchery-reared predators. Smolts were cut near the posterior portion of the gill operculum and fish were fed the ⅓ portion that included the head while the remainder of the smolt was discarded.

After a 2-week acclimation period, predators were anaesthetised in a buffered solution of MS-222, tagged for identification (Floy Tag, Seattle, Washington), measured to nearest fork length (mm), and force fed their respective smolt ration. Feeding was conducted by placing euthanized smolts down the pharynx of immobilized predators using forceps. Once fed, predators were promptly returned to their respective treatment tanks and cages for recovery. During the first 30 min of recovery, predators were observed for regurgitation. If regurgitation occurred, the predator sample was removed and a new predator from the acclimated spares was fed and added to the tank. If regurgitation was suspected beyond the 30-min observation period, a spare predator was force-fed and added to that trial as a replacement. Throughout all experimental treatments, a total of 10 regurgitations and 8 premature mortalities were observed (Table S1). Regurgitations occurred across all species, temperatures, and feed ration sizes. Although regurgitation occurred across all trials, more occurred within warm water treatments (7 in 18.5°C vs. 3 in 15.5°C). We also note that premature mortalities were observed only on wild largemouth bass, and not on hatchery subjects of either species. We hypothesize that the stress associated with electroshocking and transport affected the survival of wild largemouth bass.

At regular intervals post-ingestion, a subset of five predators from each treatment were euthanized by a single strike to the top of the skull and the digestive tracts were removed. The entire digestive tract was considered a single sample and consisted of the trachea, the pyloric cecum, stomach, pyloric sphincter, intestinal tract, and anal cavity. To remove these samples, 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. Digestive tract samples were injected with a 3 mL solution of 100% non-denatured ethanol (EtOH) to halt enzymatic digestion and stored in 200 mL conical vials. 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 h post dissection and wrapped in parafilm to prevent evaporation. Samples were then stored at room temperature until DNA extraction began. To investigate the persistence of ingested prey over time, study subjects were sacrificed, and gut contents were sampled at regular intervals after the feeding treatments, with the earliest dissections commencing 6 h post ingestion followed by regularly spaced dissections at 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, 96 h and a final dissection period at 120 h. A total of 50 predator samples per species were targeted per temperature per ration size.

2.3 | Visual diet analysis

After isolating the diet samples, we conducted a visual assessment to quantify the relative amount of prey material within the digestive tract. The two goals of this assessment were: (1) to determine if the quantity of material in the digestive tract was correlated with prey DNA quantity, and (2) to determine if the quality of digestive tract material (i.e., the ratio of tissue vs. stool) affected the molecular analyses. To conduct the visual assessment, we first removed the prey contents from the entire digestive tract (stomach and intestines) and preserved them in ethanol. While removing the prey contents, we noted whether stool was present or absent and whether it was a low, medium, or high amount relative to other samples (rank from 0 for absent to 3 for high). We used the same scoring system for undigested tissue. Each sample could therefore have a score from 0 (no stool or tissue present) to 6 (high stool and high tissue). Stool was defined as relatively large and generally dark in colour, while undigested tissue was lighter in colour and in chunks of variable size and shape. For undigested tissue, we also noted whether we could identify the prey item's taxa based on external characteristics. After visual assessment, we prepared each stomach for DNA extraction. First, we pipetted as much stool as possible into a 1.5 mL tube. If no stool was visually present, we still pipetted approximately 700 µL of ethanol from the bottom of the vial used to preserve the stomach into the tube for extraction. To sample chunks of partially digested tissue, a small piece was taken from each visible chunk to ensure thorough sampling. We recognize that our sampling approach is not fully quantitative but it represented a logistically feasible approach that should be semi-quantitative. DNA was extracted from this mixture of stool and tissue (see below).

2.4 | Molecular methods

Before extraction, stomach and intestinal contents were centrifuged for 3 min at 5724 × *g* and any ethanol was removed by pipette and discarded. Excess ethanol was then allowed to evaporate overnight. Extractions were conducted with DNA stool kits (Macherey-Nagel Nucleospin 96) modified by replacing bead-induced lysis with enzymatic lysis, using a per-sample volume of 25 µL proteinase-k with 850 µL lysis buffer ST1. Samples were incubated overnight at 56°C, and the subsequent extraction followed the standard manufacturer protocol. One negative control was included on each 96-well extraction plate. It is important to note that the samples were extracted and PCR was conducted in a laboratory space where Chinook salmon tissue is extracted and genotyped for nuclear SNPs (PCR and extraction rooms are kept separate). We are confident that contamination did not influence our findings as it is clear that our molecular results reflected known biological processes (i.e. DNA detectability decreases as digestion progresses). However, our laboratory setup would not be appropriate for a study where stomachs from wild populations were analysed because contamination could significantly impact results.

All extracted stomach samples were analysed with qPCR using an assay designed to quantify Chinook salmon DNA (Brandl et al., 2015). To prepare a standard curve, extracted genomic DNA from Chinook salmon available from Shi et al. (2023) was quantified using an assay kit (Qubit 1× HS dsDNA kit), visualized on a 2% agarose gel to determine average fragment size and calculate copy number, and then serially diluted. Each 96-well plate included seven standards in triplicate and three no-template-controls. Reactions were conducted in 10 μL volumes each containing 1× 5 μL master mix (Applied Biosystems TaqMan Universal PCR Master Mix), 0.9 μM concentration of each primer, 0.7 μM concentration of probe, and 3 μL template DNA. Thermocycling was performed using a real-time PCR system (QuantStudio 12K Flex) with the following profile: 10 min at 95°C, and then 40 cycles of 15 s at 95°C for 15 s and 60°C for 1 min. All samples were run in triplicate and the mean copy number was calculated for each sample. Amplification was considered successful if the Cq > 0 and amplification above baseline was registered for an unknown sample using the PCR system software (QuantStudio). If some replicates did not show amplification for a given sample the mean was calculated using the replicates that did amplify.

We also conducted metabarcoding on all diet samples using the MiFish 12S primer set (Miya et al., 2015). No PCR replicates were conducted for this analysis. Libraries were prepared with a two-step PCR similar to the GT-seq protocol outlined in Campbell et al. (2015). Initial PCR reactions were performed in 12 μL volumes using 2 μL of template DNA and 10 μL of PCR master mix. The PCR master mix consisted of per reaction volumes of: 6 μL master mix (Qiagen Multiplex PCR Plus), 3.4 μL sterile water and 0.3 μL each of the MiFish forward and reverse primer at 10 μM. Thermal cycling was performed as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 65°C for 15 s, and 72°C for 15 s, an extension at 72°C for 5 min, and an 8°C hold.

PCR products were indexed in a barcoding PCR as dictated by Campbell et al. (2015), modified by reducing working i05 primer concentrations to 0.5 μM and working i07 primer concentrations to 1 μM. Following this, each 96-well plate was subsequently pooled without normalizing the amount of DNA per sample to maintain a relationship with the initial concentration of DNA in the extract. A double-sided bead size selection was performed using magnetic beads (Beckman Coulter AMPure XP beads) with ratios of beads to library of 0.5× to remove non-target larger fragments and then 0.7× to remove dimer and retain the desired amplicon. Libraries were sequenced with a next-generation sequencing platform (MiSeq Illumina) using a single v2 300-cycle kit with 2× 150 bp paired-end (PE) chemistry and 15% PhiX to compensate for the low diversity library. The lane included 782 samples loaded at equal concentrations, 163 of which were not part of the current study, and 16 of which were no-template-controls (PCR negatives).

Sequencing reads were assigned to individual samples using the sequencing platform analysis software (Illumina MiSeq Analysis Software). Primers were trimmed from forward and reverse reads using open-source software (Cutadapt) and reads without primer sequences were discarded (Martin, 2011). Primer-trimmed fastq files were imported into open-source software (DADA2; Callahan et al., 2016) in R for quality filtering, chimera removal, and identifying amplicon sequence variants (ASV) using the pseudo-pool option for the core dada2 algorithm (additional parameters included truncLen = c(110, 100), maxN = 0, maxEE = c(2, 4) and truncQ = 2). Forward and reverse reads were merged and then off-target sequences that likely amplified non-fish amplicons were removed using a length filter of 166–172 bp based on the expected amplicon size for MiFish (with primers removed). Output files included a table of the number of reads per ASV per sample (ASV table) and the sequence identity for each ASV, which was used as input for a nucleotide database search (BLASTn; Camacho et al., 2009) using a local copy of the National Center for Biotechnology Information (NCBI) nucleotide database and a minimum 96 percent sequence identity (for the entire 166–172 bp amplicon). The software program taxonkit (Shen & Ren, 2021) was used to match taxonomic lineage to the BLASTn results. This taxonomy information was imported into R and custom scripts were used to assign ASV sequences to the appropriate taxonomic level. When multiple species matched the same ASV within 2% sequence identity of one another, taxonomic levels (i.e., genus, family, order) were iteratively increased until matches were unambiguous. Species-level assignments were only accepted at 98% identity, whereas matches to all other taxonomic levels were retained at 96% sequence identity. This taxonomic information was combined with samples and read count data. The minimum threshold for retaining metabarcoding data was two reads per taxon per sample. We recognize that this value is lower than many empirical studies but we felt it was appropriate because it increased the data available for the model and because this was a controlled experiment rather than an empirical study with the goal of detecting prey items in wild populations.

2.5 | Data analysis

2.5.1 | Correspondence of visual assessments and molecular gut content analysis

We hypothesized that the quality of the prey DNA would degrade over time through the process of digestion. To test this hypothesis, we used Pearson correlations to determine if the quantity of diet contents (stool + prey tissue scores) was correlated with smolt prey item read counts (metabarcoding) or the log transformed copy number (qPCR) from molecular analyses. We analysed each species separately, but we pooled gut samples across temperature and ration treatments to ensure we had a sufficient sample size at each dissection time interval post prey ingestion.

2.5.2 | Regression modelling of digestion rates

We used a generalized linear model (GLM) with a Tweedie distribution to determine what factors were most correlated with the

abundance of prey item DNA in sampled guts. Models were fit separately for the two molecular methods, with read count as the response variable for metabarcoding diet analysis and copy number as the response variable for qPCR analyses. The responses of both methods contained a substantial number of zero values as prey DNA was digested away or evacuated. Furthermore, both qPCR and metabarcoding were implemented using minimum genetic material thresholds that need be exceeded to generate positive DNA detection (>2 reads for metabarcoding, Cq > 0 for qPCR; see above). Thus, we used GLMs with a Tweedie distribution, which is a special case of the exponential distribution that is very flexible and can account for data that contains a large number of zeros (Tweedie, 1984). Tweedie GLMs also have an advantage over zero-inflated models because the zeros are handled uniformly, which improves the interpretability of model coefficients (Shono, 2008). The package "glmmTMB" (Brooks et al., 2017) was used within R (R, Development Core Team, 2021) to fit the GLM. Due to the non-normality of our model, we used quantile residuals simulated using the "DHARMa" package (Hartig, 2022) to assess model diagnostics.

We selected four factors (time post-ingestion, species, temperature, and meal size) to include in our model based on a priori hypotheses regarding their relationship with digestion rates. The first factor was time post-ingestion, which is expected to reduce DNA quantity due to the physical and chemical digestion of prey tissue (Moran et al., 2016). The second factor was species, because there are numerous behavioural and physiological differences between largemouth bass and channel catfish. These two species have substantially different feeding ecologies, but similar temperature preferences. Both largemouth bass and channel catfish prefer relatively warm temperatures in the 25–30°C range, but can tolerate a large range of temperatures (Venables et al., 1978; Wellborn, 1990). Largemouth bass are visual predators and become piscivorous as early as 5 cm in length, leading to a predominantly piscivorous life history (Davis & Lock, 2007). In contrast, channel catfish are generally classified as omnivores, feeding on fish but also plants, invertebrates, amphibians, and other organisms (Braun & Phelps, 2016; Hill et al., 1995). Carnivores typically have shorter intestines and faster digestion rates, whereas herbivores and omnivores have longer digestive tracts and slower digestion rates (reviewed in Volkoff & Rønnestad, 2020). The third factor in our model was temperature, which can affect digestion rates differently depending on gut morphology (Volkoff & Rønnestad, 2020). Therefore, as water temperatures change throughout a season, digestion rates in largemouth bass and channel catfish may not respond in the same way. In our experiment, water temperature was designated as a binary factor based on the two average temperatures of the treatment tanks: 15.5°C and 18.5°C. Higher tank temperature is expected to increase the metabolic rate of fish and subsequently increase evacuation rate (Brett & Groves, 1979). In contrast, cooler temperatures are expected to slow digestion rates, potentially prolonging DNA detection. The last factor in our model was meal size, which could influence digestion rates due to longer processing times for larger prey items (Beamish, 1972). The binary rations of 1 and 3 smolts were transformed to a continuous factor called predator-to-prey ratio (PPR) because we hypothesized that the relative size of the prey was more informative to the digestion rate than the ration size (Figure S1). PPR has been used frequently in food web and community-sized predation studies to understand metabolic constraints of predators (Woodward & Warren, 2007). We hypothesize that we would observe an inverse relationship between PPR and digestion rate, whereby a larger PPR would decrease gastric processing time. To calculate PPR, smolts were wet weighed to nearest 0.1 g and predator weights were estimated based on length-weight regressions. Predators' fork lengths were measured to nearest 1 mm and weight-at-length regressions from the literature were used to estimate individual predator weights (Henson, 1991; Keenan et al., 2011). We used the following weight-at-length regression for largemouth bass:

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

where weight in grams (w) at length in mm (l). An alternative formulation of the model was used for channel catfish:

$$w(l) = \left(\frac{l}{L}\right)^b$$

where L is equal to the standard length of a catfish at 1 kg (Keenan et al., 2011). We used this alternative formulation for Channel catfish because they are known to have a lean, longer form at higher weights compared to largemouth bass. No morphological differences were observed between the two sources of largemouth bass, so the same length-weight regression was used on hatchery and wild largemouth bass. We then divided the calculated predator mass by their respectively fed prey mass to calculate the PPR. Higher PPR means that the predator was large relative to the size of the meal. In other words, a predator of a given size that ate a small meal would have a higher PPR than the same predator that ate a large meal.

After calculating this ratio, we determined that the PPR was correlated with predator weights and that 14 catfish had much higher PPRs (>300) than all other fish in the study (Figure S1). Furthermore, these large catfish were all in the single fish ration treatment. To better compare evacuation rates between species based on the experimental treatments, we excluded these 14 large catfish from our modelling analysis.

We assessed support for different model structures in our regressions of feeding trial factors against molecular diet analysis assay outcomes (read counts for metabarcoding and copy number for qPCR) using Akaike's Information Criterion (AIC; Burnham, 1998). Our primary model selection goal was to identify the top AIC-supported regression among a larger candidate model set made up of plausible model structures. Time post-ingestion was expected to have the largest effect on the measured quantity of DNA, and therefore was included in each candidate model. Temperature, PPR, and species were included in models as main effects and as an interaction with time post-ingestion. The interactions between time and each of the other covariates were included to determine if there was an effect of any covariate on

the digestion rate. We also included an interaction term between species and PPR because we hypothesized that ration size could influence ingested prey evacuation rates differently across species due to differences in metabolism and digestive morphology. Finally, we included an interaction between species and temperature because largemouth bass and channel catfish have different physiologies. Thus, their prey digestion rates may not change proportionally to changes in temperature. Interaction terms were only included if the variable was included in the model as a main effect. We conducted model selection using the "MuMIn" package (Barton, 2022).

2.5.3 | Genetic half-life of prey DNA

While our GLM regressions on molecular diet analysis read counts (metabarcoding) or copy number (qPCR) characterize the influence of feeding trial factors on the persistence of detectable prey item DNA, we also calculated prey DNA genetic half-life values to provide a convenient single metric to facilitate DNA loss comparisons across samples. Detectability half-life provides a measure reflective of the rate that detectable prey item DNA is lost from a predator gut as prey rations are digested, degraded, and evacuated from the gut tract. We define detectability half-life as the duration it takes for smolt DNA in gut content samples to reduce to half of its initial value. To calculate the genetic half-life (HL) of prey item DNA in predator gut content samples, we used the following equation:

$$HL = \frac{ij}{log_{0.5}\frac{\Delta\, DNA_{i,j}}{DNA_{i,j}}}$$

where t is the amount of time that passed between interval (i) and interval (j) and ΔDNA is the difference in DNA quantity between interval (i) and interval (j). DNA quantities at intervals i and j were predicted from the respective top AIC-supported GLM regression models fit to read count metabarcoding) or copy number (qPCR) molecular diet analysis data described above (see: 'Regression modelling of prey DNA loss'). Because they were estimated based on the model, we will refer to them as 'GLM-estimated half-life' values. Prey item DNA half-life values were summarized across all combinations of feeding trial factors (species, temperature, and PPR). We used parametric bootstrapping (1000 samples) to estimate the 95% confidence interval for our half-life estimates based on the mean and standard deviation of the predicted DNA quantity.

To further assess the relative strength of influence of different feeding trial factors on digestion rates we calculated a half-life ratio. The half-life ratio (HL_{rat}) was calculated using the equation:

$$HL_{rat} = \frac{HL_k}{HL_l}$$

where HL_k is the half-life value for one factor at level k (e.g., species=CCF) and HL_l is the half- life value for the same factor at level l (e.g., species=LMB) when the other two covariates are equal (e.g., temperature=15.5 & PPR=low). This was repeated for every combination of factors.

3 | RESULTS

3.1 | Metabarcoding, qPCR, and visual analysis

Of the metabarcoding sequencing reads with a taxonomic assignment, approximately one-third (1,896,827) matched Chinook salmon reads. The vast majority of remaining reads matched the predator species, an expected result given the prevalence of predator DNA in predator stomachs (see Table S2 for read counts by ASV). We also detected some low-level contamination from species that should not have been present in the samples (Table S2). This is a common occurrence (e.g., Dokai et al., 2023; Larson et al., 2022) and we do not believe that it had any impact on the results of this study. Read counts from negative controls were similar to those from stomach samples taken near the end of the feeding trial (i.e., >72 h after ingestion, Table S2). Across all trials (588 samples total, 373 largemouth bass, 215 channel catfish), the average number of Chinook metabarcoding reads per sample was 3226 (SD=4139) and the average qPCR copy number for samples with positive detections was 2.18×10^8 (SD=7.8×10^8).

The number of samples with low visual scores was comparable to the number of molecular samples with few copy numbers (qPCR) or read counts (metabarcoding). We observed 219 samples (37%) with a visual score of 0, 162 samples (28%) with undetermined copy numbers for qPCR, and 50 samples (9%) with 0 Chinook reads for metabarcoding. It is possible that some metabarcoding samples that yielded small read numbers could represent false positives. If a read cut-off of 159 (the median read count of Chinook salmon in negative controls) was used, 243 samples (41%) would fall into the no detection category. This value is similar but slightly higher than the number of samples in the no detection categories for qPCR and visual analysis.

The similar numbers of negative detections with qPCR and metabarcoding when a higher cut-off was applied indicate that these two methods may have relatively similar detection thresholds in our system. We detected Chinook salmon DNA with either metabarcoding (read count threshold >159), qPCR, or both methods, in 92 of the samples with a visual score of 0, indicating that remnant DNA in the digestive track can be detected even when no discernable prey item tissue or stool is present. On average, samples with visual scores of 0 were found between 72 and 84 h into the feeding trial.

Time since ingestion had a substantial effect on molecular detectability of diet items, although the results varied by species, temperature, and ration size (Figures 1 and 2; Figure S2). In general, metabarcoding reads, qPCR DNA copy number, and visual diet content scores decreased at a consistent rate as time since prey ingestion increased. Read counts, copy number, and visual assessment values were either zero or extremely small past 72 h using either of the molecular approaches or visual assessment. Our visual analysis also indicated that the prey contents lacked any external tissue, and

were unidentifiable to species based on external characteristics, after the 12-h dissection. The two molecular diet analysis methods were correlated, with correlation coefficients of 0.65 for largemouth bass and 0.75 for channel catfish. We also found that the molecular methods were correlated with the visual scores. The metabarcoding read counts were more highly correlated with the visual scores (largemouth bass = 0.77, channel catfish = 0.84) than the log transformed qPCR copy number (largemouth bass = 0.67, channel catfish = 0.69) (Figure S3).

We also observed differences in detection metrics by predator species. Channel catfish had higher average metabarcoding read counts than largemouth bass (4139 vs. 2699) and higher qPCR DNA copy number (88.5129 million vs. 35.09 million) but lower average visual scores (1.84 vs. 2.18). The range of values in the observed data was also generally larger in channel catfish than largemouth bass, especially for the visual diet content scores (Figure 1). The raw data also indicated that the metabarcoding read counts persisted longer in treatments with channel catfish, colder temperatures, and lower PPR (Figure 2).

3.2 | Modelling the effects of different variables on prey DNA quantity

Model selection results indicated that the top AIC-supported models for metabarcoding and qPCR data were very similar. Both methods had 4 GLM models with a ΔAIC <2 (Table 2, Tables S3 and S4). The most parsimonious top AIC-supported model (i.e., the model with the fewest parameters within 2.0 AIC units of the top model) for both qPCR and metabarcoding included all four main effect variables (time, species, PPR, and temperature), and two time interactions (time by ratio, time by temperature) (Table 2, Figure 3; Figures S4 and S5). The only parameter difference between the selected models for the two molecular methods was that the metabarcoding model included an additional time by species interaction (Table 2, Figure 3). Coefficient values and standard errors for the selected models are provided in the supplementary material (Tables S5 and S6). Examination of the diagnostic plots for these top AIC-supported models indicated that these analyses failed to violate regression assumptions.

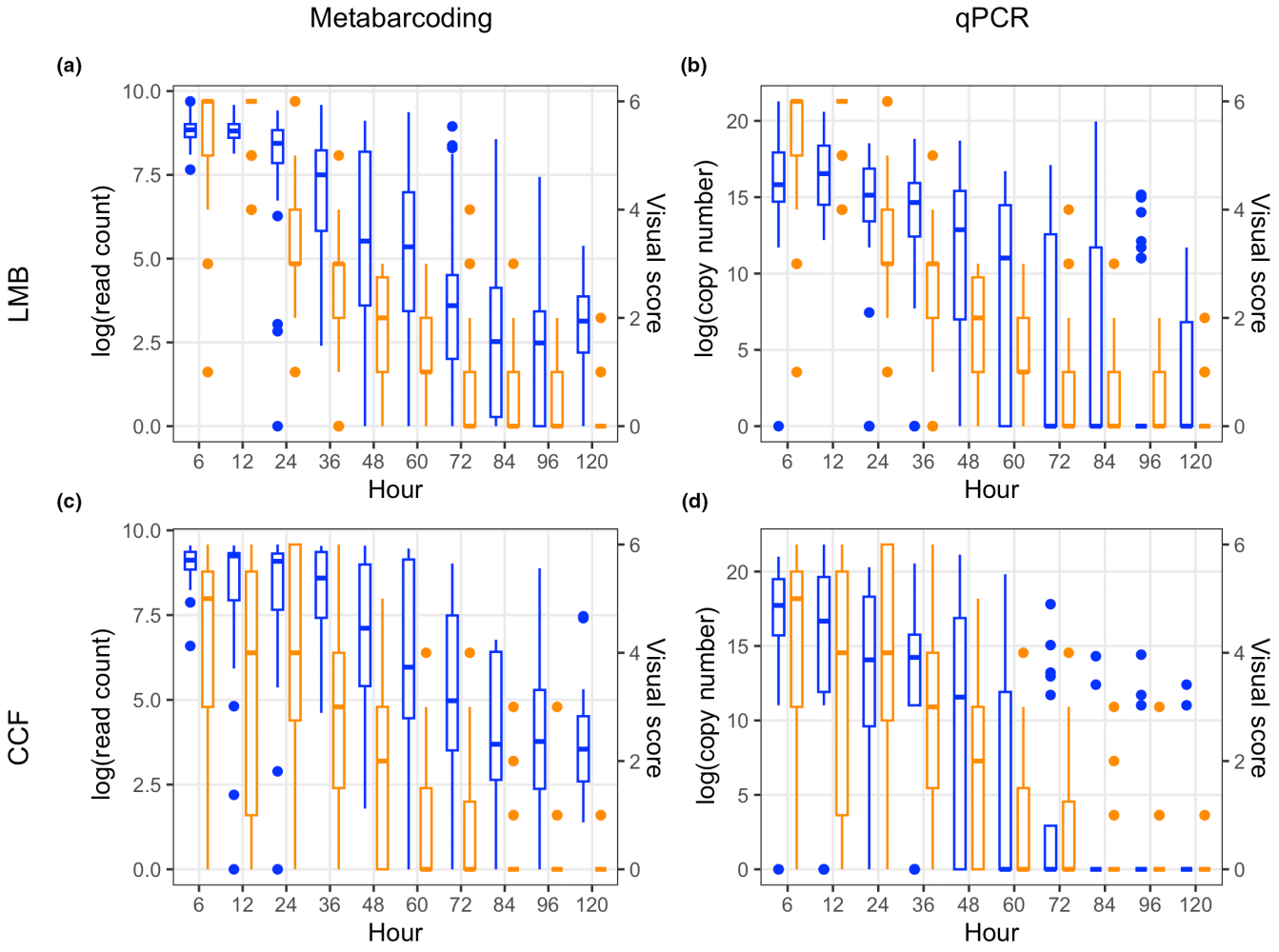

FIGURE 1 Boxplots showing observed log metabarcoding read counts (Blue – a, c), log qPCR copy number (Blue – b, d), and visual scores (Orange) of contents extracted from the digestive tracts of largemouth bass *Micropterus salmoides* (LMB, a, b) and channel catfish *Ictalurus punctatus* (CCF, c, d) at each sampling interval post-ingestion of salmon smolts during laboratory feeding trials.

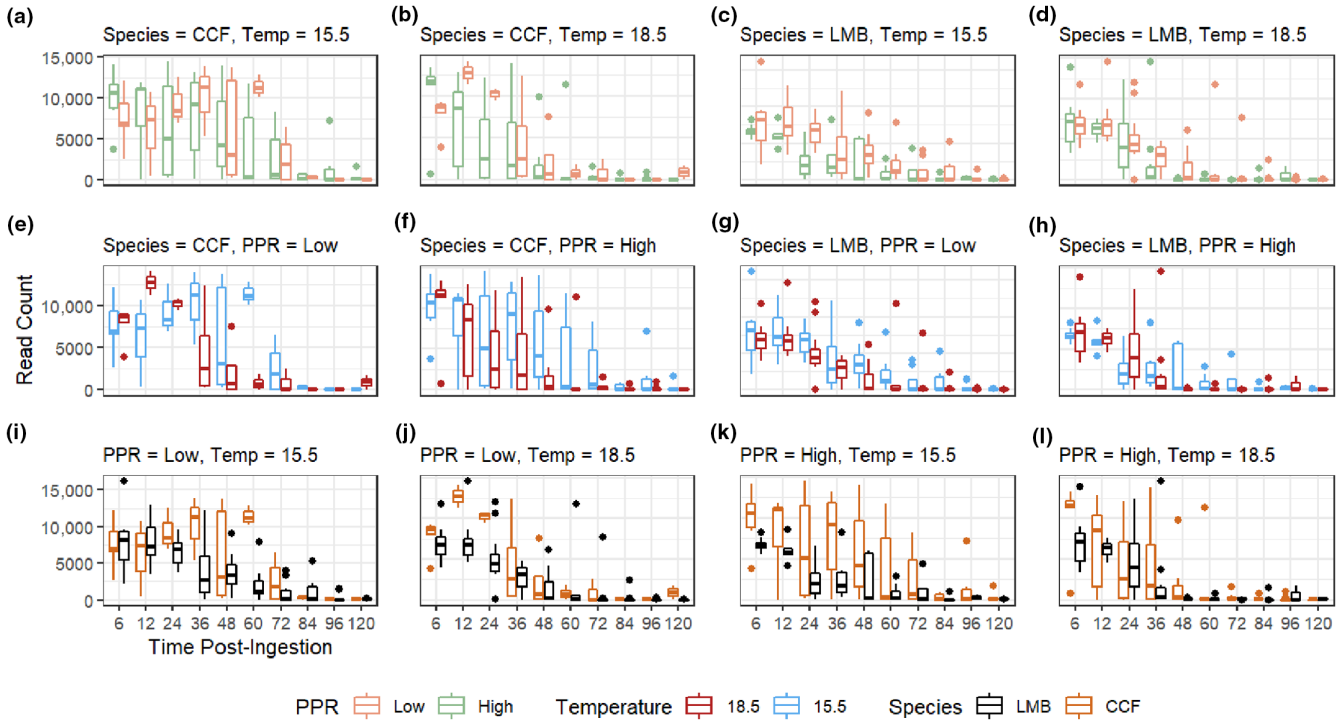

FIGURE 2 Boxplots of raw metabarcoding data (Read Count) of contents extracted from the digestive tracts of largemouth bass *Micropterus salmoides* (LMB) and channel catfish *Ictalurus punctatus* (CCF) at each sampling interval post-ingestion of salmon smolts during laboratory feeding trials to determine the effects of water temperature (15.5 vs. 18.5°C) and ration (predator–prey ratio [PPR]) on predator digestion rates. The boxplots are organized by row where only a single treatment variable is compared: (a–d) predator–prey ratio (PPR), (e–h) water temperature, (i–l) predator species (channel catfish = CCF, largemouth bass = LMB). PPR was calculated by dividing predator mass from prey mass. The values used for the low and high PPR categories were selected based on the 25th and 75th quantiles and were: low = 73.6 and high = 191.6.

TABLE 2 Model selection table for GLM models fit to metabarcoding and qPCR data from molecular diet analysis based on laboratory feeding trials to determine the effects of temperature and ration on predator digestion rates.

Method	Model	Int	Time	PPR	Species	Temp	Time: PPR	Time: Species	Time: Temp	PPR: Species	Species: Temp	DF	DAIC
Meta													
	51	8.34	−0.96	−0.43	+	+	−0.19	+	+		+	11	0.00
	27	**8.26**	**−0.95**	**−0.42**	**+**	**+**	**−0.19**	**+**	**+**			**10**	**0.33**
	59	8.32	−0.95	−0.39	+	+	−0.19	+	+	+	+	12	1.39
	39	8.23	−0.94	−0.38	+	+	−0.18	+	+	+		11	1.75
qPCR													
	37	17.36	−2.05	−0.72	+	+	−0.50		+	+		10	0.00
	24	**17.47**	**−2.02**	**−0.91**	**+**	**+**	**−0.54**		**+**			**9**	**0.82**
	39	17.40	−1.95	−0.73	+	+	−0.52	+	+	+		11	1.82
	57	17.31	−2.04	−0.71	+	+	−0.49		+	+	+	11	1.83

Note: Here we only show models with delta Akaike's information criterion (DAIC) values <2, indicating they have the most support of any model tested. The selected model, with the lowest DAIC and the fewest degrees of freedom (DF), is indicated in bold. Numeric values are presented for coefficients for continuous covariates which were included in all candidate models, whereas a '+' symbol indicates inclusion of a categorical covariate in a candidate model. "Time" is time since prey ingestion, "PPR" is predator-prey ratio, "Species" is predator species, "Temp" is water temperature, ":" indicates if there is an interaction between two covariates.

The interpretation of the prey DNA assay results from the selected metabarcoding and qPCR models were also very similar. Based on these models, at higher PPRs, which were correlated with larger predators, digestion rate of prey DNA was faster than at lower PPRs (Figure 3a,d).

These models also indicated that prey item DNA in predator gut content samples was lost more quickly at higher feeding trial temperatures (Figure 3c,f). The metabarcoding model also indicated that largemouth bass had a faster digestion rate than channel catfish

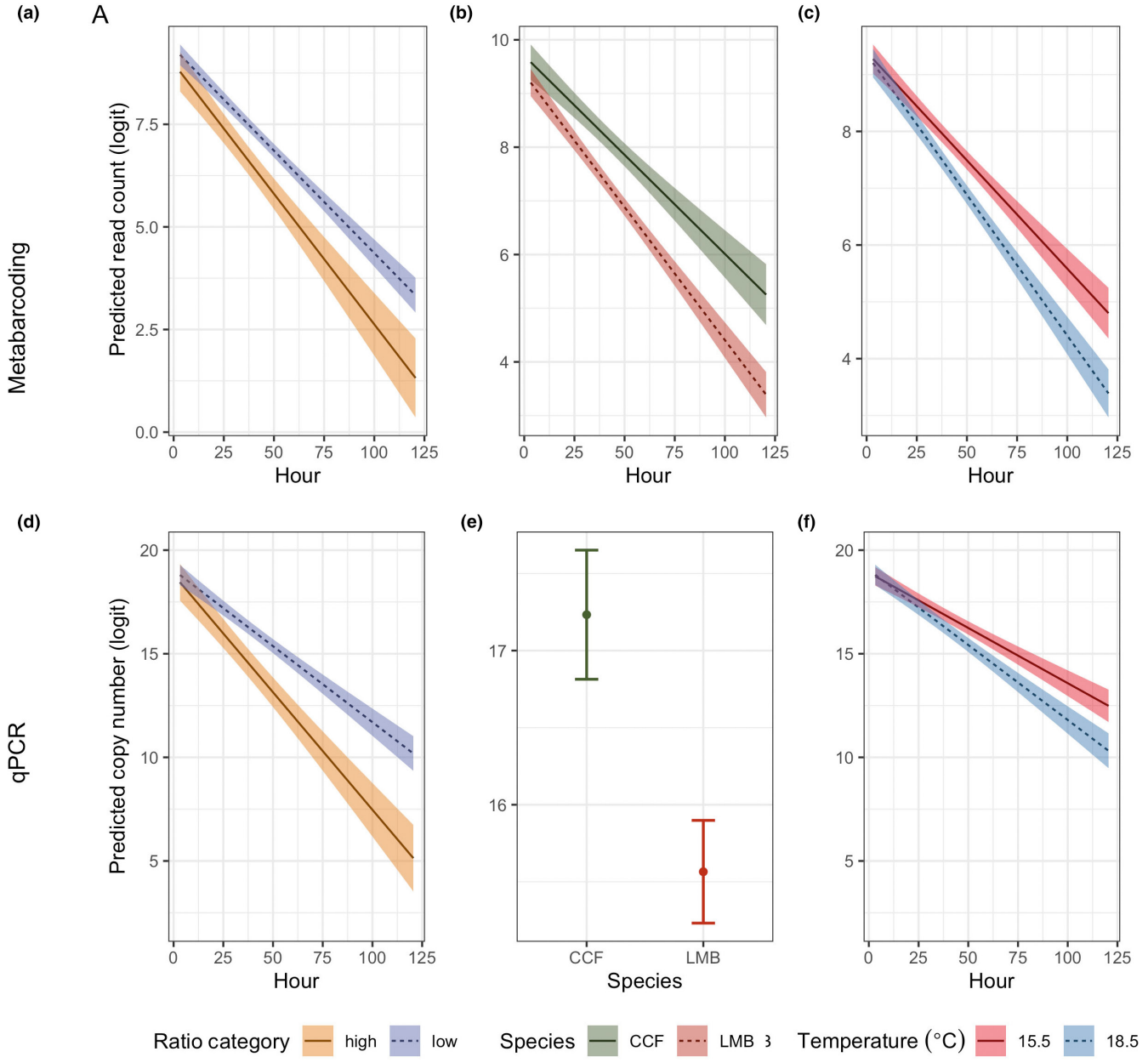

FIGURE 3 Response plots from the metabarcoding (a–c) and qPCR (d–f) generalized linear models fit to digestive tract samples from channel catfish *Ictalurus punctatus* (CCF) and largemouth bass *Micropterus salmoides* (LMB) to determine the effects of temperature (15.5 vs. 18.5°C) and ration (represented by the continuous covariate predator–prey ratio) on predator digestion rates measured using molecular methods on gut content samples from laboratory feeding trials. Note that the *y*-axis scale differs between plots and it is on the logit scale to accentuate the differences in slopes between groups. To see the effects of these covariates on the response scale, see Figures S4 and S5. PPR was calculated by dividing predator mass from prey mass and the values for PPR categories were: low = 73.6, high = 191.6.

(Figure 3b). Similarly, the qPCR model found that largemouth bass displayed lower copy numbers than channel catfish (Figure 3e). Although the qPCR model could not discern the species by time interaction, we believe the difference in copy numbers is likely due to the faster digestion rate of largemouth bass. We suspect the time by species interaction was not included in the most parsimonious top AIC-supported model because of the variability in the qPCR data (Figure 1).

3.3 | Genetic half-life

The results from the genetic half-life analysis indicated the relative impact of each factor on the loss of prey DNA from the digestive tracts of the predators in our study. Based on the GLM fitted to metabarcoding read counts of prey DNA in gut content samples, genetic half-life was shorter as PPR increased and shorter in the higher temperature treatments. Metabarcoding-based prey DNA half-lives

were systematically longer for channel catfish than for largemouth bass. For metabarcoding, the longest half-life was 348 h for channel catfish with a low PPR in the colder temperature treatment, whereas the shortest half-life was 78 h for largemouth bass with a high PPR in the warm temperature treatment (Table 3). The results for qPCR showed similar effects of PPR and temperature, however, there was much less of a difference in the half-life estimates between the two predator species. For qPCR, the longest half-life was 344 h for channel catfish in the colder temperature treatment with a low PPR whereas the shortest half-life was 97 h for largemouth bass in the warmer temperature treatment with a high PPR (Table 3).

The half-life ratio analysis provided limited evidence that PPR had the largest influence on digestion rate based on the qPCR assays, but the results from the metabarcoding analysis were more equivocal (Figure 4). The mean half-life ratio values for PPR based on the qPCR assay were all greater than 2 (Figure 4d), which was larger than the values for either the species or water temperature effect (Figure 4e,f). A value larger than 2 means that the half-life from the 'low' PPR treatments was more than twice that of the half-life from the 'high' PPR treatment. On average, these values were larger than those from the other two factors, suggesting that PPR had the largest effect on predator digestion rate. In contrast, the half-life ratios for the metabarcoding analysis were comparable for all three factors (Figure 4a–c), implying that no single factor affected digestion rate more than the others based on this assay.

4 | DISCUSSION

We found that molecular diet analysis with both metabarcoding and qPCR was able to detect Chinook salmon in predator gut samples for up to 72 h post-ingestion, whereas visual diet analysis indicated that salmon rations were only recognizable for approximately 12 h post-ingestion. These results were similar to the findings of Schooley et al. (2008) who found prey quickly became unrecognizable and that identification of larval prey needed to be conducted within 40 min of consumption. Our results are unsurprising given the large body of literature illustrating the utility of molecular diet analysis for identifying prey items that are difficult to identify visually (e.g., Carreon-Martinez et al., 2011; Matley et al., 2018; Symondson, 2002).

Although DNA detection times were generally similar across predator feeding trials (i.e., near 72 h), there was considerable variability in detectable prey DNA digestion rates inferred from GLM estimated half-lives. The largest variation was associated with meal size (PPR). Half-lives for low PPRs (i.e., large meal size relative to predator size) were 1.5–2 times those of high PPRs. Other diet studies have also consistently found that larger meal sizes increase DNA quantity (usually measured as detection rate). This finding is consistent across taxonomically diverse species including bats (Schattanek et al., 2021), piscivorous birds (Thalinger et al., 2017), and beetles (King et al., 2010). There are multiple potential explanations for why retention times are longer for larger meals including larger total surface area of prey for digestion (He & Wurtsbaugh, 1993) and limitations on maximum excretion rate (Hilton et al., 1998).

Our feeding trial experiments showed that predator–prey ratio, species, and temperature all influenced prey item loss rates in predator gut content samples. Combined, these results emphasize the importance of controlling for predator species and environmental conditions when interpreting diet item presence/absence results from molecular diet analyses. In terms of predator species, channel catfish displayed longer half-lives than largemouth bass in all trials. This is likely due to differing metabolic rates and gut

TABLE 3 Estimated half-lives (±95 confidence interval) in hours for each combination of factors included in GLM models fit to metabarcoding and qPCR data from molecular diet analysis based on laboratory feeding trials to determine the effects of temperature and ration (predator–prey ratio) on predator digestion rates.

Predator–prey ratio	15.5		18.5	
	CCF	LMB	CCF	LMB
Metabarcoding				
Low	348.35 (136.51, 1206.70)	168.08 (115.39, 266.94)	190.14 (106.44, 410.13)	119.36 (88.42, 166.27)
Medium	205.08 (120.06, 432.30)	126.00 (81.62, 203.43)	134.68 (91.34, 204.68)	95.24 (67.68, 139.56)
High	150.00 (78.48, 417.21)	105.90 (57.27, 230.08)	106.85 (64.99, 195.66)	77.63 (46.90, 132.76)
qPCR				
Low	344.12 (154.08, 924.41)	280.67 (162.74, 564.20)	212.76 (124.85, 411.70)	191.02 (123.72, 332.84)
Med	178.64 (114.64, 300.20)	168.03 (100.49, 302.08)	136.08 (95.55, 198.53)	125.41 (84.76, 194.55)
High	129.75 (76.90, 255.37)	125.20 (63.59, 320.86)	101.95 (67.22, 161.20)	96.66 (56.93, 183.31)

Note: The half-life is the amount of time that half of the prey item DNA in the digestive tract is lost due to digestion, degradation, and evacuation. Factors in the GLM included species (CCF = channel catfish *Ictalurus punctatus*, LMB = largemouth bass *Micropterus salmoides*), temperature (two levels: 15.5 and 18.5°C) and predator–prey ratio (continuous: low = 73.6, medium = 132.7, high = 191.6).

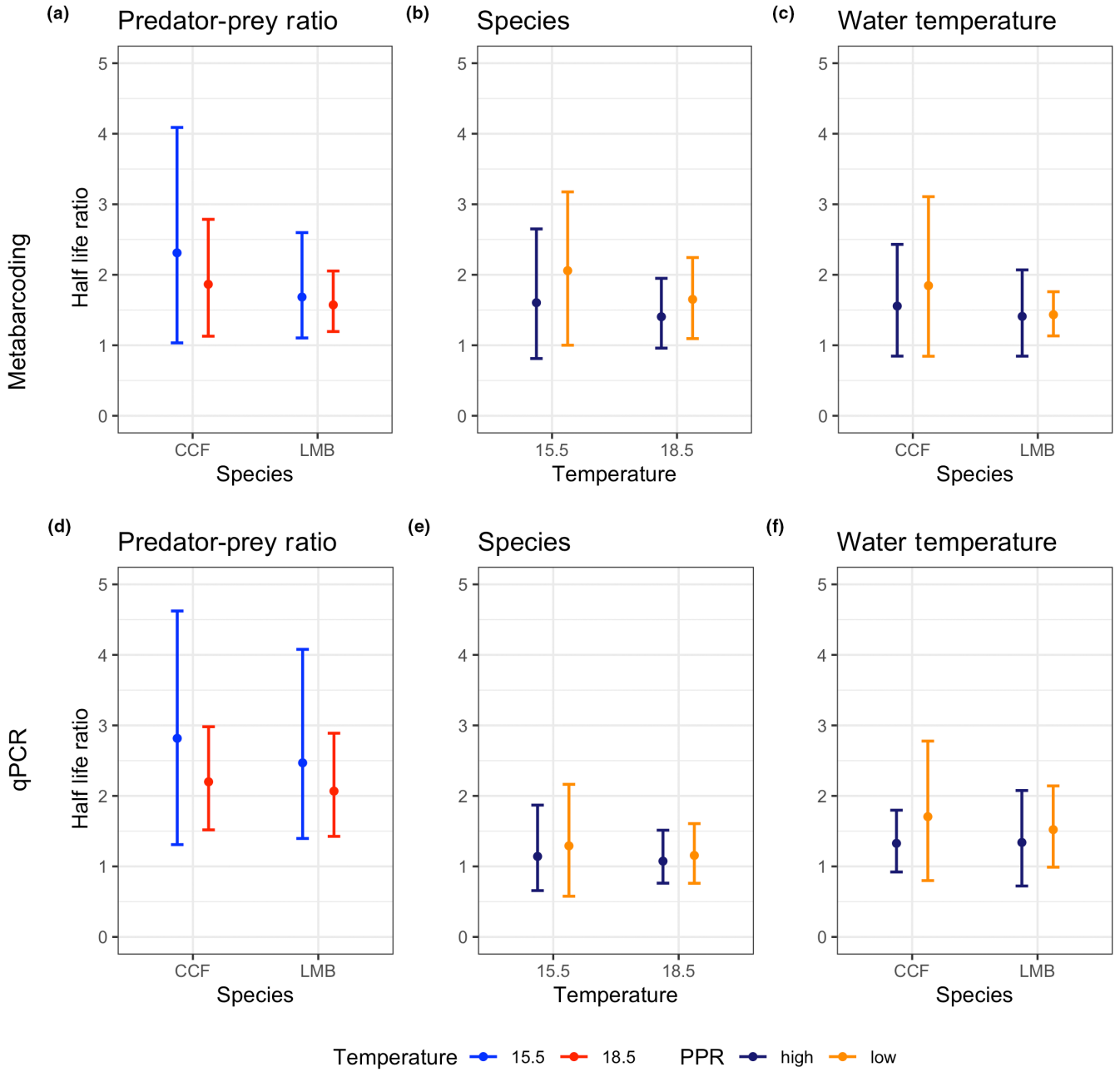

FIGURE 4 Half-life ratios calculated based on the results from the metabarcoding (a–c) and qPCR (d–f) generalized linear models (GLM) fit to digestive tract samples from channel catfish *Ictalurus punctatus* (CCF) and largemouth bass *Micropterus salmoides* (LMB) to determine the effects of temperature (15.5 vs. 18.5°C) and ration (represented by the continuous covariate predator–prey ratio) on predator digestion rates measured using molecular methods on gut content samples from laboratory feeding trials. The half-life ratio was calculated by dividing the GLM estimated half-life for one level of the factor of interest (e.g., high predator–prey ratio) by the half-life of the other level of the factor of interest (e.g., low predator–prey ratio) while keeping the other factors constant (e.g., species = CCF and temperature = 15.5). Ratios closer to 1 indicate the half-lives are more similar and that factor does not affect evacuation rate as much. PPR was calculated by dividing predator mass from prey mass and the values for PPR categories were: low = 73.6, high = 191.6.

morphology between species, as largemouth bass are fast moving, purely piscivorous apex predators, while channel catfish are slower moving omnivores with longer guts. Predictably, warmer temperatures increased digestion rates and decreased detectable prey DNA quantities in gut content samples from both predator species, consistent with results from previous molecular diet studies on fish (Carreon-Martinez et al., 2011) and other ectotherms (Hoogendoorn & Heimpel, 2001; Von Berg et al., 2008). Taken together, our results suggest that the three feeding trial variables that we examined (temperature, predator species, PPR) substantially influenced prey item DNA loss rates in predator gut contents and should be accounted for when interpreting diet analysis data.

Our finding that the presence of Chinook salmon smolt prey diet items was detectable (i.e., some samples with metabarcoding

read counts in the hundreds or thousands and/or non-zero DNA copy numbers) until roughly 72 h after ingestion was similar to results from Brandl et al. (2016), who used qPCR to detect Chinook salmon juveniles fed to striped bass (*Morone saxatilis*), another invasive predator in the Delta. However, similar types of feeding trials conducted in other systems found detectability times that differed from ours. For example, Carreon-Martinez et al. (2011), fed larval and small juvenile fish to a variety of warm water piscivorous predators native to the Laurentian Great Lakes region and found that DNA detection rates (based on COI sequencing) fell to nearly zero at 24 h. Additionally, Thalinger et al. (2017), fed piscivorous birds large quantities of multiple fish species including salmonids and found that DNA detection rates approached zero after ~32 h. Our study is comparable to Carreon-Martinez et al. (2011) in terms of predators and Thalinger et al. (2017) in terms of prey, but the time that detection rates reached ~0 were much longer in our study. It is likely that this much longer detection time window compared with Carreon-Martinez et al. (2011) was due to the much smaller, and easier to digest, prey items in that study compared to the smolts in our study. We hypothesize that differences between our results and those of Thalinger et al. (2017) were due to differing gut morphology and metabolisms of piscivorous birds in comparison to the fish in our study. Results from the studies discussed here provide strong evidence that digestion rates vary widely across predator species and prey types. However, our findings that largemouth bass, channel catfish, and striped bass (based on Brandl et al., 2016) had relatively similar digestion rates for the same prey item suggests that digestion rates from similar predator species (e.g., warm water piscivorous fish) fed similar prey may be comparable.

Another hypothesis we wanted to test in our analysis was whether DNA quantity in the digestive tract was affected by degradation. In theory, DNA in stool should be more degraded, reducing DNA quantities observed from molecular techniques (reviewed in Ando et al., 2020). We conducted a simple test of this hypothesis by using a weighting factor to account for differences in the amount of DNA between heavily digested material (i.e., stool) and less digested material (i.e., identifiable tissue). However, including this weighting factor did not improve the correlation between visual and molecular results. We hypothesize that our assays, which targeted relatively short mtDNA fragments, were not substantially affected by the low DNA quality of faecal samples, but other molecular approaches, especially those targeting nuclear DNA (Shi et al., 2023; Tende et al., 2014), are likely to be impacted. Additionally, it is important to note that our sampling for DNA analysis was not based on mass (i.e., we took a similar sized small chunk from each piece of tissue no matter the size of the tissue). Ideally, all diet contents would be homogenized and subsampled for DNA analysis, but this is logistically difficult in practice. While our approach for DNA subsampling produced results that were consistent with expectations, future studies may benefit from comparing homogenization to subsampling to determine if homogenization could further improve the quantitative results produced from molecular methods.

Another goal of our study was to compare the quantitative estimates from two molecular assays: metabarcoding and qPCR. Our results suggest that metabarcoding can perform at least as well as qPCR if targeted primers are developed for prey items. Additionally, we found that using relatively different minimum read cut-offs (2 vs. 159) had a negligible impact on our overall findings, but this may not be true for all studies. Currently, qPCR represents a cheaper and generally simpler workflow compared to high-throughput sequencing. However, while qPCR technology has been relatively static for many years, advances in high-throughput sequencing occur frequently. These advances will likely facilitate higher sequencing depths and reductions in sequencing error, which could further improve the relative performance of metabarcoding compared to qPCR. Finally, developing primers for high-throughput sequencing should be simpler since non-target amplification is less of an issue than for qPCR primers (Langlois et al., 2021), making high-throughput sequencing a potentially more attractive choice in systems where qPCR primers have not been developed.

A major simplifying aspect of our study was that it only included one prey species, but this would be extremely rare for diet studies conducted in the wild. When multiple species are present and targeted by the same primer set, quantification of each target can be substantially biased due to differences in amplification efficiency (reviewed in Kelly et al., 2019). One potential way to address this issue is to create mock communities with known amounts of DNA from each target species and then quantitatively correct for biases (Gold et al., 2022; Shelton et al., 2022). Another approach is to develop or use existing species or taxa-specific primers to reduce the number of amplification targets for metabarcoding (e.g., McCarthy et al., 2022; Min et al., 2021). Primer development can be time-consuming, but we hypothesize that the benefits of this targeted metabarcoding approach will become increasingly clear as more studies utilizing it are published. However, it is important to note that low-diversity sequencing libraries created from this targeted approach could lead to poor sequencing performance. In these cases, the libraries could be sequenced with other high-diversity libraries or with additional PhiX. Another potential concern with our analysis was the possibility of unwanted predator reads because our primer amplified both the predators and prey. Based on our results, we are confident we sequenced deep enough to ensure that we obtained appropriate estimates of Chinook salmon DNA quantity. But if this is a concern for future studies, blocking primers could be used to reduce unwanted predator reads (Ando et al., 2020). However, blocking primers can also inadvertently reduce amplification of certain prey species and choosing the correct blocking primer concentration is non-trivial (Shi et al., 2021).

If accurate quantification of multiple diet items is the goal, we suggest a multiphased approach that includes (1) screening stomach samples using broad metabarcoding primers to identify potential diet items and their approximate frequencies, (2) developing or identifying existing species-specific metabarcoding primers for each species of interest, and (3) conducting high-coverage metabarcoding using these species-specific primers. A variation on this approach

that avoids the need for species-specific primers could be to conduct broad metabarcoding then construct and screen mock communities containing species of interest to quantitatively correct for PCR bias (e.g., Shelton et al., 2022). If accurate counts of the number of individuals of a given species in diet samples is a goal, we suggest exploring DNA mixture methods that utilize allele counts at a large number of nuclear loci (Sethi et al., 2019), as these methods should be substantially more accurate than qPCR and metabarcoding for direct quantification. However, amplification of nuclear loci is more challenging than amplifying mtDNA and the methods to reliably amplify nuclear loci in lower quality samples are still under development (Shi et al., 2023). Finally, we suggest that future diet studies further explore quantifying DNA degradation to understand the influence of time since ingestion, which is an important component of interpreting diet data through molecular assays. Shi et al. (2023) amplified nuclear markers on the same samples described in this study and found a consistent relationship between time since ingestion and the percentage of on-target reads, which is an indirect measure of DNA quality. Another potential approach to indirectly quantify DNA quality is to utilize primers with different amplicon sizes and compare their amplification success (e.g., Uiterwaal & DeLong, 2020). While these approaches are intriguing, future research using controlled experiments is necessary to better understand the relationship between time since digestion and indirect measures of DNA quality.

4.1 | Conservation implications and conclusions

The utility of molecular methods for detecting the presence/absence of diet items that could not be detected by visual inspection has been clear for many years, and molecular diet analysis has been used numerous times in this context to inform management and conservation (e.g., Egeter et al., 2019; Ford et al., 2016; Schreier et al., 2016). However, translating molecular diet analysis results into quantitative estimates that can be used to directly assess the impacts of predation and inform more holistic bioenergetics models has been difficult. Our study provides important information on the variables influencing digestion rates of non-native piscivores feeding on Chinook salmon that can be used to estimate daily consumption rates and integrated into predation impact assessments for the Delta. Of particular interest, we have shown species-specific differences in digestion rates, which could have implications for data interpretation and bioenergetic model extrapolation. Relative to largemouth bass, channel catfish had slower digestion rates, which is correlated with reduced metabolisms at common temperatures (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, 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. To estimate the total number of salmon smolts consumed by different predators during the spring outmigration period, accurate estimates of species metabolisms, or digestion rates, are critical.

Although it would be impossible to repeat our in-depth feeding trials for every species in every ecosystem, recent research with spiders has suggested that it may be possible to estimate molecular digestion rates using characteristics of the environment, predator and prey. Uiterwaal and DeLong (2020) found that DNA half-lives (i.e., digestion rates) can be calculated for a variety of predator and prey items using a relatively small set of variables that includes amplicon length, ambient temperature, prey mass, and predator mass. It is unclear whether this approach would work for other taxa, but the results from spiders are encouraging. A valuable future study would be to combine modelling and meta-analysis approaches, such as Uiterwaal and DeLong (2020), with targeted experimental feeding trials to better understand digestion rates. Results from studies such as these can then be directly utilized to improve the ability of molecular diet studies to quantitatively estimate the impacts of predation and thus better inform management and conservation. To create more general models for fish digestion rates, we suggest that future research includes both a meta-analysis and a study that conducts feeding trials containing multiple prey species with different characteristics to assess variable digestion rates across species.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequence data can be found on NCBI in the SRA under accession number PRJNA983053. All other data produced in this study

are either presented in the supplementary materials or available on DRYAD (https://doi.org/10.5061/dryad.fj6q5740x).

BENEFIT-SHARING STATEMENT

Molecular diet analysis is an important tool for understanding ecological processes and can rapidly provide data in data poor systems. This manuscript is useful for improving the utility of molecular diet analysis, which will hopefully be helpful in many systems around the world. We will continue to share the results of this work as broadly as possible and are ready and willing to provide technical support to other laboratories conducting this type of work.

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SUPPORTING INFORMATION

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