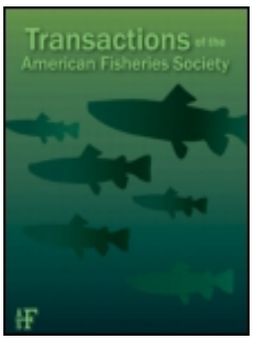

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NOTE

Generation of Quantitative Polymerase Chain Reaction Detectability Half-Lives and Comparison of Sampling Protocols for Genetic Diet Studies of San Francisco Estuary Fishes

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Abstract

Several factors affect the probability of genetic analyses to detect prey in predator gut contents, including biological differences in the prey and predator species as well as differences in sampling and laboratory methodologies. Understanding these biases allows researchers to more appropriately put genetic prey detections in an ecological context. In this study, we determined the detectability half-lives of DNA from two prey species in the guts of two predators. The half-life detectability of juvenile Chinook Salmon *Oncorhynchus tshawytscha* in Striped Bass *Morone saxatilis* was 66.2 h, and that of larval Delta Smelt *Hypomesus transpacificus* in Mississippi Silverside *Menidia audens* was 26.4 h. Additionally, we performed a series of laboratory trials to examine the effects of variables in sample collection and preservation methodologies on the detectability of prey. Differences between methodologies were minimal, providing confidence that laboratory analyses will not be greatly affected by inconsistencies in field sampling procedures. Injecting a 95% solution of ethanol into the stomach via the esophagus immediately following collection and placing the fish on ice in the field prior to freezing at –20°C in the laboratory is a protocol readily applied in the field that will provide consistent results.

The San Francisco Estuary (SFE) provides water to approximately 25 million California residents as well as irrigation to more than 3.5 million acres of farmland (Carle 2004). The largest estuary on the West Coast, the SFE is host to one federally endangered fish species, Central Valley winter-run Chinook Salmon *Oncorhynchus tshawytscha*, and four federally threatened fish species, including Delta Smelt *Hypomesus transpacificus* (Sommer et al. 2007) and Central Valley spring-run Chinook Salmon (Yoshiyama et al. 1998). There are a number of causes of fishery decline in the SFE, including habitat alterations and the collapse of the lower trophic food web. Two top-down effects have also been identified as significant stressors to imperiled fishes: predation by nonnative fish, and entrainment in water diversion facilities (Sommer et al. 2007).

Though entrainment has been the focus of much research (Brown et al. 1996, 2009; Kimmerer 2008), predation studies that utilize gut contents remain limited in number and scope (but see Stevens 1966; Nobriga 2007; Nobriga and Feyrer 2008; Baerwald et al. 2012). Two predator species from the Delta have been hypothesized to have considerable effects on high-profile prey. First, predation by nonnative Striped Bass *Morone saxatilis* on juvenile Chinook Salmon is of interest because survival rates for Chinook Salmon are low throughout the SFE (Newman and Brandes 2010) and predation rates may be augmented by altered flows (Lindley and Mohr 2003;

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Cavallo et al. 2015). Second, predation by Mississippi Silversides *Menidia audens* on early life stage Delta Smelt (Bennett 2005; Baerwald et al. 2012) has received attention because the two species' niches overlap (Bennett and Moyle 1996) and because it has been shown that Mississippi Silversides eat Delta Smelt in the wild (Baerwald et al. 2012). Predation by Mississippi Silversides likely occurs on early life stage Delta Smelt, which are difficult to detect visually. One study has shown that the remains of soft-tissue prey like larval fish may be visually unidentifiable 30–60 min postingestion (Schooley et al. 2008). The lower sensitivity of visual gut content studies in these scenarios likely leads to underestimating predation (Deagle et al. 2005), so genetic approaches are particularly effective for detecting predation in these cases (King et al. 2008). Genetic techniques also provide an advantage when looking for rare prey. Delta Smelt and Central Valley spring- and winter-run Chinook Salmon are relatively rare in the ecosystem, so lengthening the postingestion detection period of these prey increases the likelihood of detection. For example, in captive feeding trials, soft-tissue prey can be detected for 12 h in Green Sunfish *Lepomis cyanellus* (Ley et al. 2013) and for more than 25 h in Whiting *Merlangius merlangus* (Hunter et al. 2012) in at least half of genetically assayed predators. For these reasons, genetic analysis has become a complementary or, in some circumstances, favored approach to visual prey identification (Greenstone et al. 2014).

We used species-specific quantitative PCR (qPCR) to detect the presence of target DNA. If present, the DNA is amplified and emits a fluorescent signal. Quantification cycle (Cq) is the number of qPCR cycles completed before the fluorescent signal crosses a predetermined threshold. Quantification cycle reflects the number of copies of target DNA that are present and detectable in a sample, though it has been shown that the genetic signal from different species are not always representative of the biomass of that species consumed (Deagle and Tollit 2007; Deagle et al. 2010; Bowles et al. 2011). As a consequence, most genetic studies confine their results to detection or nondetection of prey (Thomas et al. 2013). To advance genetic diet studies beyond detection, a number of factors should be taken into consideration that can be categorized as those relating to the biology of the predators, prey, and their environment, and those relating to sampling and laboratory methodologies.

Detections cannot be directly compared between different predator–prey pairs due to the natural biological variation between them. These differences are rooted in the morphological and physiological characteristics of the predators and prey themselves, such as surface area-to-volume ratio of the prey (Salvanes et al. 1995), prey tissue properties such as hardness and fat and protein content (Thomas et al. 2013), and predator digestion and metabolic rates, which are dependent on water temperature. Captive feeding trials can be useful for quantifying these biases so that a correction can be made to normalize their effects (Thomas et al. 2013). Detectability half-life (T50) is the metric often used to normalize the differences in detectability among predator and prey pairings (Greenstone et al. 2014). Detectability half-life is a measure of the time after feeding at which prey remains can be detected in half of the assayed predators. This metric is particularly useful because it mediates many of the sources of biological bias into one comparable number.

Relatively few half-life studies have been performed on fish (but see Baerwald et al. 2012; Hunter et al. 2012; Ley et al. 2013; Albaina et al. 2014). We chose Mississippi Silversides and Striped Bass because they predate on high-profile native fish of conservation concern in the SFE, and because these fish are anatomically distinct. Striped Bass have a longer and more complex gastrointestinal tract (hereafter, "GI tract"), including a pyloric sphincter, pyloric cecum, and a distinct intestine, while Mississippi Silversides lack a true stomach and have a relatively simple GI tract (Shoup and Hill 1997). Examining both large- and small-bodied predator fishes allows us to assess the potential range of detectability half-lives possible for SFE predators, and provides a launching point for further studies.

Consistent sampling, preservation, and laboratory methodologies are important in DNA analyses of gut contents because digestive enzymes and the ephemeral nature of DNA put diet samples at risk of degradation (King et al. 2008). Suboptimal preservation techniques may reduce the comparability of results as well as limit the sensitivity of detection methods. Previous genetic gut content studies in fish have used ethanol, freezing, or a combination of the two to preserve samples (Carreon-Martinez et al. 2011; Baerwald et al. 2012), though details of their method optimization process were not documented. Preservation methods for different-sized fish may vary as will the approach taken when handling these species in the field, so we examined the effectiveness of a number of preservation techniques for both a large-bodied fish (Striped Bass) and a small-bodied fish (Mississippi Silverside). For both species, our primary goal was to determine how to maximize the preservation of genetic material by testing various combinations of sample processing and storage times, coupled with different storage temperatures. We chose the methods we tested because they were likely to adequately preserve DNA while limiting the potential for contamination, and because they were easy to implement in the field setting.

The goal of the half-life experiments was to understand the biological biases in the genetic detection of prey species. Specifically, (1) what are the detectability half-lives of the species of interest, and (2) how do these half-lives affect our interpretations of a positive detection within and between species. The purpose of the preservation method experiments was to lend insight into how to minimize methodological biases from field preservation methods and laboratory procedures. Specifically, (1) what combinations of ethanol application and cooling or freezing minimizes DNA degradation (i.e., increases the sensitivity of the detection method), and (2) does the length of time the sample spent on ice or was frozen affect the detection sensitivity of the assays.

METHODS

Detectability Half-Life

Half-life of juvenile Chinook Salmon in Striped Bass.—Sub-adult Striped Bass (180–250 mm FL) were moved from the Freshwater Fish Company (Elk Grove, California) to 6-ft flow-through tanks at the Center for Aquatic Biology and Aquaculture (CABA) at the University of California–Davis (UC–Davis). For 3 d prior to the start of the experiment, we withheld food and allowed the fish to acclimate to the tanks. Water temperatures were held constant at 18°C (±0.5°C) throughout the acclimation and trial periods. Juvenile Chinook Salmon (48–96 mm FL) were obtained from the Feather River Hatchery (Oroville, California) the morning of the feeding trial and kept in tanks with aeration. The median FL ratio of Chinook Salmon to Striped Bass was 0.33. Striped Bass were anesthetized individually using water saturated with CO_2. When fully anesthetized, the Striped Bass were Floy tagged and force-fed a single whole euthanized Chinook Salmon with forceps. After feeding, the Striped Bass were allowed to recover individually in an oxygenated holding tank while being closely monitored for regurgitation and signs of poor recovery. None of the Striped Bass had problems with regurgitation. After the recovery period, consecutive fish were grouped in tanks by digestion time period. Digestion periods for each individual were determined from the Floy tags. Two Striped Bass were euthanized immediately following feeding ($t = 0$), followed by samples (n = 9–11/time point) taken at 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, and 168 h postfeeding. Upon completion of each tank's digestion period, all Striped Bass were euthanized and their stomachs were injected with 3 mL of a 95% solution of ethanol to halt enzymatic activity. The Striped Bass were put in bags individually and frozen at –20°C until dissection.

All dissections were performed in 1 d (2–8 d posteuthanization). The stomachs and intestines were removed using DNA-sterile dissection techniques, and their contents were emptied into Qiagen lysis buffer (4.5 mL ATL, 0.5 mL proteinase K; Qiagen, Valencia, California) and incubated at 55°C for 2 d to dissolve the tissues. Lysed tissue was centrifuged at 1,000 × g for 15 min to aggregate the solid material before removing 100 µL of the supernatant and adding it to 100 µL lysis buffer to dilute the solution in order to avoid clogging the extraction filter plate. Extraction of DNA was performed with a Qiagen DNeasy Blood and Tissue kit on the Qiagen BioRobot. Salmon DNA was detected using a previously designed species-specific qPCR assay (Brandl et al. 2014). Sample DNA was preamplified in a primer-limited environment for 14 cycles, and high throughput qPCR was performed on the Biomark system (Fluidigm, South San Francisco, California) with four no-template (negative) controls (Brandl et al. 2014). Samples were determined to be positive for the presence of prey DNA if the qPCR amplification plots showed a logarithmic signal in fewer than 40 cycles, with the baseline threshold set above the no-template controls. To be considered positive for the presence of prey DNA, at least four of six technical replicates must have displayed unambiguous amplification. To disqualify ambiguous amplification plots, reactions with a ΔRn (fluorescence emission relative to reference dye) of less than 0.05 were removed. The half-life was determined using Probit regression in R (Payton et al. 2003).

Half-life of larval Delta Smelt in Mississippi Silversides.—The Mississippi Silverside feeding trial was conducted in much the same way as the Striped Bass feeding trial. Wild Mississippi Silversides (61–96 mm FL) were collected from the SFE and brought to CABA for the experiment. After an acclimatization period of 10 d and a fasting period of 2 d, the Mississippi Silversides were anaesthetized with 150 mg/mL MS-222 (tricaine methanesulfonate) and force-fed single euthanized larval Delta Smelt (10–12 mm TL) with a single-use glass pipette. The median FL ratio of Delta Smelt to Striped Bass was 0.15. Mississippi Silversides were observed for regurgitation then housed in tanks according to their time point (0, 2, 4, 8, 12, 18, 24, 36, 48, or 65 h postingestion). Few fish had issues with regurgitation, and those that did were later reused depending on their condition. For each time point, 10–12 fish were dissected and the GI tracts were removed and preserved in lysis buffer. These GI tract samples were homogenized in a bead mill (Qiagen TissueLyser II; Qiagen), DNA was extracted, and Delta Smelt DNA was detected using a qPCR assay (Baerwald et al. 2011). Laboratory protocols and the half-life calculation were performed as above.

Testing DNA Preservation Methods

DNA preservation in gut contents of large-bodied fish.—We used Mississippi Silversides as the prey species for Striped Bass because they are easier to obtain than Chinook Salmon and are a known prey item for Striped Bass in the SFE (Feyrer et al. 2007). We fed Mississippi Silverside tissue to Striped Bass and subjected each group to a different preservation technique. Whole Mississippi Silversides were euthanized and homogenized to create a tissue slurry. The homogenization of prey tissue was necessary to standardize the volume of starting material so that qPCR results could be compared among samples. Striped Bass were anaesthetized with MS-222, and 3 mL of the slurry was fed to them using a single-use serological pipette. We used DNA extracted from Mississippi Silverside fin tissue (N = 3) as a positive control. For the treatments (N = 5–6 fish/method), Striped Bass were euthanized 4 h after feeding and one of the following preservation methods was applied (Table 1): (1) immediate dissection and emptying of gut contents into room temperature 95% ethanol; (2) immediate dissection and entire GI tract, with contents, placed in room temperature 95% ethanol; (3) injection of room temperature 95% ethanol into the stomach via the esophagus and whole Striped Bass stored on wet ice for 8 h before freezing at –20°C for 24 h; or (4) injection of room temperature 95% ethanol into the stomach via the esophagus and whole Striped Bass snap frozen by

immersing the fish in 95% ethanol that had been cooled with dry ice (–78°C). After snap freezing, the fish from this treatment were placed on wet ice for 8 h and then frozen at –20°C for 24 h. For methods 3 and 4, the guts were put into ethanol after dissection. Methods 2, 3, and 4 were designed to be scenarios that would simulate possible field methods; hereafter, these three methods will be collectively called the "field methods." Because the possibility for contamination is too great with field dissections, method 1 was intended as an optimal treatment to be used as a comparison. For all treatments, ethanol was decanted and the tissues were homogenized using a Qiagen TissueRuptor (Qiagen), and 5 mL of lysis buffer was added to stabilize the DNA and dissolve the tissue. Tissue samples were incubated for 2 d at 55°C, and DNA extractions were performed in the same manner as the T50 experiments. Amplifications were performed on a Chromo4 real-time PCR machine for 40 cycles (Brandl et al. 2014). Two technical replicates were performed for each sample and their Cq values averaged. Three Delta Smelt positive controls and four no-template controls were included per plate. Sample Cq numbers were compared using one-way ANOVA in R, and pairwise differences were compared using Tukey's honestly significant difference (HSD) test in R ($P < 0.05$).

Effect of duration of ethanol and freezing on quantification cycle in small-bodied fish.—Sampling and preserving small-bodied fish requires a different approach than that used for large-bodied fish. To simulate possible field scenarios, fish were preserved whole. The primary question addressed in this experiment was whether the length of time a sample is frozen or left at room temperature postcapture affects the degradation and detectability of the prey DNA. Similar to the detectability half-life trial for Mississippi Silversides, we force-fed whole larval Delta Smelt to individual Mississippi Silversides. For the treatments, 4 h after feeding, Mississippi Silversides were euthanized and whole fish were submerged in room temperature 95% ethanol for (Table 1) one of the following: (1) 18 h, (2) 5 d, (3) 18 h followed by 5 d frozen at –20°C, (4) 5 d followed by 5 d frozen at –20°C, (5) 18 h followed by 2 months frozen at –20°C, or (6) 18 h followed by 2 months frozen at –20°C, then thawed for 2 h under an incandescent light (simulating a visual diet assessment under a dissecting microscope) prior to genetic analysis. Dissections were performed at the end of each treatment by removing the whole gut and homogenizing it in the same manner as described for the Mississippi Silverside feeding trial experiment. We performed DNA extractions and detections as stated above as well. As with the Striped Bass preservation methods, we compared Cq values with a one-way ANOVA, followed by pairwise comparisons with Tukey's HSD to test for differences between preservation methods.

RESULTS

Detectability Half-Life

For Striped Bass, juvenile Chinook Salmon were detected in 100% of the samples for the first 36 h, with the proportion positive decreasing until there were no detections at the 96, 108, or 168 h time points (Figure 1A). Two of ten samples were positive at 120 h postingestion. It is not clear what may have caused these positive detections, though towards the end of the experiment, fish in this tank had a fungal infection, possibly slowing digestion rates in these two individuals. The Probit regression indicated that the half-life of Chinook Salmon in the Striped Bass guts was 66.2 h. Delta Smelt DNA was detectable in 100% of Mississippi Silversides for the first 12 h, followed by a decline with no detections at 65 h (Figure 1B). The detectability half-life for larval Delta Smelt in Mississippi Silverside guts was 26.4 h.

Preservation Methods

DNA preservation in gut contents of large-bodied fish.—The average Cq for the Striped Bass preservation methods ranged from 18 to 27 cycles (Figure 2). Some of the treatments were significantly different based on the Cq values (ANOVA: $F = 13.73$, $p = 4.7 \times 10^{-6}$). The Tukey's test indicated that the detection of prey DNA from positive controls did not differ significantly from method 1 (gut contents immediately removed and stored in 95% ethanol). These two methods were significantly different than the field methods (methods 2, 3, and 4; $P < 0.01$). Differences among the three field methods were not significant.

Effect of duration of ethanol and freezing on quantification cycle in small-bodied fish.—The average Cq for the Mississippi Silverside preservation methods ranged from 27 to 32 cycles, with the positive control DNA amplifying at 18 cycles (Figure 3). The ANOVA indicated a difference between the control and treatments (ANOVA: $F = 17.42$, $p = 1.05 \times 10^{-8}$), but the Tukey's test indicated there were no differences among the preservation methods tested ($P < 0.01$).

DISCUSSION

Detectability Half-Life

Baerwald et al. (2012) reported a Delta Smelt DNA half-life in Mississippi Silverside guts of 17.5 h. The current study was a similar experiment, though we used field-relevant prey life stage, a different tank temperature, and more time points to investigate the half-life at a finer scale. Using the same laboratory detection method, we estimated a DNA half-life of 26.4 h. The difference observed between these studies indicates that even for a given prey and predator combination, there may be considerable variability in detectability half-life. It is unclear which variable created the difference—surface area-to-volume ratio of the prey, prey tissue hardness, prey composition, the tank water temperature, or a combination of these factors. Further experiments are warranted to more clearly delineate these differences.

TABLE 1. List of gut preservation methods tested.

Species	Method	Abbreviated name in text	Method description
Striped Bass		Control	DNA extracted from fin clips
Striped Bass	1	Gut contents in ethanol	Immediate dissection and emptying of gut contents into 95% ethanol
Striped Bass	2	Gut in ethanol	Immediate dissection and entire GI tract placed in 95% ethanol
Striped Bass	3	Inject ethanol, wet ice	Injection of 95% ethanol into the stomach, whole fish stored on wet ice for 8 h before freezing at –20°C and dissected the next day
Striped Bass	4	Inject ethanol, snap freeze	Injection of 95% ethanol into the stomach; whole fish frozen by immersing the fish in 95% ethanol that has been cooled with dry ice until it is frozen; after freezing, the fish were placed on wet ice for 8 h before being frozen at –20°C and dissected the next day
Mississippi Silverside		Control	DNA extracted from a fin clip
Mississippi Silverside	1	18-h ethanol	Whole fish placed in room temperature 95% ethanol for 18 h
Mississippi Silverside	2	5-d ethanol	Whole fish placed in room temperature 95% ethanol for 5 d
Mississippi Silverside	3	18 h, 5 d frozen	Whole fish placed in room temperature 95% ethanol for 18 h followed by 5 d frozen at –20°C
Mississippi Silverside	4	5-d ethanol, 5 d frozen	Whole fish placed in room temperature 95% ethanol for 5 d followed by 5 d frozen at –20°C
Mississippi Silverside	5	18-h ethanol, 60 d frozen	Whole fish placed in room temp 95% ethanol 18 h followed by 2 months frozen at –20°C
Mississippi Silverside	6	18-h ethanol, 60 d frozen, 2 h under lamp	Whole fish placed in room temperature 95% ethanol for 18 h, then stored in a –20°C freezer for 2 months and thawed for 2 h under an incandescent light (simulating a visual diet assessment prior to genetic analysis)

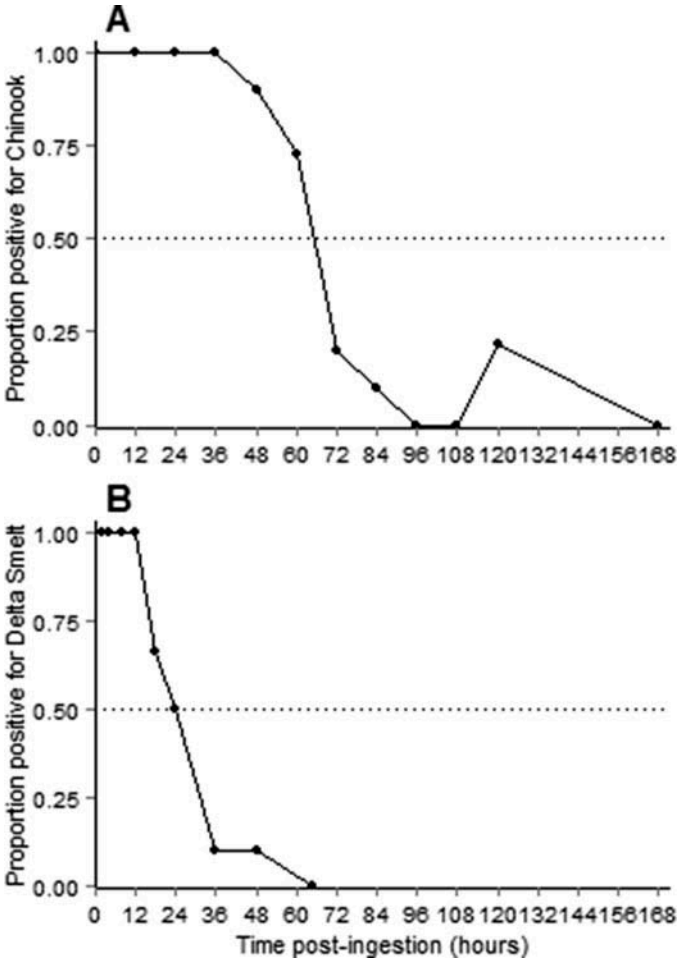

FIGURE 1. Proportion of predators for each time point with detectable prey DNA present. The top panel is the detection probability of juvenile Chinook Salmon in Striped Bass. The bottom panel shows the probability of detecting larval Delta Smelt in Mississippi Silverside.

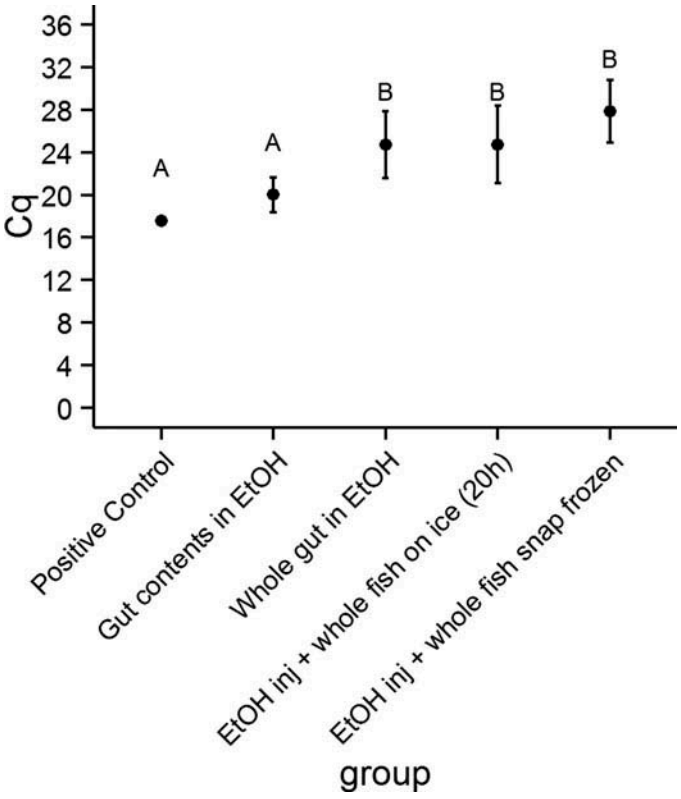

FIGURE 2. Comparisons among Cq values for each Striped Bass preservation method. Bars represent 95% confidence intervals. The treatments fell into two statistically significant groups, labeled A and B.

The intent of the Striped Bass and Mississippi Silverside detectability half-life feeding trials was not for direct comparison with each other. Nonetheless, the laboratory methodology and experimental tank conditions were the same for both feeding trials. The differences between these two half-life experiments are attributable to biological differences between the predators and prey—factors such as digestion and metabolic rates of the predator, surface area-to-volume ratio of the prey, and prey composition. The T50 values for these two scenarios varied considerably—26.4 and 66.4 h—reflecting the biological differences between the two predator–prey pairs.

Knowledge of the detectability half-life for each predator–prey pair is important because it provides a way to normalize detection rates between predators and possibly compare predator impacts on prey populations. For example, a common metric used in genetic diet studies is the proportion of the sample population that was positive for a specific prey species. This metric may be applied to assess minimum overall impact of a predator population on a prey species. For example, because multiple prey of the same species cannot be detected genetically, Albaina et al. (2014) used the conservative assumption that one detection indicates one individual prey eaten. In this way, the minimum effect a predator population had on a prey population was estimated by multiplying the proportion of the population positive for the prey by the abundance estimate for the predator in that area. However, in order to compare the proportion positive from more than one predator–prey pair, the T50 numbers must be known for both, to normalize the detection periods. For example, if T50 is known for two prey species in a predator species, the frequency of detection of the prey species can be normalized by dividing the proportion positive by their T50 value. The result is a value that indicates the relative amount of these two prey in the predator. In this way it is possible to estimate the relative importance of each prey to the predator (Greenstone et al. 2014). Likewise, it is possible to estimate the relative impact of two different predators on a common prey after normalizing the proportion positive by their respective T50 numbers (Greenstone et al. 2010).

Another implication of the different half-lives of our two predator–prey scenarios is the ecological interpretations for positive detections in field studies. Given the shorter detection period (27 h) and lower mobility of individual Mississippi

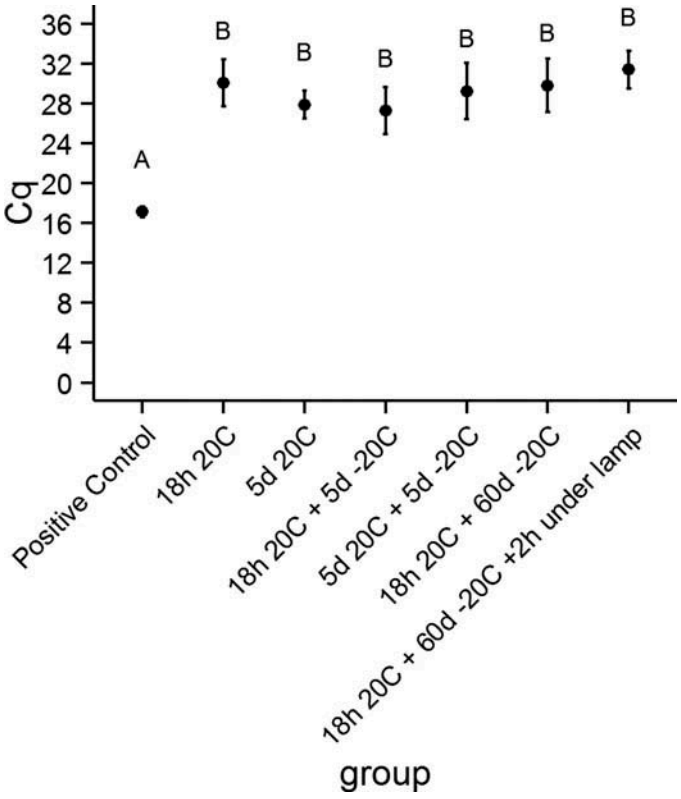

FIGURE 3. Comparisons among Cq values for Mississippi Silversides with 95% confidence intervals. The treatments fell into two statistically significant groups, labeled A and B.

Silversides relative to Striped Bass (Moyle 2002), the habitat where they are captured is more likely to be representative of the habitat where predation took place. The seasonal movements of Striped Bass are well documented (Moyle 2002), as are their movements in response to changing temperatures in some populations (Tupper and Able 2000). Since a detection can be made in Striped Bass for 66 h postingestion in half of fish guts assayed, it may not be reasonable to assume that the predation event occurred near where the fish was captured, complicating the interpretation of diet data as it relates to habitat at small spatial scales. Population-specific details of these movements must be taken into consideration when linking diet data with habitat of capture.

DNA Preservation of Gut Contents for Large-Bodied Fish

An important consideration for genetic gut content studies is the level of preparation and care that is needed to maintain a forensics-level environment, free of contaminant DNA. Care was given when designing the field methods to test field scenarios that maintain a contaminant-free environment as well as being easy to implement in the field. Gut contents in ethanol (Striped Bass method 1) was used as a best-case comparison and is not a feasible field method because of the risk of contamination. Method 1 and the control were not different and had significantly lower Cq than the other methods, indicating that rapid contact with ethanol was key to preserving the samples. The field methods (2, 3, and 4) had immediate contact with ethanol, though not in the same quantity as method 1. For the field methods, there was no significant difference between them, so ease of implementation was the primary criterion for selecting the best method. In method 2, the whole gut was put in ethanol, so on-site dissections would be required, making this is a less desirable option. When comparing methods 3 and 4, snap freezing did not provide any advantage over using wet ice for the initial cooling of the sample. Therefore, injecting ethanol and putting the fish on ice was the best compromise of efficiency and DNA quality.

Effect of Duration of Ethanol and Freezing on Quantification Cycle in Small-Bodied Fish

The positive control was detected much more easily than the experimental treatments, though the results for different preservation methods were not significantly different from each other. We hypothesize that rapid penetration of ethanol in the initial phase was the primary factor in the success of these preservation methods, as we observed that the length of time these samples were frozen had no effect on the ability to detect the prey DNA.

Taken together, the preservation method experiments indicate that the more quickly ethanol comes in contact with tissue, the less the target DNA will degrade. We believe freezing is a necessary step for long-term preservation, but the lengths of time we tested did not affect sample quality.

Other Methodological Considerations

The longevity and consistency of detections between experiments may also vary depending on other methodological issues, such as differences in PCR primer efficiencies or varying levels of PCR inhibitors in the gut environment. Equalizing or quantifying primer efficiencies (De Barba et al. 2013) and quantifying PCR inhibition between samples (King et al. 2009) should be done to minimize the differences between the genetic signal and the mass of prey actually consumed. These biases are project and fish specific, so they should be considered separately for every study system.

Final Remarks

The primary advantage of genetic techniques in diet studies is the high accuracy and sensitivity of detections and the length of time prey can be detected versus visual methods. As the body of literature grows for these types of studies, emphasis is being placed on obtaining more quantitative measures of predator diets, including predicting half-life values. Removing systematic biases in laboratory and field protocols will be an important step, but making corrections for biological biases for different fishes will be the most difficult task. These advances will increase the utility of genetic analyses, but it is likely that visual analysis will remain an important

method for studies that require data such as number or mass of prey. Our experiments demonstrate that future diet studies should carefully characterize the sensitivity of their assays with the specific predator–prey combinations in order to normalize positive detections in field samples. When properly implemented, genetic methods are better suited for detecting rare prey and for estimating the relative frequency of the presence of prey of all life stages in a predator's guts. Ultimately, we expect to see the prevalence of genetic diet studies to increase as the methods evolve.

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