Use of Real Time Quantitative Polymerase Chain Reaction to Detect Delta Smelt DNA in the Stomach Contents of Predators

Technical Review Report
January 2011

Bradley Cavallo, Gregg Schumer, and Phil Gaskill

Introduction

Top-down predation effects are an important part of the Pelagic Organism Decline (POD) conceptual model, and these effects are theorized to have substantial impacts on threatened delta smelt and other pelagic fishes (Sommer et al. 2007). Particular interest has been placed on Mississippi silversides because their abundance has increased over recent years, and they readily consume delta smelt larvae in captivity (Bennett 2005). Consequently, there is a need to evaluate the degree to which Mississippi silversides and other larval fish predators affect delta smelt recruitment and how predation rates are influenced by different habitat variables.

Traditionally, studies examining larval fish predation have been limited to visual identification of predator stomach contents, resulting in poor detection rates and small sample sizes (e.g., Schooley et al. 2008, Braley et al. 2010). However, DNA-based tools have gained prominence across multiple systems in the field of predation ecology (King et al. 2008). In order to more accurately assess the effects of predation on delta smelt populations Cramer Fish Sciences (CFS), in collaboration with the Department of Water Resources (DWR) and the Genetic Variation Laboratory at UC Davis (GVL), developed a novel Real Time Quantitative Polymerase Chain Reaction (qPCR) assay for the detection of delta smelt (*Hypomesus transpacificus*) DNA in the stomach contents of potential predators. Our qPCR based method of detection enabled us to conduct a more accurate assessment of predation on larval, juvenile, and pre-spawning adult delta smelt.

The study detailed in this report included experiments to model the degradation of delta smelt DNA in the guts of captive Mississippi silversides and to determine the sensitivity of the assay using dilution experiments. The likelihood of false positives was also tested using genetic samples from multiple fish species around the Sacramento-San Joaquin Delta. The final assay was then used to analyze stomach contents from wild Mississippi silversides and other predators sampled during the delta smelt breeding season from areas in the lower Sacramento deepwater ship channel. Further application of this new approach will likely enable many unanswered questions about predation on larval fish to be addressed.

A manuscript describing the qPCR application to detect delta smelt DNA has been completed and is in review for publication in the journal Molecular Ecology (Baerwald et al. *in review*). A second manuscript is being written to describe the use of the qPCR technique as a method for evaluating the presence and frequency of Mississippi silverside (*Menidia audens*) predation on delta smelt in the Sacramento-San Joaquin delta. In addition, CFS and GVL have submitted a Record of Invention to the UC Davis Office of Technology Transfer with the intent to file for patent protection of the delta smelt species-specific primer and probe sequences used in the qPCR assay.

Methods

Assay Design

A species-specific primer and probe set was designed for the identification of *Hypomesus transpacificus* (delta smelt) DNA utilizing a 5′ exonuclease (TaqMan™) assay or qPCR. The

primer and probe set was developed to produce a highly specific assay yielding no cross-reactivity with the DNA of closely related species. The specificity of the primer and probe set, combined with the sensitivity of the qPCR assay, yields a method for the definitive detection of *Hypomesus transpacificus* DNA. The primer and probe sequences were derived from mitochondrial DNA localized within a conserved region of the Cytochrome B (CytB) gene of delta smelt. A BLAST search was done on the NCBI nucleotide database to ensure that the CytB DNA template for the primer and probe design had no known homology with other identified nucleotides. From this conserved region of the CytB gene the following forward primer, reverse primer, and species-specific probe were designed to perform a qPCR assay:

Forward primer: [5’ AATGGCCAACCTTCGGAAA 3’]

Reverse primer: [5’ GARATATTRGAGGGTGCAGG 3’]

Species-specific probe: [5’ (6FAM) CCCATCCCCTCCTGAAAATTACCAACG (BHQ1a-6FAM) 3’]

The delta smelt primer and probe set was validated for specificity by testing for cross-reactivity with 22 common Sacramento-San Joaquin Delta region fish species (Table 1), and was determined to be species-specific and highly sensitive to detecting the presence of delta smelt DNA.

Table 1. Common Sacramento-San Joaquin Delta region fish species tested for cross-reactivity, listed by scientific and corresponding common names.

Scientific name	Common name
Alosa sapidissima	American shad
Parcina macrolepida	Bigscale logperch
Pomoxis nigromaculatus	Black crappie
Lepomis machrochirus	Bluegill
Cyprinus carpio	Carp
Ictalarus punctatus	Channel catfish
Micropterus salmodoides	Largemouth bass
Spirinchus thaleichthys	Longfin smelt
Menidia beryllina	Mississippi silverside
Gambusia affinis	Mosquito fish
Clupea pallasii	Pacific herring
Cottus asper	Prickly sculpin
Lepomis gibbosus	Pumpkinseed
Catostomus occidentalis	Sacramento sucker
Tridentiger bifaciatus	Shimofuri goby
Pogonichthys maerolepidotus	Sacramento splittail
Morone saxatilis	Striped bass
Dorosa petenense	Threadfin shad
Hypomesus nipponensis	Wakasagi smelt
Ameiurus catus	White catfish
Pomoxis annularis	White crappie
Acanthogobius flavimanus	Yellowfin goby

Feeding trial

For the feeding experiments, Mississippi silversides were placed in small holding tanks. After a period of time for acclimation and fasting the silversides were fed equal quantities of delta smelt tissue (larvae or chopped up adults from the refugia population). Then, 24 silversides were recovered from the holding tanks, euthanized, and dissected to remove the entire digestive tract at 1, 3, 6, 9, 24, 36, 48, and 60 hours post ingestion. Gut samples were analyzed and the rate of detection of delta smelt DNA was plotted against time in the gut. For the negative controls, ten samples of silverside tissue were sent to the lab for blind analysis. In lieu of conducting positive controls, a sensitivity analysis was conducted. Varying ratios of delta smelt tissue to silverside tissue (1:100, 1:1000, 1:10000, 1:100000; 10 replicates each) were analyzed to quantify the sensitivity of the assay.

Field sampling

During the months of March, April, and May 2010, we conducted field sampling at several locations within the Sacramento-San Joaquin Delta region utilizing a variety of techniques including: hook and line, electrofishing, beach seine, and Kodiak trawl. To acquire sufficient samples, we collaborated with East Bay Municipal Utility District (EBMUD), UC Davis/Moyle Lab, DWR seine, and CDFG trawl programs. Sample locations included: B&W Marina Mokelumne River for hook and line sampling, North Fork Mokelumne, Suisun Marsh, and Sacramento deepwater ship channel north of Rio Vista (Table 2). Adult Mississippi silversides were collected via beach seine from stations near Sherman Island and the Sacramento deep water ship channel at times when DFG's 20mm larval fish survey found larval delta smelt (March-May 2010). A total of 897 fish from 22 different species were collected during sampling for this study (Table 3; Figure 1). Of the 897 fish collected 684 or 76.3% were Mississippi silverside the remaining 213 fish were members of the other species listed in Table 3.

Table 2. Sample method, team, location, and dates.

Sample method	Sampling team	Location	Dates
Hook and line	CFS	B&W Marina Mokelumne River	19, 24, 26 March, 16 April
Electrofishing	CFS/EBMUD	North Fork Mokelumne River	19, 24 May
Beach seine	DWR	Deepwater ship channel	14, 19, 21, 22, 23, 26, 28, 29 April
Beach seine	UC Davis/Moyle Lab	Suisun Marsh	11, 13 May
Kodiak trawl	CDFG	Deepwater ship channel	7, 8 April

Table 3. Species collected, by common name and number collected.

Common name	# collected
Mississippi silverside	684
Yellowfin goby	95
Striped bass	29
Largemouth bass	18
Redear sunfish	12
Spotted bass	10
Sunfish (*Lepomis spp.*)	11
Sculpin	8
Redeye bass	5
Smallmouth bass	5
Shimofuri goby	4
American shad	3
Bullhead catfish	2
White catfish	2
Chinook salmon	2
Stickleback	2
Green sunfish	2
Black bass (*Micropterus spp.*)	1
Threadfin shad	1
Warmouth	1

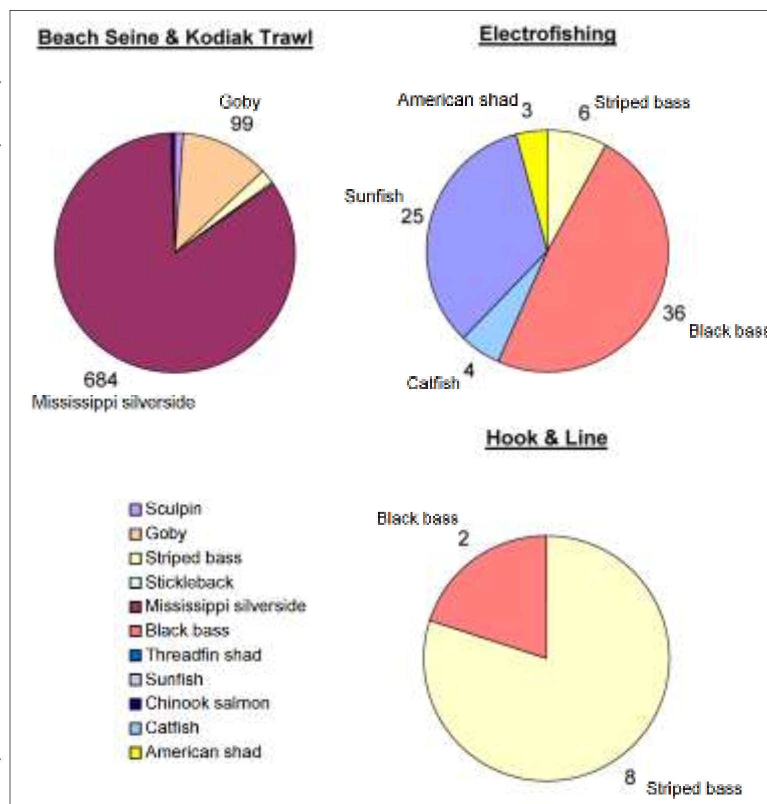

Figure 1. Species collected, by method and number collected.

Laboratory analysis

Samples from both the feeding trial and the field collections were processed at the DWR West Sacramento lab and at the GVL in the following manner: 1) each fish was initially stored in a collection jar containing 95% ethanol and kept at room temperature to preserve the tissues until dissection, 2) dissection of the entire digestive tract was performed using sterile equipment for each individual sample to eliminate cross contamination, 3) dissected stomachs were stored in individual 1.5ml tubes in 95% ethanol at -20°C until DNA extraction, 4) total DNA was extracted from each sample using Qiagen Blood and Tissue extraction kits, and 5) the extracted DNA was analyzed for the presence of delta smelt DNA using the above described qPCR assay. All samples were initially tested in duplicate for the presence of delta smelt DNA. All samples that tested positive for delta smelt DNA were tested again 10 times each. Samples were determined to be positive when delta smelt DNA was detected at or below the limit of detection in a minimum of 8 of the 10 replicates.

Results and Discussion

Assay design

The delta smelt qPCR assay was validated by testing for cross-reactivity with 22 other common Sacramento-San Joaquin Delta region fish species. It was determined that the primer and probe

sequences used for the qPCR assay to detect the presence or absence of delta smelt DNA are highly species-specific and do not cross react or produce false positives. We have established a molecular-based method of detection and taxonomic identification for delta smelt DNA in picogram quantities within a variety of mixed and pure sample types. The qPCR method affords many advantages over traditional visual taxonomic identification methods currently in use. Current visual identification techniques require a taxonomic expert to separate debris and non-target organisms from target organisms. Once separated, target organisms must be subjected to painstaking visual identification, typically using a dissecting microscope. This traditional process of sampling for aquatic organisms is slow, labor intensive, requires intact specimens, and is expensive. In contrast, the identification technique utilizing the qPCR assay is fast, requires very little time and labor to perform, is highly sensitive to minute quantities of DNA, and is relatively inexpensive.

Feeding trial

It was determined that delta smelt DNA from the stomachs of Mississippi silversides is within the technique's limit of detection for at most up to 24 hours after ingestion (Figure 2). On average, at some point between 9 and 24 hours the delta smelt DNA degrades too much to be detected. As recently as 2008, it has been reported that larval remains within the stomachs of predators are readily identifiable at 15 minutes post ingestion but not identifiable after 60 minutes post ingestion using traditional visual identification techniques (Schooley et al. 2008). Thus, using the qPCR assay for the detection of delta smelt DNA translates to at least a 10-fold increase in the detection window versus visual identification. By design, this feeding trial did not include time points between 9 and 24 hours. It is recommended that further feeding trials be conducted for the 9 to 24 hour time period in order to more precisely identify the limit of detection.

It was further determined that the assay is highly sensitive, and capable of detecting as little as 0.1 picograms of delta smelt DNA in a pure (unmixed) sample or within a mixed sample containing 100 nanograms of *Menidia beryllina* (Mississippi silverside) DNA. Using the data from the sensitivity study, we determined the limit of detection for the qPCR assay to be a C(t) of 35 or below (Figure 2). Any sample with a C(t) of higher than 35 was not considered positive.

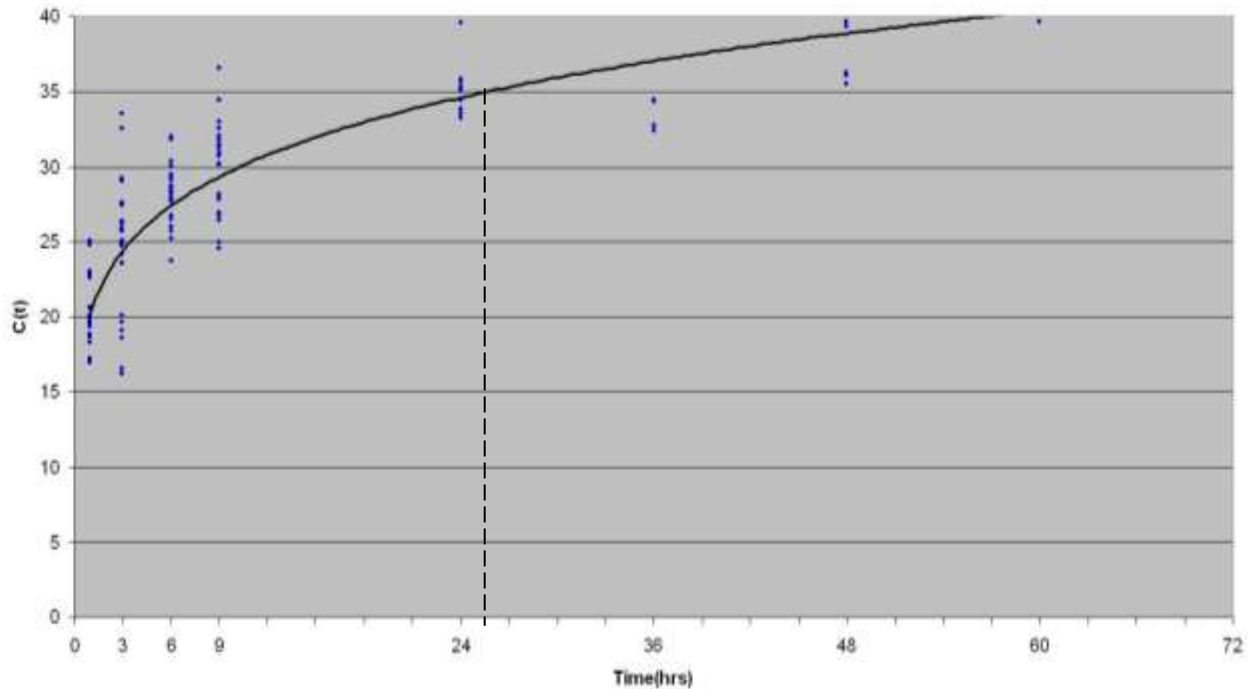

Figure 2. Detection of delta smelt DNA by PCR cycle and time post ingestion. PCR cycle number C(t) is on the y-axis and time post ingestion in hours is on the x-axis. Blue dots indicate the number of PCR cycles at which delta smelt DNA was detected in the stomach contents of individual Mississippi silversides, and the black line is the best fit to the data. The limit of detection is indicated by the vertical dotted line at the intercept of 35 PCR cycles and the curve fit to the data.

Field Sampling

The field study results show that there is a high incidence of delta smelt predation by Mississippi silversides in the Deepwater Ship Channel/Cache Slough area, but none in adjacent seine samples. Of the 37 stomachs analyzed from CDFG Kodiak trawls at sites 719-SKT, 719-SKT2, and 716-SKT2, 14 (37.5%) tested positive for the presence of delta smelt DNA (Figure 3). This represents a single day of the CDFG Kodiak trawl as there was no delta smelt DNA detected from day 2 of the CDFG Kodiak trawl (during which 7 total samples were collected). Of the more than 600 samples collected at beach seine stations adjacent to the deepwater ship channel, 1 Mississippi silverside and 1 Chinook salmon showed the presence of delta smelt DNA – a positive detection rate of 0.3% (Figure 3). An additional 210 samples from the Suisun Marsh, B&W Marina Mokelumne River, and North Fork Mokelumne were analyzed for the presence of delta smelt DNA and none was detected. These data suggest that Mississippi silversides are an effective open water predator of delta smelt. No delta smelt predation was observed among bass and sunfish populations but this result is inconclusive due to insufficient sample size.

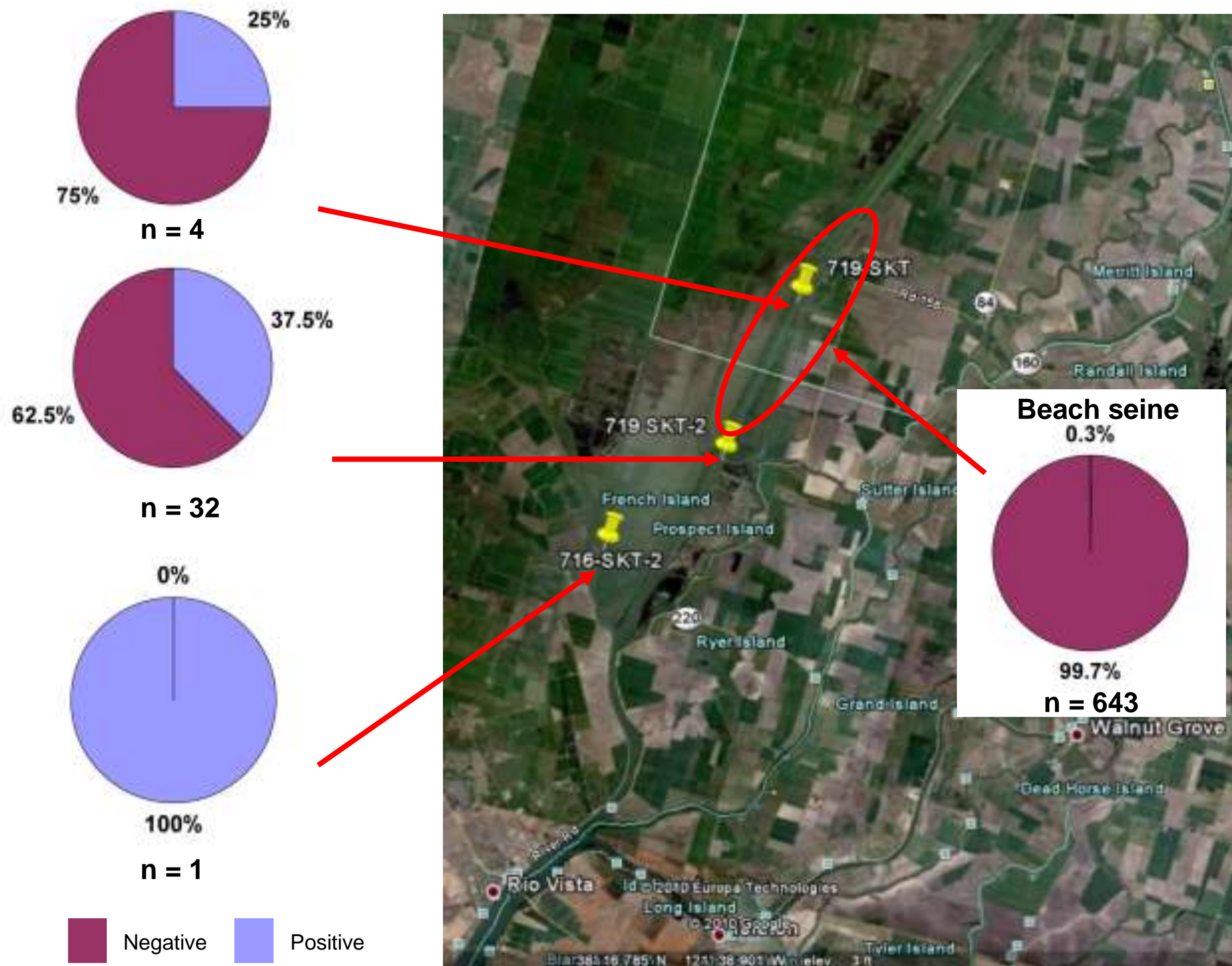

Figure 3. Number and percentage of Mississippi silverside samples from the deepwater ship channel testing positive for the presence of delta smelt, by location. Data for 719-SKT, 719 SKT-2, and 716-SKT-2 are from the CDFG Kodiak trawl

Conclusion

The study results demonstrate a low probability of the qPCR assay reporting false positives, and indicate that our qPCR based method is a reliable tool for detecting delta smelt predation when applied to field samples of Mississippi silversides. Our results also hint at potential patterns in larval delta smelt predation by Mississippi silversides. Of 643 silversides captured during beach seine sampling in April, 2010, only one tested positive for delta smelt DNA in its digestive tract. However, of the silversides sampled from the CDFG Kodiak trawl, 37.5% were positive for delta smelt DNA. Thus, nearly all of our positive detections of larval delta smelt predation occurred in the channel. These data suggest that Mississippi silversides are an effective open water predator of delta smelt.

In attempting to evaluate delta smelt predation by other species (e.g., striped bass, centrarchids), the principal limitation was the difficulty of capturing specimens, particularly in areas known to host spawning delta smelt. To address this, as well as to further assess delta smelt predation by

Mississippi silversides, CFS has proposed a study for 2011 which will build upon the successful elements of the study reported here and will provide an aggressive assessment of Mississippi silverside, striped bass and centrarchid predation of delta smelt in the north Sacramento-San Joaquin Delta.

References

Baerwald, M.R., G. Schumer, B. Schreier, and B. May. In review. TaqMan assays for the identification of delta smelt (*Hypomesus transpacificus*) and Wakasagi smelt (*Hypomesus nipponensis*).

Bennet, W.A. 2005. Critical assessment of the delta smelt population in the San Francisco Estuary, California. San Francisco Estuary and Watershed Science 3:1. Available online at http://repositories.cdlib.org/jmie/sfews/vol3/iss2/art1.

Braley, M.S., D. Goldsworthy, B. Page, M. Steer, and J.J. Austin. 2010. Assessing morphological and DNA-based diet analysis techniques in a generalist predator, the arrow squid *Nototodarus gouldi*. Molecular Ecology Resources 10:466-474.

King, R.A., D.S. Read, M. Traugott, and W.O.C. Symondson. 2008. Molecular analysis of predation: a review of best practice for DNA-based approaches. Molecular Ecology 17:947-963.

Schooley, J.D., A.P. Karam, B.R. Kesner, P.C. Marsh, C.A. Pacey, and D.J. Thornbrugh. 2008. Detection of larval remains after consumption by fishes. Transactions of the American Fisheries Society 137:1044-1049.

Sommer, T.R., C. Armor, R.D. Baxter, R. Breuer, L.R. Brown, M. Chotkowski, S. Culberson, F. Feyrer, M. Gingras, B. Herbold, W.J. Kimmerer, A. Mueller-Solger, M.L. Nobriga, and K. Souza. 2007. The collapse of pelagic fishes in the upper San Francisco Estuary. Fisheries 32:270-277.