



# Profiles of digestive enzymes of two competing planktivores, silver carp and gizzard shad, differ

Jon J. Amberg<sup>1</sup> · Nathan R. Jensen<sup>2</sup> · Richard A. Erickson<sup>1</sup> · Blake Sauey<sup>1</sup> · Craig Jackson<sup>1</sup>

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## Abstract

Typically, studies in digestive physiology in fish focus on a few enzymes and provide insight into the specific processes of the enzyme in a targeted species. Comparative studies assessing a wide number of digestive enzymes on fishes that compete for food resources are lacking, especially in the context of an introduced species. It is generally thought that the invasive silver carp (SVC; *Hypophthalmichthys molitrix*) directly compete for food resources with the native gizzard shad (GZS; *Dorosoma cepedianum*) in waters where they coexist. We compared 19 digestive enzymes between SVC and GZS throughout a year and in two rivers in the Midwestern United States: Illinois River and Wabash River. All digestive enzymes analyzed were detected in both SVC and GZS in both rivers. However, the profiles of the digestive enzymes varied by species. Alkaline phosphatase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl- $\beta$ -glucosaminidase were all much higher in SVC than in GZS. Differences between digestive enzyme profiles were also observed between rivers and months. This study demonstrates the utility of using an ecological approach to compare physiological features in fishes.

**Keywords** Asian carp · Silver carp · Digestive physiology

## Introduction

The study of digestive enzymes is vital to understanding the nutritional needs of an animal and can provide insight beyond the presence and activities of an enzyme. The presence and activities of digestive enzymes are key components of an organism's ability to break down food into the basic nutritional components required for maintenance, growth and health, and their presence is dependent on the presence of their substrate (Smith 1989). Typically, these studies in fish focus on a few enzymes (Dabrowski and Glogowski 1977; Bitterlich 1985; Das and Tripathi 1991; Infante and Cahu 1994; Cahu and Infante 1994; Hidalgo et al. 1999; Harpaz and Uni 1999; German et al. 2004) in a single species and provide insight into specific processes such as protein digestion. Comparative studies assessing a wide number of digestive enzymes on fishes that compete

for food resources are lacking, especially in the context of an introduced species.

Silver carp (SVC; *Hypophthalmichthys molitrix*) and gizzard shad (GZS; *Dorosoma cepedianum*) provide an ideal comparison for two fishes that compete for food resources in systems where they coexist (Irons et al. 2007; Sampson et al. 2008). SVC were imported into North America in the 1970s and subsequently escaped into the nearby Mississippi River. Their populations expanded throughout much of the Mississippi River drainage. Well-established populations are found in both the Illinois and Wabash Rivers. Filter-feeding adult SVC primarily feed on zooplankton and phytoplankton, which is a niche few adult native fishes in the Illinois and Wabash Rivers occupy. However, GZS is one species of fish native to this region that does feed on zooplankton and phytoplankton and stable isotope studies have indicated that they directly compete with SVC for food (Irons et al. 2007; Sampson et al. 2008). Both SVC and GZS lack true stomachs, but GZS do have a gizzard and cecae, whereas SVC do not. SVC have a significant impact on native fishes by sequestering essential nutrients, like fatty acids (Gutreuter et al. 2011). However, GZS have been found to feed on detritus, while SVC have not (Yako et al. 1996; Ye et al. 2014). Contributions from nutritional resources such as detritus,

✉ Jon J. Amberg  
jamberg@usgs.gov

<sup>1</sup> U.S. Geological Survey, 2630 Fanta Reed Rd, La Crosse, WI 54603, USA

<sup>2</sup> Fish and Wildlife, Kootenai Tribe of Idaho, PO BOX 1269, Bonners Ferry, ID 83805, USA

bacteria, and cyanobacteria are not typically well identified (or well characterized) through stable isotope analysis, but differences among these food resources may be observed though changes in gut enzyme composition and activities.

A better understanding of digestion and nutritional physiology in SVC is expected to improve our ability to predict their ecological effects. We compared 19 digestive enzymes between SVC and GZS on a temporal and spatial scale using a commercially available kit previously found to be useful in assessing digestive enzymes in mussels and other invertebrates (Sauey et al. 2015; Sauey et al. 2016). We hypothesize that the profile of digestive enzymes will differ among months and that some enzymes will be more active in SVC than in GZS. The objectives of this study were: (1) to determine if a commercially available kit can be used to assess the activities of 19 different digestive enzymes; (2) to characterize the digestive enzyme profiles of SVC and GZS; (3) to determine seasonal changes in digestive enzymes for both of these species; and (4) to compare digestive enzyme profiles between these two species captured from two separate rivers.

## Materials and methods

**Animals and sample collection.** To identify temporal changes in digestive enzyme activity, we captured six SVC and six GZS by electroshocking from the Illinois River near Havana, Illinois (40°18'26.22" N and 90°04'10.84"W) during both August and September of 2010 and each of March, May, and June of 2011. These fish were collected concurrently with those captured and analyzed for intestinal microbiome reported in Ye et al. (2014). To compare digestive enzyme activity between rivers within a species, we captured five SVC and six GZS by electroshocking from the Wabash River near Lafayette, Indiana (40°25'48.99" N and 86°93'49.55"W), in June two days after fish were captured from the Illinois River. We only sampled the Wabash River in June due to a malfunctioning of the electroshocking boat at a time similar to those when fish were captured from the Illinois River. We chose these two rivers because of the high abundance of both species and the consistent seasonal changes in hydrological conditions (USGS National Water Information System; <https://waterdata.usgs.gov/nwis>). Upon capture, all fish were immediately euthanized with Tricaine-S (Western Chemical, Inc., Ferndale, Washington, USA). The gastrointestinal tract (GIT) was removed from each fish and individual anterior gut contents were collected, flash-frozen, and stored at -80 °C until further processing. In this manuscript, we define the anterior gut in SVC as the section posterior to the duct connecting the gall bladder and anterior to the first distal loop, while the section posterior to the gizzard with caeca was considered the anterior gut in GZS.

**Digestive enzyme sample.** All GIT content samples were diluted with de-ionized water. Samples were individually homogenized with a Tissue Tearor™ (Cole-Palmer, Vernon Hills, IL, USA) and centrifuged at 13,000 × g for 10 minutes at 4 °C. Supernatants were retained for subsequent analysis and pellets were discarded. Total protein concentration of each supernatant was determined by BCA assay (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions at a wavelength of 562 nm on a MultiSkan Spectrum plate reader (Thermo Scientific, Rockford, IL, USA). All supernatants were then diluted with de-ionized water to a final total protein concentration of 1.0 µg µL<sup>-1</sup>. A buffer was not used because the commercial kit contains salts to buffer the enzymatic reactions.

**Validation of the screening assay.** A commercially available colorimetric kit for digestive enzymes (api<sup>®</sup>ZYM test kit, bioMérieux, Inc. Durham, NC) was validated with serially diluted intestinal contents. The wells of a strip were loaded with 6.00, 3.00, 1.50, 0.75, 0.38, or 0 µg total protein of the supernatant. One test strip was used for each concentration of total protein. Each strip was loaded with the supernatant and processed according to the manufacturer's instructions. Following incubation at 37 °C for 4 h, 60 µL of the contents from each well was individually transferred to a 96-well plate. Absorbance was measured by spectrophotometry at 400, 450, 500, 550, 600, and 650 nm using a MultiSkan Spectrum plate reader. Absorbance of each well for each protein concentration was adjusted for the blank at each respective wavelength. Standard curves were developed, and the wavelength that provided the best fit model ( $R^2 \geq 0.95$ ) was used for further analysis of each enzyme. Those enzymes that did not provide an appropriate model were only confirmed as present or absent. This process was repeated using the supernatant of a second SVC and GZS to verify that the appropriate wavelengths were chosen for analysis.

**Screening enzyme assay.** We assessed 19 digestive enzymes from individual fish using the api<sup>®</sup>ZYM test kit. For each fish, equal amounts of total protein (3.0 µg) were loaded into each of the 20 wells of a test strip. One test strip was used for each fish. Absorbance at the appropriate wavelength (determined during the validation process described above) was used to quantify the concentration of each specific enzyme using the equation  $(Abs_x - B)/C = \text{Quantity (a.u.)}$ , where  $Abs_x$  is the absorbance at wavelength x and B and C are values determined from the linear model at wavelength x (B is the y-intercept and C is the slope). This concentration is referred to as an arbitrary concentration (a.u.) and was used in all subsequent analysis.

**Statistical analysis.** Total length (TL), mass, and condition factor (K) of fish are expressed as mean values ( $\pm$  SD). K was the ratio between the mass of the fish and its length. Differences in K within a species among months

were determined using ANOVA and Tukey’s pairwise comparisons. A t-test was used to compare differences in condition factor between rivers. Only fish captured during June were used to compare between the Illinois and Wabash Rivers. These analyses were performed using R (Legendre and Anderson 1999) with a significance level of  $\alpha < 0.05$ .

To compare enzyme profile, we used multivariate analysis to examine if enzyme activities differed by species, month, and river. Specifically, a distance-based redundancy analysis (dbRDA) was used (Kuczynski et al. 2010). A type of multivariable regression, dbRDA, generalizes redundancy analysis (RDA) to allow for non-Euclidean dissimilarities using Bray–Curtis dissimilarity (Oksanen et al. 2015). Enzyme activities were the response variables, and species, year, and site were the predictor variables. The regression included interaction terms among all of the predictor variables. The capscale function from the vegan package (Oksanen et al. 2015) in R (R Core Team 2016) was used. Data was visualized using the ggplot2 package (Wickham 2009) in R.

## Results

**Validation of enzyme assay.** All 19 enzymes included in the api<sup>®</sup>ZYM test kit were present in both GZS and SVC from the Wabash River and Illinois River. We were able to generate positive response curves of each of the nine enzymes that was useful for semi-quantification. Enzymes that had responses which could be semi-quantified included according to the api<sup>®</sup>ZYM label were alkaline phosphatase (ALP), esterase (EST), valine arylamidase (VAL), cysteine arylamidase (CYS), trypsin (TRY), acid phosphatase (ACP), naphthol-AS-BI-phosphohydrolase (NAP),  $\alpha$ -glucosidase ( $\alpha$ GS), and N-acetyl- $\beta$ -glucosaminidase (NAG). All other enzymes could not be semi-quantified; therefore, we classified them as present or absent.

**Morphometric comparisons of fish.** *Temporal.* Differences in condition factor were observed in GZS among months ( $P = 0.001$ ,  $F = 6.276$ ,  $df = 4$ ). GZS captured in March had a lower condition factor than other months (Table 1). The condition factor increased from March ( $1.30 \pm 0.14$ ) to August and September ( $1.58 \pm 0.05$  and  $1.57 \pm 0.10$ , respectively). Differences were also observed in SVC condition factor among months ( $P = 0.021$ ,  $F = 3.517$ ,  $df = 4$ ). Pairwise comparisons only indicated that the condition factor in SVC captured in March was different from those captured in September.

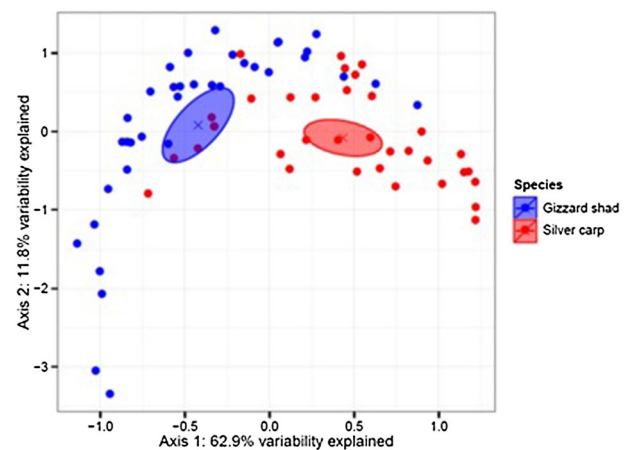
*Spatial.* No significant difference in condition factor was found between GZS captured from either the Illinois River or the Wabash River ( $P = 0.086$ ,  $t = 1.903$ ,  $df = 10$ ). Mean TL of GZS was  $246 \pm 45$  mm and  $229 \pm 45$  mm from the Wabash River and Illinois River, respectively. The mass of GZS was also similar between the Wabash River and Illinois

**Table 1** Percent variability explained from a distance-based redundancy analysis

Axis <sup>a</sup>	% Variability explained <sup>b</sup>	F-statistic	P-value
1	62.9	35.8	<0.001
2	11.8	6.69	<0.001
3	10.8	6.13	<0.001
4	5.8	3.28	0.005
5	3.8	2.14	0.044

<sup>a</sup>Axes 6 to 11 did not explain a significant amount of variability and are not included in the table

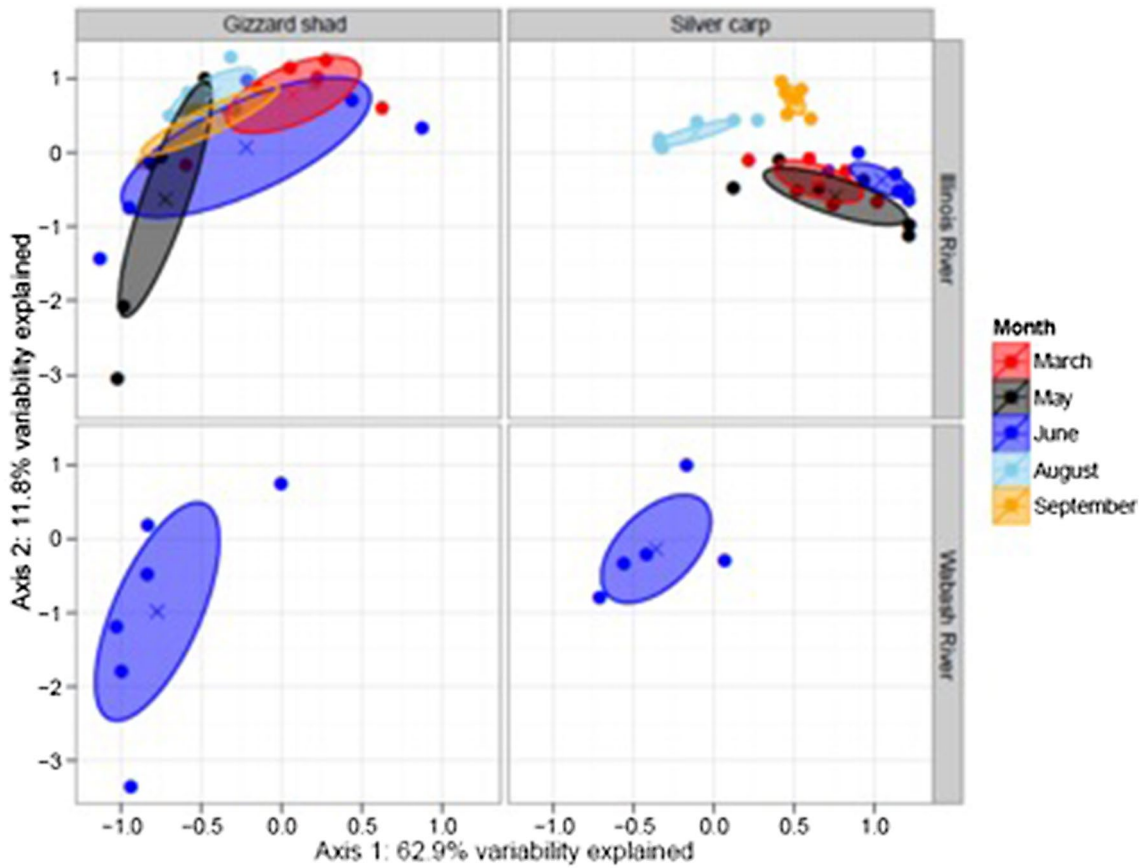
<sup>b</sup>Variability is the amount of variation in enzyme activities that can be reduced to fewer axes (or dimensions of data) while being constrained by species, month, and river



**Fig. 1** Graphical representation of the distance-based redundancy analysis for digestive enzymes in SVC (red) and GZS (blue). X symbols indicate the means for each species and centroids indicate 95% confidence intervals

River,  $217 \pm 126$  g and  $192 \pm 94$  g, respectively. There was no significant difference in the condition factor between SVC captured contemporaneously from both of these rivers ( $P = 0.573$ ,  $F = -0.585$ ,  $df = 9$ ). SVC from the Wabash River were  $474 \pm 125$  mm in TL and weighed  $1702 \pm 1153$  g, whereas SVC from the Illinois were  $454 \pm 125$  mm long and weighed  $1374 \pm 841$  g.

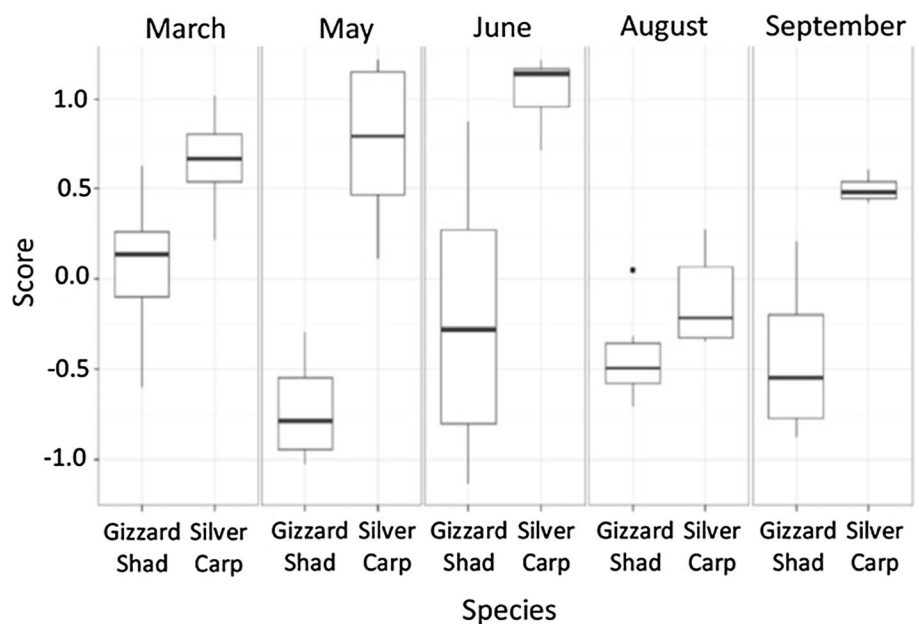
*Digestive enzyme richness and diversity.* Enzyme activities varied by species, month, and river and an interaction occurred among all three parameters ( $P = 0.001$ ,  $F = 5.176$ ,  $df = 11$ ). A clear separation between the species was observed (Fig. 1). Enzymes in both species varied by month (Figs. 2 and 3). Enzymes in SVC were similar to those in GZS during August (Fig. 2). Enzymes in SVC from the Illinois River differed from those of SVC captured from the Wabash River, whereas enzymes were similar in GZS for both rivers in June (Fig. 2). Overall, SVC had higher enzyme



**Fig. 2** Graphical representation of the distance-based redundancy analysis for digestive enzymes in SVC and GZS from the Illinois and Wabash Rivers during March, May, June, August, and September. *X*

symbols indicate the means for each species and centroids indicate 95% confidence intervals

**Fig. 3** Box plot of scores from the first axis of the distance-based redundancy analysis for digestive enzymes in SVC and GZS captured from the Illinois River in March, May, June, August, and September. *The horizontal line in the box represents the median. Boxes represent 25 to 75% of the data and the whiskers indicate 0 to 25% and 75 to 100% of the data. Dots indicate outliers*



activities than GZS, except for CYS (Figs. 3 and 4). The activity of 4 enzymes (NAP, ACP, NAG, VAL) was notably greater in SVC than in GZS (Fig. 4). NAP was prominent in SVC when compared to levels in GZS with NAG as the second most prominent enzyme (Fig. 4). The two phosphatases, ACP and ALP, were also greater in SVC when compared with levels observed in GZS (Fig. 4).

## Discussion

Our study was designed to determine if differences in digestive enzymes exist between two planktivorous fishes with dietary overlap, GZS and SVC. We were able to demonstrate that a commercial enzyme kit was useful for the determination of the presence or absence of 19 digestive enzymes in both species.

We determined that the activities of the digestive enzymes of SVC were different from those of GZS. These differences in enzyme activities could be related to the specific resources that the fish are consuming. SVC are known to directly compete with GZS for food resources (Irons et al. 2007; Sampson et al. 2008). SVC have significantly adapted to food resources in the Illinois River since their invasion in the 1990s (Irons et al. 2007; Sass et al. 2014) and it is plausible that SVC are now consuming cyanobacteria. In fish collected concurrently with these fish, Ye et al. (2014) found that SVC had high amounts of cyanobacteria within their anterior guts and few in their hindgut. GZS may have switched to detritus. Ye et al. (2014) found no cyanobacteria and high numbers of, and a high diversity of, microbes within their anterior guts. It has been reported that GZS will consume detritus when plankton is limited (Yako et al. 1996). Shifts such as these in diet may explain the differences in digestive enzymes between these two species. Our data, along with Ye et al. (2014), suggest SVC may be taking

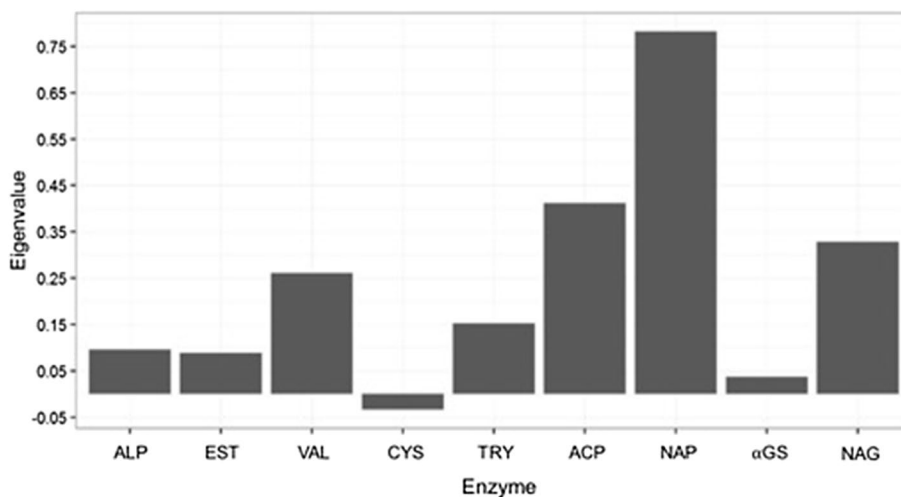
advantage of cyanobacteria as a food resource that GZS are not accessing.

The difference in primary productivity, and hence secondary productivity, between the two rivers studied likely has a role in the differences observed in enzyme activity/presence between SVC and GZS in these rivers. Headwaters of the Illinois River drain a highly urban area and receive a significant amount of treated wastewater from the City of Chicago, IL. Further downstream, the Illinois River watershed is dominated by crop agriculture and a series of navigation pools or impoundments. Thus, it is a very productive system that supports the greatest density of SVC in the world (Sass et al. 2009). The Wabash River is a free-flowing system without impoundments and drains agricultural fields. Similar to the Illinois River, the Wabash River is highly productive, but lacks impoundments for nutrient accumulation and cycling. Primary productivity in the Wabash River is likely more closely aligned with pulsed addition of key nutrients such as nitrogen and phosphorus associated with agriculture land runoff than with the nutrient cycling which likely occurs in the impounded sections of the Illinois River.

Phosphatases and proteases appear to be important groups of enzymes for SVC. The phosphatases NAP, ACP, and ALP were greater in SVC than in GZS, which may suggest that SVC have a higher requirement for phosphorus than GZS. Similar to the phosphatases, the proteases VAL and TRY were also greater in SVC. These elevated levels may be required by SVC to support their rapid growth rate (Williamson and Garvey 2005). Rapid growth would require greater amounts of phosphorus for the production of bone, and protein used in muscle. Unfortunately, the dietary requirements for SVC and GZS are unknown and should be further studied.

The differences in the activities of digestive enzymes (i.e. NAP, NAG, ACP, and VAL) suggest that the efficiency of digestion between SVC and GZS is dissimilar. The increased

**Fig. 4** Relative activity of individual digestive enzymes for SVC and GZS. Bars that are positive indicate greater activity in SVC, while negative bars indicate greater activity in GZS. The height of the bar indicates the significance of that enzyme



activity observed in SVC could be due to their rapid gut transit time (Opuszynski and Shireman 1991). The rapid evacuation rate of SVC would require quick digestion and rapid nutrient uptake of foodstuffs to maintain high growth rates (Williamson and Garvey 2005). Enteric microbes could also be responsible for the higher levels of certain digestive enzymes in SVC. Numerous microbes that may play a role in digestion have been found in the anterior gut of SVC (Ye et al. 2014).

## Conclusion

In summary, we found that the api<sup>®</sup>ZYM test kit was a valuable tool for uncovering trends in activities of 19 digestive enzymes in SVC and GZS. Enzyme activities differed temporally and spatially within each species. Our results suggest that SVC may feed on different food sources at different times relative to native planktivorous species, even if they occupy similar trophic positions. Our research suggests that SVC digestive enzymes such as trypsin and alkaline phosphatase, which vary significantly over time and in comparison to native GZS, represent valuable biochemical and physiological differences between two species known to have dietary overlap. It is important to continue gathering information about seasonal digestive enzyme profiles of invasive SVC relative to those in native species to provide insight into the magnitude of overlap between their diets. This is important if management actions to reduce SVC populations allow GZS to shift back to other resources.

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