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Liver Glycogen as a Sensitive Indicator of Food Limitation in Delta Smelt

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Abstract

Assessing habitat quality is a major goal of conservationists and restoration practitioners, but to associate habitat quality with biomarkers of vagile animals, the biomarkers must respond rapidly. Here, we identified a biomarker capable of rapidly detecting food limitation in the imperiled Delta Smelt (*Hypomesus transpacificus*), a pelagic fish endemic to the San Francisco Estuary (SFE). We conducted an experiment with fed and unfed treatments of hatchery-raised, sub-adult Delta Smelt that were sampled at 12 time points: 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, and 21 days. We then compared four biomarkers using Day 21 fish: RNA/DNA in liver, triglycerides in liver, glycogen in liver, and glycogen in muscle. Of the liver endpoints, glycogen had the largest difference between treatments at Day 21, so we compared it to muscle glycogen across all time points. Liver glycogen declined by 60% after 1 day of fasting and remained depressed in the fasting treatment across all subsequent time points. Muscle glycogen also responded rapidly, taking only 2 days to decline by 39% in the fasting treatment, but the difference was inconsistent across subsequent time points. When applied to hatchery-released Delta Smelt collected from the SFE, liver glycogen concentrations were less than half that of the fed hatchery fish, consistent with the hypothesis of food limitation in the wild, but also several other potential causes. This study highlights the utility of liver glycogen as an indicator of recent foraging success in Delta Smelt.

Keywords Fish · Fasting · Biochemistry · Energy reserves · Biomarker

Introduction

Many fishes experience periods of food limitation due to declines in food supply, high water temperature, estivation, or migration. In some cases, individuals do not survive these periods, resulting in population declines (Theilacker 1986; Hurst 2007; Le Pape and Bonhommeau 2015; Saulnier et al. 2020). Therefore, considerable interest exists in detecting food limitation in wild fish populations, particularly in commercial or imperiled species. However, due to differences in physiology and life history strategies, fish have evolved species-specific responses to food limitation (e.g., mobilization of preferred energy stores) which can change based on environmental factors, making them difficult to predict even within a species (Valtonen 1974; Coban and Sen 2011; Vornanen et al. 2011; Liew et al. 2012; Jiao et al. 2020). For example, although most fishes utilize carbohydrates as a primary source of energy during initial stages of food limitation, Goldfish prioritize protein metabolism (Storer 1967; Liew et al. 2012), while Mudskippers initially metabolize lipids (Lim and Ip 1989). Variation in the temperature

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among fasting studies adds further complexity, as biomarkers of food limitation respond more quickly at higher temperatures (Brown et al. 2004; Volkoff and Rønnestad 2020; Pham et al. 2022). Given the large variation in the responses of fish to fasting and among fasting studies, identifying the initial responses to food limitation and their timings necessitates species and temperature-specific experiments.

A variety of indicators are used to assess nutritional condition in fish, at scales ranging from molecular to whole body. RNA/DNA is a proxy for recent growth because RNA concentration increases with increasing protein synthesis, while DNA concentration remains constant. RNA/DNA can respond to changes in feeding and growth within 1–3 days (Bulow 1970; Buckley et al. 1999; Yandi and Altinok 2018) and is widely used to assess recent nutritional condition, and therefore habitat quality (e.g., Tanaka et al. 2008). While RNA/DNA is typically measured in muscle, it can also be measured in the liver. For example, liver RNA/DNA decreased within 2 weeks of fasting in juvenile Copper Rockfish (12 °C; Hack et al. 2019) and after 3 weeks of fasting in Zebrafish (28 °C; Fan et al. 2019). More recent methods quantify the expression of genes related to growth or energy metabolism to evaluate nutritional stress. For example, the expression of insulin was reduced after 3 days of fasting in Yangtze Sturgeon (Zhang et al. 2022), and transcriptional activity of genes controlling lipid metabolism differed between fasted and fed European Sea Bass after 15 days (Rimoldi et al. 2016).

Quantifying energy stores like carbohydrates and lipids can also provide valuable insight into nutritional status. Glycogen, a major form of carbohydrate storage in fishes, can be a sensitive biomarker to food limitation (Navarro and Gutiérrez 1995; Hemre et al. 2002; Furné et al. 2012). The main sites of glycogen storage are the liver and skeletal muscle; the liver maintains blood glucose levels through glycogenolysis and muscle glycogen serves as a form of local energy (Rossi et al. 2015; Soengas and Aldegunde 2002). Some fishes utilize liver glycogen over muscle glycogen during periods of food limitation (Barcellos et al. 2010; Navarro et al. 1992), while some favor muscle glycogen (Lim and Ip 1989), and others utilize both simultaneously (Black and Love 1986; Mehner and Weiser 1994). Triglycerides are the most readily available lipid reserve, with studies showing increased plasma triglyceride levels following 1–3 days of fasting in European Sea Bass and Rainbow Trout (22–24 °C; Pérez-Jiménez et al. 2007; Bermejo-Poza et al. 2020). Triglycerides and glycogen in the liver can both respond quickly to food limitation, with lower concentrations of both energy stores observed in Nile Tilapia after just 1 day of fasting at 30 °C (Wang et al. 2019).

Condition factor, hepatosomatic index, and stomach fullness are widely used gravimetric indicators of nutritional status. Condition factor reflects the "plumpness" of a fish and is sensitive to fasting because weight generally responds to fasting more readily than length (Weatherley and Gill 1981; Hvas et al. 2021). The liver is especially sensitive to fasting, responding through glycogen depletion, hepatocyte atrophy, mitochondrial enlargement, and necrosis (Weis 1972; Storch and Juario 1983; Panserat et al. 2019). These responses result in a faster decrease in hepatosomatic index, as measured by the liver weight relative to the body weight, compared to condition factor. For example, differences between fed and unfed juvenile Nile Tilapia were detected after 2 weeks using hepatosomatic index, while condition factor took 3 weeks to respond (25 °C; Abdel-Tawwab et al. 2006). Stomach fullness, or the ratio of stomach content weight to fish weight, quantifies recent foraging success and has been widely used to study feeding habits and diet composition (Nemerson and Able 2004; Amundsen and Sánchez-Hernández 2019; Hedden et al. 2022). However, stomach fullness is best paired with other biomarkers because it can overestimate the importance of slowly digested prey or indigestible remains, and it provides an instantaneous snapshot of food consumption that may not represent overall nutritional status of the fish (Hyslop 1980; Amundsen and Sánchez-Hernández 2019).

One fish for which biomarkers sensitive to food limitation have yet to be validated is the Delta Smelt, *Hypomesus transpacificus*—a small, pelagic, imperiled species endemic to the San Francisco Estuary (SFE). The SFE is formed by the confluence of the Sacramento and San Joaquin rivers and the Pacific Ocean in California, USA. Despite high nutrient concentrations in the SFE, phytoplankton, zooplankton, and pelagic fish, including the Delta Smelt, have all exhibited similarly timed, negative exponential declines in abundance beginning in the early 1970s (Hammock et al. 2019a). The loss of pelagic primary productivity and resultant prey scarcity is one of the factors implicated in the decline of pelagic fish (e.g., Feyrer et al. 2003; Sommer et al. 2007).

A recent study by Hammock et al. (2020) compared the sensitivities—defined as the time taken for a biomarker to respond to a stressor at a constant water temperature of 16 °C—of many biomarkers of food limitation in Delta Smelt, including condition factor, hepatosomatic index, RNA/DNA in muscle, triglycerides in muscle, and histopathologic responses in the liver. Hepatosomatic index was the most sensitive measure of food limitation examined, declining significantly after 4 days of fasting, followed by condition factor at 7 days. This was the rationale for subsequently modeling hepatosomatic index and condition factor of Delta Smelt as a function of environmental variables such as water temperature, salinity, and zooplankton abundance (Hammock et al. 2021). However, Delta Smelt could conceivably swim a considerable distance in 4 days given their estimated swimming speed of 0.72 km/h (Swanson et al. 1998), especially if aided by currents (Bennett and Burau 2015). Thus, even the most sensitive biomarkers give

individuals time to move among habitats of varying quality, obscuring the relationship between the level of hepatosomatic index or condition factor and the point of collection. Moreover, the two biochemical biomarkers examined in the muscle by Hammock et al. (2020) were particularly insensitive, with responses to fasting first occurring after 28 days for RNA/DNA and 14 days for triglycerides, and inconsistently thereafter.

The present study is divided into three parts. First, we ran an experiment similar to that of Hammock et al. (2020) in which Delta Smelt were either fasted or fed. We then compared the responses of four biochemical biomarkers to fasting: RNA/DNA in liver, triglycerides in liver, glycogen in liver, and glycogen in dorsal muscle. Given the insensitivity of RNA/DNA and triglycerides in dorsal muscle observed in Hammock et al. (2020), our aim was to identify biomarkers that are highly sensitive to fasting in Delta Smelt for use in comparing habitats from which Delta Smelt are collected. Second, we summarized the work to date on biomarkers of food limitation for Delta Smelt in terms of their sensitivities and dynamic ranges (dynamic range is the extent to which a biomarker responds linearly to a stressor). Finally, we applied the most sensitive of the four biomarkers to hatchery-raised Delta Smelt that were released into the wild (hereafter Supplemental Delta Smelt) and eventually recaptured. In the future, applying highly sensitive biomarkers to Delta Smelt collected from the wild will provide insight into habitat suitability, which will assist in restoration and conservation efforts.

Materials and Methods

Fasting Experiment

The fasting experiment was conducted at the UC Davis Fish Conservation and Culture Laboratory (FCCL) near Byron, CA, USA. Inasmuch as possible, this experiment followed Hammock et al. (2020; i.e., the same building, tanks, feed, feeding rates, life stage, water temperature, etc.). On Sept 16, 2020, 800 sub-adult Delta Smelt were divided equally among eight, circular black tanks with working volumes of 290 L (100 fish/tank). The fish were given 2 weeks to acclimate to their new surroundings. During this period, fish in all tanks were fed to satiation following standard FCCL feeding protocol with Bio-vita Crum #1 (Bio-Oregon, Longview, WA). Five-micron particle filters were placed on the water inlets to each tank to eliminate any potential food in the inflow. Filters were changed weekly during the acclimation period and experiment to maintain sufficient flow through the filters, which slowly clogged with particles through time. After the acclimation period ended on Oct 1, we randomly assigned four tanks to the "No Feeding" treatment, and four to the "Feeding" treatment. The "No Feeding" treatment tanks were provided no food for the duration of the experiment. The "Feeding" treatment tanks were fed to satiation as usual. Fish were 149 days post-hatch (dph) when the treatments were imposed (in Hammock et al. (2020), the fish were 157 dph when fasting began). Fish were sampled between ~ 10:30 am and 12:30 pm on the following time points: Day 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, and 21. We prioritized sampling early time points to focus on indicators of mild food limitation, as our previous study was successful in identifying several indicators of moderate to severe food limitation which were observed at later time points (Hammock et al. 2020). We ended the experiment at 21 days to avoid inflicting starvation-induced mortality, which began after 21 days in Hammock et al. (2020).

Test replicates were observed daily, mortalities were removed when present, and only live fish were sampled and analyzed for biomarkers. Water quality was measured approximately every 3 days throughout the experiment. Parameters included dissolved oxygen, salinity, pH, total ammonia–nitrogen, nitrite, and nitrate. Water temperature was measured hourly using HOBO temperature loggers placed in the tanks (Onset, MA, USA). Three fish from each tank were sampled on Day 0 of the experiment. Five fish were sampled from each tank for the remainder of the time points, except for Day 21, on which 15 fish were sampled from each tank. Thus, 68 fish were sampled from each tank, and 544 fish were sampled in total. Sampled fish were caught with an aquarium net, euthanized with an overdose of buffered Tricaine methanesulfonate (MS-222), blotted dry on a paper towel, wrapped in aluminum foil, and flash-frozen in liquid nitrogen. The study protocol was approved on May 14, 2020, by the University of California, Davis, Institutional Animal Care and Use Committee (protocol # 21,737).

Application to Supplemental Delta Smelt

From Dec 2021 through Feb 2022, more than fifty thousand adult Delta Smelt, raised at FCCL, were released into the SFE to evaluate survival, distribution, and reproductive success for future supplementation to the wild population (Hung et al. 2022). From Dec 2021 through Mar 2022, 75 of these fish were recaptured during routine agency fish monitoring trawls and confirmed to be of hatchery origin based on adipose fin clips. Of the 75 Supplemental fish, 69 were flash-frozen immediately following collection, but the other 6 were kept live in buckets for several hours until they could be flash-frozen. We note the Supplemental fish were not euthanized with MS-222 prior to flash freezing, unlike the fish from the fasting experiment. All 75 Supplemental fish were transported to UC Davis in liquid nitrogen for analysis.

Sample Processing

We dissected 619 Delta Smelt (544 experimental fish and 75 Supplemental fish) following a similar protocol to Teh et al. (2016). Briefly, Delta Smelt were removed from liquid nitrogen, photographed, measured for fork length, and weighed on an analytical balance (± 0.01 mg). Liver and dorsal muscle were excised as the fish thawed over 5–10 min, weighed on an analytical balance (± 0.01 mg), and then again flash-frozen in liquid nitrogen for storage. Tissues were stored at − 80 °C until processing for biochemistry. For Supplemental fish, gonads were also excised during the dissection process, weighed on an analytical balance, and fixed in 10% phosphate-buffered formalin. They were then sectioned using a microtome and stained with eosin and hematoxylin for histopathological assessment of sex and sexual maturity.

Bioassays for Experimental Fish

The four bioassays conducted on experimental fish were RNA/DNA in liver, triglycerides in liver, glycogen in liver, and glycogen in dorsal muscle. We focused on biochemical responses in the liver, the center of many metabolic processes. However, muscle glycogen was of particular interest because Delta Smelt have small livers, which are useful for histopathology (e.g., Teh et al. 2020). Liver histopathology can leave little to no tissue for biochemical assays, especially for younger life stages. The body weight of Delta Smelt from our experiment was small (mean 0.4 g), so we had insufficient tissue to run all four assays for every time point on individual fish. Therefore, we identified the most promising bioassays by initially comparing each bioassay endpoint using Day 21 fish, making the assumption that the assays showing the lowest *P*-values on Day 21 would likely be the most sensitive to fasting. For the 21-day time point, liver and muscle samples from five fish per tank were pooled, resulting in three different samples from each of the eight tanks (i.e., 12 pooled samples of 5 fish each for 60 fish of each treatment). The four assays were run on the pooled samples, with liver glycogen having the lowest *P*-value between the two treatments of the three liver assays. We therefore ran glycogen bioassays on liver for the remaining time points. Although the muscle glycogen endpoint was not as promising as liver glycogen based on the Day 21 results, we ran it for all time points as tissue limitation was not an issue.

Liver RNA/DNA

RNA/DNA in liver was measured using the ethidium bromide fluorometric technique reported by Caldarone et al. (2001). Samples were evaluated using a microplate reader (Tecan Infinite M200).

Liver Triglycerides

We measured liver triglyceride concentration using an adipogenesis assay kit (Catalog #K610-100, Biovision, CA, USA), as per the manufacturer's instructions and standardized to protein concentration that we determined following Lowry et al. (1951). Samples were evaluated using a microplate reader (Tecan Infinite M200). Triglyceride concentration is reported in nmol of triglyceride per mg of protein.

Liver and Muscle Glycogen

Muscle glycogen was measured for all experimental Delta Smelt, while liver glycogen was measured for all experimental and Supplemental Delta Smelt. Muscle and liver tissue were homogenized in ice-cold Tris–EDTA buffer (5 mM Tris–HCl, 0.5 mM EDTA, pH 7.5) to reach a homogenate concentration of 1 mg tissue/20 µL Tris–EDTA buffer. Homogenates were then boiled at 100 °C for 10 min to denature enzymes that could alter glycogen concentrations. Homogenates were centrifuged and the supernatants collected and stored at − 80 °C until glycogen measurement. Glycogen concentration was measured following the colorimetric method reported by Roehrig and Allred (1974) with modifications to reagent quantities, incubation temperature, and incubation length. Briefly, 10 µL of homogenate was incubated with 7 units amyloglucosidase in 0.05 M sodium acetate buffer pH 4.5 (Sigma-Aldrich #10,115) at 60 °C for 30 min. Samples were then incubated in 1 unit glucose oxidase (Sigma-Aldrich #G7141), 2.5 purpurogallin units peroxidase (Sigma-Aldrich #P6782), and 0.125 mg o-dianisidine (Spectrum # TCI-D3864) in 0.1 M sodium phosphate buffer pH 6 at 37 °C for 30 min and read on a spectrophotometer at 500 nm (Tecan Infinite M200). We used aliquots of 0–7 µg of bovine liver glycogen (Sigma-Aldrich # G0885) and D-(+)-Glucose (Sigma-Aldrich # G8270) to develop a standard curve. Samples were run in duplicate when sufficient homogenate was available. Glycogen is reported in µg of glycogen per mg of tissue.

Statistical Analyses

Day 21: Liver RNA/DNA, Liver Triglycerides, Liver Glycogen, and Muscle Glycogen

The measurements from Day 21 fish were analyzed with four ANOVAs, one for each of the four bioassays. Each ANOVA included two predictors: *treatment* (Feeding and No Feeding) and *tank* (tanks 1 through 8). *Tank* was included as a random effect to account for the multiple measurements from the same tank. The liver and muscle glycogen variables were $\log_{10}$-transformed to address

heterogeneity of variance (i.e., far greater variance in the Feeding than the No Feeding treatment).

Day 1–21: LIVER and Muscle Glycogen

Separate factorial ANOVAs were performed on the liver and muscle glycogen results following Hammock et al. (2020). For both ANOVAs, predictors included *day*, *treatment*, a *day* by *treatment* interaction, *body weight*, a *body weight* by *treatment* interaction, and *tank* as a random effect. The *day* by *treatment* interaction was to account for any changing influence of treatment during the experiment. That is, we expected the influence of fasting to increase as the experiment progressed, from no treatment effect at Day 0 to a strong treatment effect by Day 21. The body weight of individuals was included as a predictor to test whether larger fish had more glycogen-rich tissue. The *body weight* by *treatment* interaction tested the possibility that fish size had less influence on glycogen concentration in the No Feeding treatment (i.e., pervasive glycogen depletion, regardless of fish size). Interactions between *day* and *treatment* were deconstructed using planned linear contrasts (i.e., "test slices" in JMP at each time point). Both liver and muscle glycogen were $\log_{10}$-transformed to account for the heterogeneity of variance apparent in plots of the residuals (higher variance in the Feeding treatment).

Application to Supplemental Delta Smelt

We were interested in comparing the Supplemental Delta Smelt to the fed and fasted experimental Delta Smelt to assess if the Supplemental fish appeared to be receiving sufficient nutrition in the wild. Therefore, the liver glycogen concentrations of fish from the Feeding and No Feeding treatments and Supplemental Delta Smelt were compared with an ANOVA. Because liver glycogen was stable from Day 1 to 14 and then appeared to decline from Day 14 to 21, the No Feeding treatment was divided into Day 1–14 and Day 21. Day 0 fish, from before the treatments were imposed, were excluded from the analysis. To account for the potential loss of liver glycogen while the Supplemental fish were held in buckets for several hours, we analyzed these six "bucket" fish separately from the other Supplemental Delta Smelt. Thus, there were five treatments: Feeding (Day 1–21), No Feeding (Day 1–14), No Feeding (Day 21), Supplemental, and Bucket. The predictors included *group* (the five treatments) and *body weight*. Liver glycogen was $\log_{10}$-transformed to account for the heterogeneity of variance between groups, as above. In our preliminary analysis, we found no clear patterns in liver glycogen across characteristics such as sex, sexual maturity, and age, indicating that comparing the Supplemental fish, which were released as adults, to the sub-adults from our experiment is reasonable. However, given larger sample size or age range, this could change, as is seen in other species (Chang and Idler 1960; Valtonen 1974; Ng et al. 1986; Coban and Sen 2011). All analyses were performed in JMP Pro 16.

Results

Water Quality

Water quality was maintained throughout the experiment at standard levels used by the FCCL to culture Delta Smelt (i.e., freshwater, 16 °C; Table 1). Water quality measured during the collection of Supplemental Delta Smelt was far more variable, with water temperature ranging from 8.5 to 16.5 °C and salinity from 0.06 to 3.1 (Table 1). Dissolved oxygen was sufficient for supporting fish and similar between the two groups (Table 1).

Mortality

In the present study, mortality rates were 18% and 11% for the Feeding and No Feeding treatments at termination on Day 21 (excluding the acclimation period). In Hammock et al. (2020), mortality rates were ~ 20% for both the Feeding and No Feeding treatments on Day 21, at which point

Table 1 Water quality from the fasting experiment and recapture of Supplemental Delta Smelt

	Experimental				Supplemental			
Water quality parameter	Mean	SD	Min	Max	Mean	SD	Min	Max
Temperature (°C)	15.9	0.20	15.5	16.3	11.9	2.25	8.5	16.5
Salinity	0.31	0.06	0.30	0.60	0.80	0.90	0.06	3.05
Dissolved oxygen (mg/L)	9.82	0.22	9.58	10.5	10.1	0.69	8.32	11.0
pH	8.13	0.07	8.02	8.24				
Total ammonia–nitrogen (mg/L)	0.02	0.02	0.00	0.06				
Nitrite (mg/L)	0.01	0.02	0.00	0.06				
Nitrate (mg/L)	0.92	0.32	0.24	1.41				

mortality began increasing in the No Feeding treatment above control rates. Thus, mortality rates were somewhat lower than our previous experiment, and we avoided inducing mortality due to starvation.

Day 21: Liver RNA/DNA, Liver Triglycerides, Liver Glycogen, and Muscle Glycogen

On Day 21, the sample means of all four biomarkers were higher in the Feeding treatment (Fig. 1). Specifically, liver RNA/DNA was higher in the Feeding treatment by 2.1-fold (ANOVA, $F_{1,\ 6} = 30.57$, $P = 0.0015$, Fig. 1a). Liver triglycerides (nmol/mg protein) were higher in the Feeding treatment by 2.2-fold (ANOVA, $F_{1,\ 6} = 19.95$, $P = 0.0043$, Fig. 1b). Liver glycogen (µg glycogen/mg tissue) was 4.9-fold higher in the Feeding treatment (ANOVA, $F_{1,\ 6} = 60.52$, $P = 0.0002$, Fig. 1c). Finally, muscle glycogen (µg glycogen/mg tissue) was 1.4-fold higher in the Feeding treatment, although not significantly so (ANOVA, $F_{1,\ 6} = 4.2498$, $P = 0.0865$, Fig. 1d). Thus, liver glycogen showed the largest difference at the final time point and the lowest *P*-value, and was therefore considered the most promising of the liver endpoints as a sensitive biomarker of fasting.

Day 0–21: Liver and Muscle Glycogen

For liver glycogen, there was an interaction between *day* and *treatment* (ANOVA, $F_{11,\ 379.3} = 2.45$, $P < 0.0057$), as the influence of the No Feeding treatment increased as the experiment progressed (Fig. 2a). In the No Feeding treatment, liver glycogen declined rapidly from Day 0 to Day 1, was fairly stable from Day 1 through Day 14, and then declined again on Day 21 (Fig. 2a). In contrast, in the Feeding treatment, mean liver glycogen was consistently higher through time, did not show a clear negative or positive trend, and varied widely. Based on the series of linear contrasts, *treatment* (Feeding and No Feeding) became significant on Day 1 (linear contrast, $P = 0.0002$) and stayed significant for the remainder of the experiment (Fig. 2a). Liver glycogen concentrations below ~ 8 µg glycogen/mg tissue indicate moderate food limitation in sub-adult, hatchery-raised Delta Smelt, as demonstrated by sample means from the No Feeding treatment on Days 1–14 (range 4.7–7.6). A concentration below 2.5 µg glycogen/mg tissue indicates more severe starvation, as seen on Day 21. There was also a significant interaction between *body weight* and *treatment* (ANOVA, $F_{1,380.6} = 14.01$, $P = 0.0002$). Liver glycogen increased strongly with body weight in the Feeding treatment, but body weight had little to no influence on liver glycogen in the No Feeding treatment (Fig. 3).

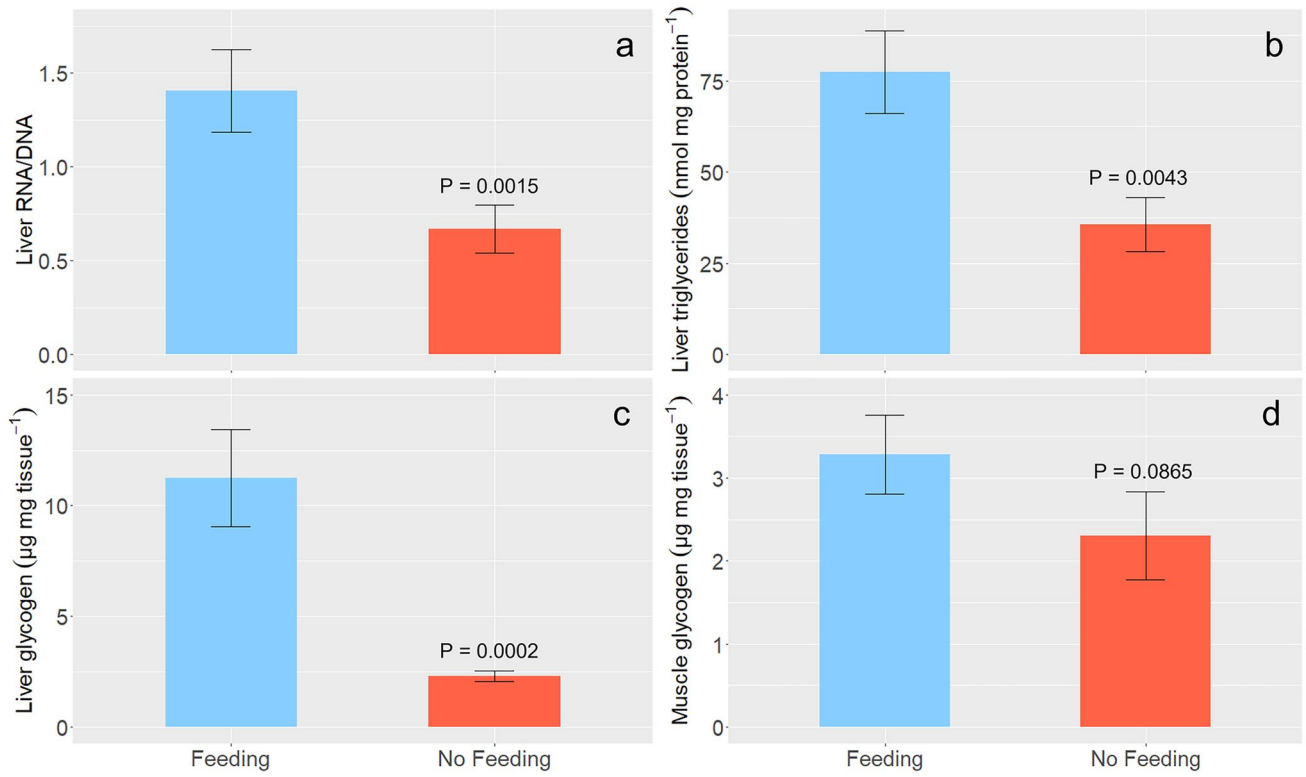

Fig. 1 Mean liver RNA/DNA (**a**), liver triglycerides (**b**), liver glycogen (**c**), and muscle glycogen (**d**) for Delta Smelt from the Feeding and No Feeding treatments on Day 21. Error bars are ± SE

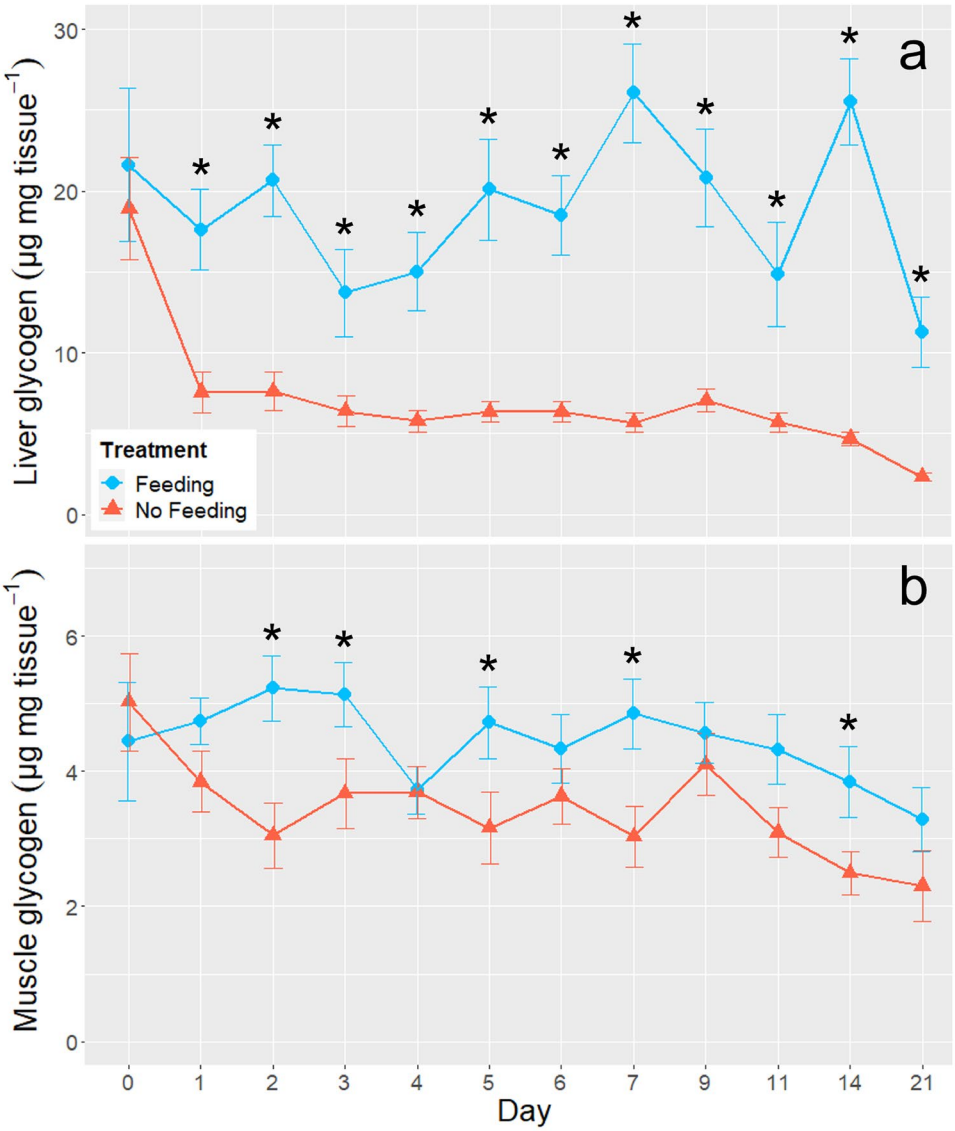

Fig. 2 Mean liver (**a**) and muscle (**b**) glycogen concentration ± SE in the Feeding and No Feeding treatments through time. The Feeding treatment is shown in blue circles, while the No Feeding treatment is shown in red triangles. Significant differences, based on linear contrasts, are indicated by asterisks. The *x*-axis is not to scale

Muscle glycogen was lower overall in the No Feeding than in the Feeding treatment (ANOVA, $F_{1,\ 6.7} = 19.27$, $P = 0.0035$), but there was not a significant interaction between *day* and *treatment* (Feeding vs No Feeding; ANOVA, $F_{11,\ 397.5} = 1.27$, $P = 0.24$). The linear contrasts showed that the treatment differences were inconsistent through time, with a significant influence of treatment on Days 2, 3, 5, 7, and 14, but non-significant differences on Days 0, 1, 4, 6, 9, 11, and 21 (Fig. 2). In contrast to liver glycogen, there was no interaction between body weight and treatment (ANOVA, $F_{1,\ 401.4} = 0.38$, $P = 0.54$).

Comparing Biomarkers of Food Limitation

As demonstrated in the current study, biochemically measured liver glycogen is a highly sensitive biomarker of food limitation, but it has a relatively narrow dynamic range. That is, it responded rapidly to fasting, but it stayed fairly constant from Days 1 through 14, making it of little use distinguishing between mild and moderate food limitation (Figs. 2 and 4). However, Hammock et al. (2020) and Lewis et al. (In progress) have identified biomarkers with wider dynamic ranges. For instance, condition factor combined the widest dynamic range—Day 7 through 56—with the third best sensitivity (7 days; Fig. 4). Hepatosomatic index also provided a valuable combination of sensitivity (4 days) and dynamic range (Day 4–21). The histological biomarkers in liver (i.e., hepatocyte area, single cell necrosis, autophagosomes, and glycogen depletion) were most effective for detecting moderate to severe starvation. Otolith-based growth reconstructions could also be used to detect periods of moderate to severe starvation (Lewis et al. 2021). This tool is used retrospectively, meaning that a fish could be sampled at 56 days into the fasting experiment,

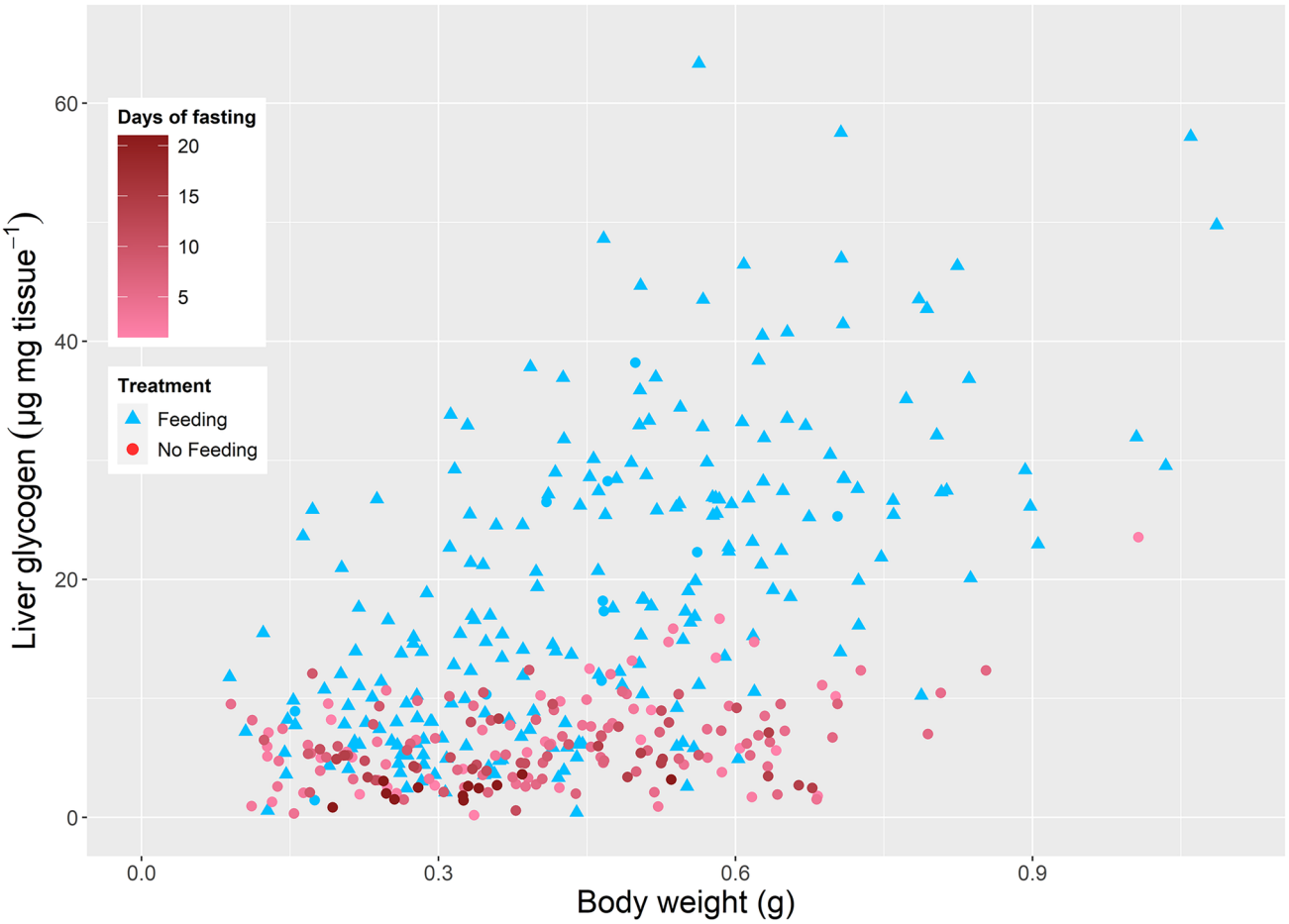

Fig. 3 Interaction between body weight and treatment (Feeding and No Feeding) on liver glycogen concentration. The Feeding treatment is shown as blue triangles and the No Feeding treatment is shown as red circles. Fish sampled on Day 0 from the No Feeding tanks are shown as blue circles because they were recently fed when sampled (i.e., treatments had not yet been imposed). Days of fasting are represented by the shade of red, with the shade darkening as the length of fast increases

with daily growth histories constructed for its entire life. This approach detected up to a 40% decline in growth due to fasting that was detected within 11 days after fasting began (Lewis et al. In progress).

Application to Supplemental Delta Smelt

Liver glycogen concentrations of the Supplemental fish were significantly lower than the Feeding treatment fish (ANOVA, $F_{4,\ 135.5} = 70.5$, $P < 0.0001$, Fig. 5), with an average liver glycogen concentration less than half that of the Feeding fish. The Supplemental fish were also statistically indistinguishable from both the No Feeding (Days 1–14) and the No Feeding (Day 21) fish (Fig. 5, $P = 0.3706$ and 0.3476, respectively). The mean liver glycogen concentration of the Bucket fish was roughly half that of the Supplemental fish, although the difference was not significant (Fig. 5, $P = 0.6645$).

Discussion

Given the relative insensitivity of biomarkers currently used to detect food limitation in Delta Smelt, our goal was to identify more sensitive biomarkers that could be used to better assess local habitat quality. In fishes, there exist three common metabolic strategies during periods of food limitation: rapid glycogen depletion, partial protection of glycogen reserves (i.e., glycogen preserved rather than metabolized), and complete protection of glycogen reserves (Sheridan and Mommsen 1991; Soengas et al. 1996; Pérez-Jiménez et al. 2007). In Delta Smelt, liver glycogen was rapidly depleted initially with the concentration dropping 60% from Day 0 to Day 1 in the No Feeding treatment. This is consistent with other studies that report liver glycogen depletion in various fishes in as little as 1 day (Mehner and Wieser 1994; Soengas et al. 1996; Rossi et al. 2015; Wang et al. 2019). However, from Day 1 through 14, sub-adult Delta Smelt partially protected

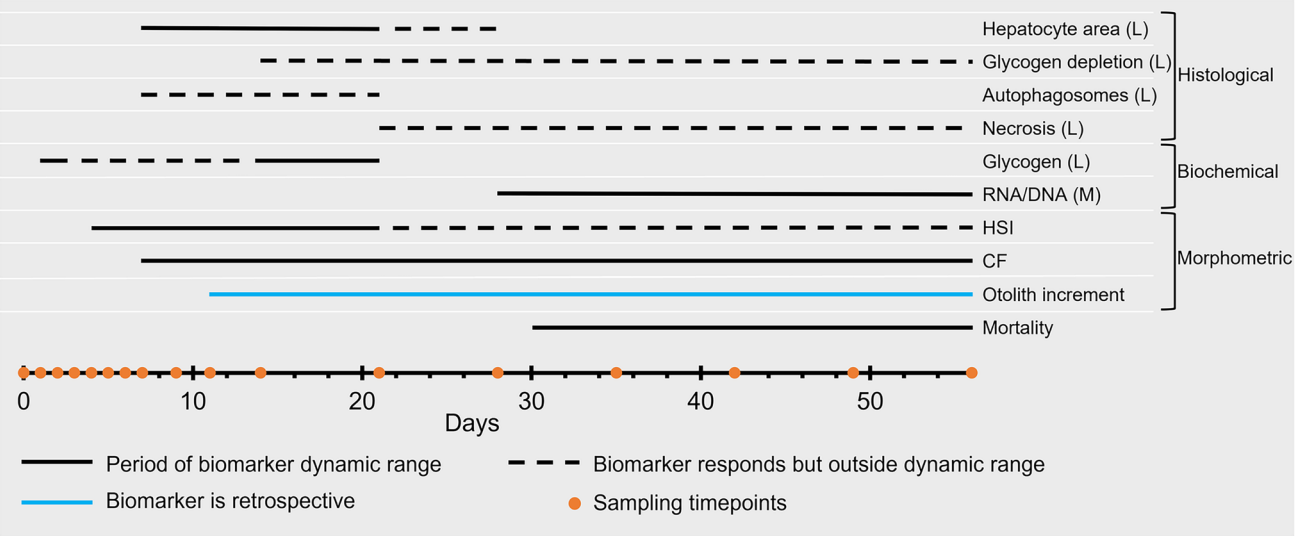

Fig. 4 Comparison of biomarkers for detecting food limitation in subadult Delta Smelt, including results from the present study, Hammock et al. (2020), and Lewis et al. (In progress). Fasting increases in duration from left (0 days) to right (56 days). Dynamic range refers to the period over which the biomarker changes in response to increasingly severe food limitation. Sensitivity is the time taken for the biomarker to respond significantly to fasting at constant water temperature (16 °C). "L" is liver tissue and "M" is muscle tissue. Orange dots represent days on which fish were sampled, but not every biomarker was measured at every time point. Muscle glycogen and muscle triglycerides were excluded from this figure due to their insensitivity and inconsistent response to fasting, while liver RNA/DNA and liver triglycerides were excluded because data was only available for one day of sampling (Day 21)

remaining liver glycogen, until it was nearly depleted by Day 21. With little difference between fish fasted from 1 day to 2 weeks, liver glycogen appears ineffective for differentiating between mild and moderate food limitation in Delta Smelt. Similar partial protection of liver glycogen has been observed in Black Carp, where glycogen was depleted in the first 3 days of fasting and then remained constant for a week or more (Dai et al. 2022). However, liver glycogen could be useful in differentiating between moderate and severe food limitation, since it declined from Day 14 to 21. In contrast to liver glycogen, muscle glycogen was almost fully protected throughout the 21 days of fasting. Muscle glycogen is similarly conserved during the first 21 days of fasting in Rainbow Trout (Harmon et al. 2011), Brown Trout (Navarro et al. 1992), and Jundiá (Barcellos et al. 2010). Thus, our study demonstrates that biochemically measuring liver glycogen concentration

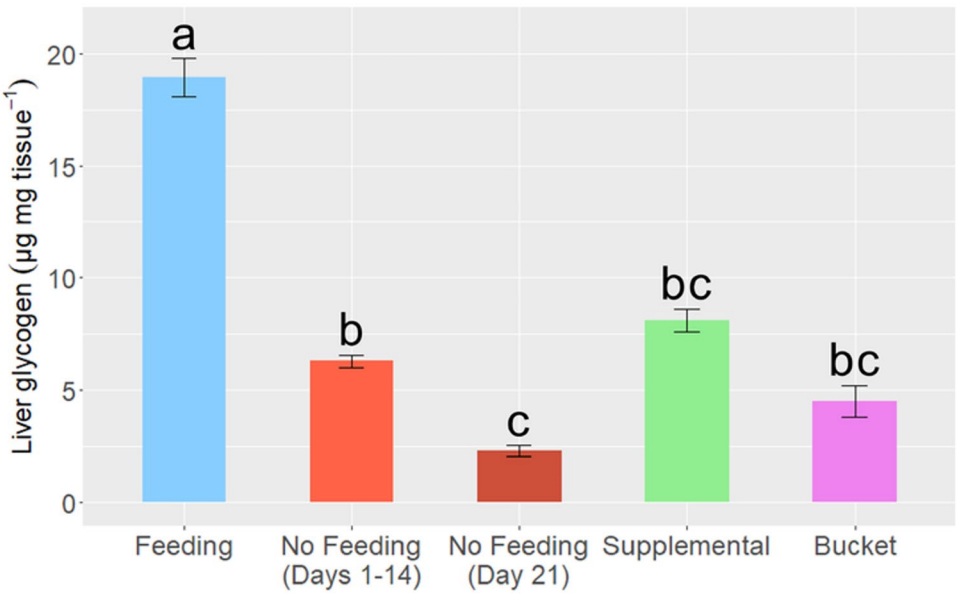

Fig. 5 Mean liver glycogen concentrations for Feeding, No Feeding (Days 1–14, or mild to moderate food limitation), No Feeding (Day 21, or severe food limitation), Supplemental, and "Bucket" Delta Smelt, ± SE. Supplemental Delta Smelt were collected from the San Francisco Estuary and flash-frozen on boats, and Bucket fish are Supplemental Delta Smelt that were kept live in a bucket for several hours before flash-freezing, potentially depressing their glycogen stores. Treatments with different letters are significantly different based on a Tukey HSD test

is highly sensitive to detecting food limitation, albeit with a narrow dynamic range until moderate levels of starvation are reached (~ Day 14). While muscle glycogen responded to fasting, it was too protected to have utility as a biomarker of food limitation in Delta Smelt.

Variance was considerably higher among individuals in the Feeding treatment than the No Feeding treatment in terms of liver glycogen. This difference can be largely explained by the interaction between body weight and treatment (Feeding and No Feeding), in which liver glycogen increased with fish weight in the Feeding treatment but not in the No Feeding treatment (Fig. 3). However, the cause of this interaction is uncertain. Larger fish may have outcompeted smaller fish for food in the Feeding treatment, leading to higher glycogen concentrations in the livers of larger fish. Competition for food is common in aquaculture, resulting in size grading and separation to encourage growth of smaller fish and reduce size variability (Magnuson 1962; Saoud et al. 2005; Torrans and Ott 2018). In the No Feeding treatment, a competitive advantage for food was presumably impossible, possibly resulting in little to no influence of body weight on liver glycogen. However, food competition cannot entirely account for the treatment by body weight interaction, because there were average-sized fish in the Feeding treatment with low liver glycogen values. Another possibility is that this variation in liver glycogen is due to phenotypic variation among individuals. Whatever the cause, future studies will need sufficiently large sample sizes to offset the variance apparent in a well-fed population.

A shortcoming of glycogen concentration as a biomarker of nutritional stress is its limited specificity. In addition to food limitation, glycogen can deplete due to handling or toxic stress (Haux et al. 1985; Vijayan and Moon 1992; Hemre and Krogdahl 1996). For example, muscle glycogen declines after 5 min of chase and capture in European Sea Bass (Samaras et al. 2016). Rapid depletion of muscle glycogen during sampling may therefore have hindered our ability to detect differences between treatments, along with the maintenance of minimum levels of muscle glycogen throughout our 21-day experiment. However, Delta Smelt may not exhibit this depletion since muscle glycogen remains constant after 1 h of handling stress in Atlantic Salmon (Hemre and Krogdahl 1996). Handling stress can also deplete liver glycogen, as seen in the Supplemental fish held in buckets for several hours, though the decline was not statistically significant (Fig. 5).

Both the lack of specificity and narrow dynamic range of liver glycogen can be addressed by using the biomarker in combination with other endpoints. For example, condition factor declined throughout most of our previous 56-day experiment, allowing for the differentiation among degrees of food limitation stress (Hammock et al. 2020). In addition, autophagosomes are indicators of moderate food limitation stress, and necrosis in the liver indicates severe starvation (Fig. S1, Hammock et al. 2020). Stomach fullness may help with interpretation as well, given its specificity and sensitivity to food limitation (Hobbs et al. 2006; Hammock et al. 2019b). However, even stomach fullness can be difficult to use as a sole indication of nutritional status because it is influenced by variables besides foraging success, such as water temperature and prey digestibility, and only represents recent foraging success of the fish (Robinson et al. 2010; Fall and Fiksen 2020). Identifying stressors is always difficult when interpreting biomarkers of wild or Supplemental fish due, for example, to the variability of habitat quality, migration patterns, and life history strategies (Hook et al. 2014). Nevertheless, using a combination of well-characterized biomarkers with differing sensitivities and dynamic ranges can aid interpretation.

Progression of Starvation in Delta Smelt

The results from this study, Hammock et al. (2020), and Lewis et al. (In progress) yield a more comprehensive understanding of the progression of starvation in sub-adult Delta Smelt and how it compares to other teleosts. Here, we demonstrate that liver glycogen drops substantially on the first day of fasting, stabilizes for 2 weeks, and then declines again from Days 15 to 21. In Hammock et al. (2020), hepatosomatic index declined after 4 days, likely due to a combination of glycogen loss, water loss, and autophagosomal degradation. Hepatosomatic index seems especially sensitive to fasting in Delta Smelt, since it typically responds to fasting after 2 weeks in other fishes, even at higher temperatures than in our study (22–29 °C; Uchida et al. 2003; Abdel-Tawwab et al. 2006; Barcellos et al. 2010; Xu et al. 2019; Bermejo-Poza et al. 2020). Condition factor was also fairly responsive in Delta Smelt, declining below control levels at 7 days in Hammock et al. (2020), consistent with declines seen after 1 week in Rainbow Trout at 23 °C (Bermejo-Poza et al. 2020), and 2 weeks in Atlantic Salmon and Mozambique Tilapia at 28 °C and 12 °C, respectively (Uchida et al. 2003; Hvas et al. 2021). Moving forward, applying the liver glycogen biomarker—which is far more sensitive than hepatosomatic index and condition factor—to wild caught Delta Smelt could inform management actions. For example, measuring the liver glycogen of wild Delta Smelt before and after opening the Suisun Marsh Salinity Control Gate could determine whether the action improves Delta Smelt nutritional condition (e.g., Sommer et al. 2020). Nevertheless, biomarkers like condition factor with wide dynamic ranges will remain useful, even if they lack the sensitivity of liver glycogen.

Due to their role in metabolism, hepatocytes shrink in response to fasting, with the timing usually associated with the mobilization of hepatic energy stores (Power et al.

2000; Séité et al. 2019). Our results are consistent with this timeline as hepatocyte area declined after 7 days of fasting (Hammock et al. 2020), following the rapid depletion of liver glycogen described in the present study. The timing of the decline in hepatocyte size is identical to that of Milkfish, albeit at much higher temperatures (26–30 °C; Storch and Juario 1983). Autophagosomes were apparent in the liver after 7 days of fasting in Delta Smelt (Fig. S1), likely to digest hepatocyte organelles that became superfluous without food. This is slower than the appearance of autophagosomes in Zebrafish liver after 2 days of fasting combined with cold stress at 11 °C, and faster than Rainbow Trout muscle after 2 weeks of fasting at 18 °C (Seilez et al. 2010; Lu et al. 2019). The near-complete depletion of liver glycogen observed in the present study from Days 15 to 21 corresponds with moderate to severe glycogen depletion scores observed histologically beginning at 14 days in Hammock et al. (2020).

In the present study, the timing of the near-complete depletion in liver glycogen on Day 21 in the No Feeding treatment coincided with initial signs of severe starvation in our previous work. In Hammock et al. (2020), hepatosomatic index in the No Feeding treatment stopped decreasing after Day 21, and mortality began increasing. The plateau in hepatosomatic index also correlated with the disappearance of hepatic autophagosomes and the onset of necrosis in the liver, both on Day 21 (Fig. S1). This indicates that organelles available for digestion were exhausted and autophagy could no longer extend hepatocyte survival (Hammock et al. 2020). Liver necrosis occurred considerably sooner in Delta Smelt than in Rainbow Trout, in which it took 70 days of fasting to become apparent, though this experiment was run at 8–10 °C (Karatas et al. 2021). Thus, despite its relative insensitivity, histopathology may remain useful, because lesions like single-cell necrosis in the liver indicate severe stress (Fig. 4). Whether severely starved fish in the wild could be detected, however, is questionable given that mortality occurs shortly after this stage in laboratory conditions and additional stressors are present in the field (e.g., predators). Thus, biomarkers that are sensitive to food limitation like liver glycogen may be more practical for field applications and for delicate species like Delta Smelt.

Triglycerides in the liver also decreased by Day 21 in the current study but may have declined earlier in the fasting period. There is evidence of rapid triglyceride depletion in other fishes, though at higher temperatures (e.g., 1 day in Nile Tilapia at 30 °C, Wang et al. (2019); 1 week in Rainbow Trout at 23 °C, Bermejo-Poza et al. (2020)). Declines in RNA/DNA in liver were also observed on Day 21 in the present study, which is similar to reductions occurring after 2–3 weeks of fasting in other fishes at 12–28 °C (e.g., Hack et al. 2019; Fan et al. 2019). This suggests that RNA/DNA in liver is more sensitive to food limitation than in dorsal muscle in Delta Smelt, in which differences between fed and fasted treatments first became detectable at 28 days (Hammock et al. 2020).

Application to Supplemental Delta Smelt

The fish from the Feeding treatment had liver glycogen levels roughly two times higher than the Supplemental fish. In fact, the Supplemental fish had liver glycogen levels that were statistically indistinguishable from both No Feeding categories (Days 1–14 and 21). Given that the Supplemental fish were more mature than the fish in the Feeding treatment, that liver glycogen increases with size, and that water temperature was lower in the wild (decreasing metabolic demand), these results suggest that the Supplemental fish experienced food limitation in the SFE. However, this assumes that healthy Delta Smelt in the wild would have similar liver glycogen levels to that of fed hatchery fish, which may be unrealistic. For example, given that the Supplemental Delta Smelt were sampled from winter to spring, migration and spawning may have increased energy expenditure. Moreover, decreases in glycogen could have been caused by the stress of collection in a trawl, which could last up to 10 min, in comparison to our faster (< 1 min) netting of hatchery fish. The apparent food limitation in the Supplemental Delta Smelt could also have been caused by the stress of the supplementation process or difficulty adjusting to the prey field in the wild. Because Delta Smelt were released repeatedly over several months, the time-at-liberty for these released fish is unknown, so we are unable to identify how long individuals were in the SFE. In the future, if fish are released in distinguishable batches, liver glycogen could assist in determining which culture practices or release locations result in nutritionally robust fish in the wild. For example, acclimation to live prey could improve foraging performance (e.g., Ellis et al. 2002, Paszkowski and Olla 1985; Brown and Day 2002). Nonetheless, the low glycogen levels of the Supplemental fish are consistent with the well-established hypothesis that pelagic fish are prey limited in the SFE (e.g., Feyrer et al. 2003; Slater and Baxter 2014).

Conclusion

The results of our 21-day fasting study demonstrate that liver glycogen is highly sensitive to food limitation, with only 1 day of fasting at 16 °C resulting in a significant decline in liver glycogen. However, liver glycogen has limited specificity and a narrow dynamic range, so a suite of biomarkers would help assess the severity of nutritional stress when applied to fish caught from the wild.

For example, biochemically measured liver glycogen and stomach fullness can detect mild food limitation, while autophagosomes in the liver, muscle RNA/DNA, and liver necrosis indicate moderate to severe starvation. Supplemental Delta Smelt collected from the wild exhibited half the liver glycogen concentrations as fed hatchery fish, which is consistent with the hypothesis of pelagic food limitation in the wild, but several other potential causes are possible, as discussed. This study demonstrates the potential of liver glycogen to assess habitat suitability, and therefore inform decisions regarding conservation and restoration.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12237-023-01282-y.

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Data Availability Data are available on request from the authors.

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