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Effects of nutritional deprivation on juvenile green sturgeon growth and thermal tolerance

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Introduction

In the San Francisco Estuary (SFE), declines in the abundance of native fish species have been linked to altered food webs and reduced food availability (Moyle 2002; Feyrer et al. 2003; Kimmerer 2004). This is particularly concerning for protected green sturgeon (*Acipenser medirostris* Ayres, 1854) populations restricted to the SFE during early life stages. Green sturgeon are composed of at least two genetically distinct and protected populations (Israel et al. 2009): the Northern and Southern Distinct Population Segments (DPS), and spawn only in Oregon and California, USA. Details of the current distribution and spawning locations of these fish can be found in Beamesderfer et al. (2007). Briefly, anadromous adult green sturgeon spend most of their lives in the marine environment, with seasonal migrations between natal freshwater spawning grounds and the ocean. Adults of the Northern DPS, which is classified as a species of concern by the National Oceanic and Atmospheric Administration (NOAA) of the USA, spawn in rivers north of the Eel River of northwest California (Adams et al. 2007). The Southern DPS is classified as threatened under the Endangered Species Act, and all suspected and confirmed spawning locations are within the watershed of the Sacramento and San Joaquin rivers (Adams et al. 2007). As juvenile green sturgeon are intolerant of full strength seawater until they are 0.5 to 1.5 years old (Allen and Cech 2007; Allen et al. 2009, 2011), habitat is restricted to the fresh to brackish water sections of the SFE for early life stages.

The SFE is made up of two major Californian rivers, the Sacramento and San Joaquin, and their drainage to the Pacific Ocean through the San Francisco Bay. Since the 1800's, this watershed has been highly modified through hydraulic gold mining in the Sierra Nevada mountains, followed by consumption and diversion of water to support urbanization and agriculture, introduction of sewage input, and active management through environmental policy (Atwater et al. 1979; Cloern and Jassby 2012). International shipping, climate change and ecosystem alterations have created conditions conducive to pervasive invasions by non-native species, which now characterize the entire system (Atwater et al. 1979; Cloern and Jassby 2012). These ecosystem-wide changes are directly attributable to population declines of native aquatic biota associated with all trophic levels (Cloern and Jassby 2012), resulting in large shifts in the composition of biological

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communities (Winder and Jassby 2010). These shifts in communities, concurrent with four decades of altered nutrient concentrations and loadings (Gilbert et al. 2011), habitat structure, and physicochemical characteristics of the SFE have resulted in a restructuring of the entire food web (Alpine and Cloern 1992; Jassby 2008; Cloern and Jassby 2012). This altered food web has been linked to declines in many SFE fish species (Moyle 2002; Feyrer et al. 2003; Kimmerer 2004), e.g. the listed Delta smelt (*Hypomesus transpacificus* McAllister, 1963), and likely has impacted other listed species such as green sturgeon through reduced habitat quality and altered interspecies interactions, such as prey availability.

Though the rarity and protected status of green sturgeon has precluded detailed study of their natural diet, young green sturgeon feed exclusively on benthic organisms, including shrimp, crabs, worms, amphipods, isopods, clams and small fishes (EPIC 2001). The recent colonization and domination of the SFE benthos by *Corbula amurensis* Schrenck, 1861 (the Asian overbite clam) (Feyrer et al. 2003; Cloern and Jassby 2012) has displaced many of these prey species (Nichols et al. 1990; Gilbert et al. 2011; Greene et al. 2011; Cloern and Jassby 2012). Other sturgeon species have been shown to successfully adjust their diet to altered prey availability (Brosse et al. 2000; Usvyatsov et al. 2012), and the Asian overbite clam has been identified as a major component of the diet of the sympatric white sturgeon (Kogut 2008). However, in sturgeons, manipulation of diet ration or nutritional content has been shown to affect growth, energetic status (Hung and Lutes 1987; Cui et al. 1997; Hung et al. 1997; Deng et al. 2009; Han et al. 2012; Haller et al. 2015) and the cellular response to heat stress (Deng et al. 2009; Han et al. 2012; Wang et al. 2013). Furthermore, diet composition and nutritional status have been shown to influence swimming capacity (Wagner et al. 2004; Chatelier et al. 2006; Wilson et al. 2007; Gingerich et al. 2010; Pettersson et al. 2010; Killen et al. 2014; Silva et al. 2014), osmoregulation (Kirschner 1995; Tseng and Hwang 2008; Haller et al. 2015), growth at high temperatures (Glencross and Rutherford 2010; Zhou et al. 2013) and tolerance of high temperatures (Akhtar et al. 2011; Kumar et al. 2014; Patterson and Green 2014; Tejpal et al. 2014) for a wide range of fish species.

Little is known of the effects of nutritional status on high temperature tolerance in young green sturgeon confined to the SFE, a system where water flow patterns and temperature regimes have been drastically altered for the last century (Lund et al. 2010; Moyle et al. 2010; Cloern and Jassby 2012). Due to water diversion for municipal and agricultural uses, annual water flow into the SFE has been reduced to ca. 60 % of the natural freshwater inflow (Lund et al. 2010), which has resulted in increasing summer water temperatures (Moyle et al. 2010) and upstream salinities (Cloern and Jassby 2012). Climate change is projected to further increase temperatures of the SFE and the Sacramento River at a rate of 0.1 to 0.3 °C per decade, depending on the location and climate model applied (Cloern et al. 2011). Intolerably high water temperatures during the late summer, coupled with low nutritional energy supply, may threaten young sturgeon before they develop the osmoregulatory capacity to access cooler seawater.

Therefore, we investigated how nutritional deprivation, through manipulation of feed ration, affected temperature sensitivity and tolerance in juvenile green sturgeon 100 days post hatch (dph). We also quantified the cellular heat shock protein 70 (HSP70) response of feed-restricted juvenile green sturgeon to acute heating to evaluate the ability of nutritionally deprived sturgeon to immobilize a cellular stress response.

Methods

Animal acquisition

Green sturgeon were F2 offspring of F1 broodstock from wild-caught Klamath River Northern DPS sturgeon. Broodstock were matured at 18.5 (±0.5) °C, then artificially-spawned at 15 (±0.5) °C under aquaculture conditions at the Center for Aquatic Biology and Aquaculture (CABA), University of California Davis (UCD) (Van Eenennaam et al. 2008, 2012). Sturgeon were held in tanks or experimental holding containers supplied with aerated degassed well water through all rearing from egg incubation through experimental treatments and tests. Eggs were incubated in 15 (±0.5) °C well water until hatch in early April, 2011, after which larvae were transferred to 18.5 (±0.5) °C well water. Post-hatch sturgeon were fed commercial feeds (depending on fish size: Soft-Moist #0 to #3 crumble, Rangen Inc., Buhl, ID, USA; SCD 1.0 to 2.0 mm sinking pellet, Skretting, Tooele, UT, USA) using 24-h automatic feeders (Cui et al. 1997) according to the manufacturer's

recommendations. All fish were held under a natural photoperiod from April to August, and under these rearing conditions it took ca. 4 months for fish to reach the experimental target size (ca. 60 g and 105 days post hatch (dph)).

Feed ration treatments

Once fish reached the target mass [56.9±0.2 g (mean ±SD, n=480)], they were allocated to sixteen, total-mass-matched replicate tanks of thirty sturgeon. Tanks were circular (90 l, fiberglass) and supplied with 5 l min^{-1}, flow-through, well water. There were four replicate tanks for each feed ration treatment of 0.25, 0.5, 1.0, and 2.0 % body mass per day (%BM day^{-1}). For all treatments sturgeon were fed a salmonid commercial diet (SCD 2.0 mm sinking pellet, Skretting) composed of 5.5 % moisture, 52.0 % crude protein, and 16.0 % crude lipid. Temperature and dissolved O_2 were maintained at 18–20.5 °C and 7.5–9 mg l^{-1}, respectively. Photoperiod was maintained on a 14 h light: 10 h dark schedule.

After allocation to treatment tanks, fish were reared on one of four feed ration treatments for 3 weeks. Feed ration treatments were based on previously determined optimal feed rations of 2.0 % BM day^{-1} for similarly aged and sized juvenile white sturgeon (*Acipenser transmontanus* Richardson, 1836) (Hung and Lutes 1987). Each entire tank of fish was batch-weighed weekly to assess growth and adjust feed amount according to changes in tank biomass (Hung and Lutes 1987).

Growth assessment

A tank biomass-based growth trial was performed during the first 2 weeks of feed ration treatments to assess the effects of feed ration treatment on green sturgeon growth and nutritional status. Fish were fasted for 20 to 24 h before and after mass measurements to allow evacuation of food from the gastrointestinal tract and facilitate recovery from handling stress. Whole tank biomass was measured, and mean individual body mass (MBM) estimates were calculated from tank biomass and tank fish counts after a 24 h starvation period. Specific growth rate (SGR, %BM day^{-1}) and feed efficiency (FE) were calculated according to the following equations:

$$\mathrm{SGR} = 100^{*}\ln(\mathrm{FMBM}/\mathrm{IMBM})/14$$
$$\mathrm{FE} = 100^{*}(\mathrm{FMBM}/\mathrm{IMBM})/\mathrm{TF},$$

where FMBM and IMBM are mean final and initial body mass (g) per fish in a treatment tank, respectively; TF is average total amount fed (g) per fish in the tank over the 2 week experiment. This growth assessment protocol was based on a previous feed ration studies on white sturgeon, which showed maximal diet ration effects on growth and nutritional status at 2 weeks of diet ration treatment (Cui et al. 1997).

Nutritional status

Concurrent with the growth assessment at the end of the 2-week tank biomass growth trial, six fish were removed from each tank and euthanized in 0.5 g l^{-1} tricaine methanesulfonate (MS-222, Argent Inc., Redwood, WA, USA) dissolved in well water and buffered with 2.5 g l^{-1} sodium bicarbonate (buffered MS-222) for determination of nutritional status. Three fish were used for plasma metabolite quantification, while the other three fish were used for proximate analysis (Jones 1984).

The plasma metabolites measured included plasma protein, glucose and triglycerides. Blood samples were drawn into heparinized vacutainers via caudal puncture, then centrifuged at 4500 g for 5 min at room temperature in order to separate plasma and red blood cells. Plasma from the three fish from the same tank was then pooled and stored at −80 °C for approximately 3 months until assays were performed. Plasma samples were assayed in duplicate for plasma glucose (GAGO20, Sigma-Aldrich, Saint Louis, MO, USA), protein (TP0200, Sigma-Aldrich) and triglycerides (TAG, TR0100, Sigma-Aldrich) using commercially produced assay kits. The fish used for plasma metabolite measurements were also measured for BM and total length (TL, cm), and Fulton's condition factor (K; Fulton (1902)) was calculated from these measurements according to the formula:

$$\mathrm{K} = 100^{*}\left(\mathrm{BM}/\mathrm{TL}^{3}\right).$$

The final three fish terminally sampled from each tank at the end of the feed ration treatments were prepared for whole body proximate composition analysis, which was used to calculate whole body energy (WBe). After euthanization, fish were frozen in plastic bags with the triplicate fish from one tank pooled into one bag, and

stored at −20 °C until sample processing. Frozen samples of the pooled (n=3) fish per bag (i.e. per tank) were cut into small pieces, and ground in a Wiley mill (Hobart, Troy, NH). Grindings were thoroughly mixed, then placed into a freeze drying container and stored at −20 °C until freeze drying. Ground samples were freeze dried for 68 h, then mixed in a commercial kitchen blender, and finally stored in Whirl-Pack® bags (Nasco, Ft. Wilkinson, WI, USA) until proximate analysis. Duplicate determinations of whole body proximate moisture (WBmoist, %) and crude protein (WBprot, %), lipid (WBlip, %) and ash (WBash, %) were then performed on the ground carcasses according to Association of Official Analytical Chemists standards (Jones 1984). Calculations of WBe (mJ kg^{-1}) were as in Deng et al. (2003) using the formula:

$$\mathrm{WBe}\ 4.18 \times \left(5.65 \times \mathrm{WBprot} \times 100^{-1} + 9.4 \times \mathrm{WBlip} \times 100^{-1} + 4.23 \times \mathrm{WBnfe} \times 100^{-1}\right),$$

where WBnfe is nitrogen free extract, and calculated as:

$$\mathrm{WBnfe} = 100 - \mathrm{WBprot} - \mathrm{WBash} - \mathrm{WBlip} - \mathrm{WBmoist}.$$

High temperature tolerance

During week three of this study, high temperature tolerance was assessed by measuring critical thermal maximum as well as the response of constitutive and induced heat shock protein 70 (HSC/HSP70) expression to warming.

CTmax

To assess whole organism thermal tolerance, critical thermal maximum (CTmax) tests were performed according to the methods of Cox (1974) and Fangue et al. (2006) for one or two fish per ration treatment tank (n=7 fish per treatment). After a 24 h fasting period, an individual fish was acclimated for 30 min in an aerated 30 l glass aquarium filled with 18.5 to 19 °C water. Temperature was then increased at a rate of 0.3 °C min^{-1} under the control of a YSI 72 Proportional Temperature Controller (YSI, Yellow Springs, OH, USA), while gill ventilation movements were monitored. The CTmax of an individual fish was determined as the temperature at which gill ventilation movements ceased, which is an end point commonly used in CTmax tests on fishes (Fangue and Bennett 2003), including green sturgeon (Beitinger and Bennett 2000; Sardella et al. 2008). Immediately after gill movement ceased, the fish was removed to an aerated 45 l tank supplied with 18–19.1 °C flow-through, water, where fish recovered for 24 h. No mortalities occurred during the CTmax test or during the 24 h recovery period, after which, fish were euthanized with an overdose of buffered MS-222, and BM and TL were measured.

Heat shock protein levels

HSC/HSP70 protein levels in response to acute heat shock were quantified to determine effects of feed ration on the magnitude of cellular-level stress response and the temperature at which a cellular-level stress response is mobilized. Tissue protein levels of HSC/HSP70 were quantified based on the discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method (Laemmli 1970) followed by chemiluminescence staining of the secondary antibody according to the methods of Todgham et al. (2005) and Deng et al. (2009).

Twelve fish per feed ration treatment (three from each of the four replicate tanks) were acutely exposed to one of four temperatures. Fish were directly transferred from their treatment tanks to 12 l aerated, plastic buckets at 18 (handling control), 24, 26, or 28 °C, where they remained for a 2-h exposure. Exposure time was based on previous findings of increased levels of HSP70 protein after a 2-h exposure (Dietz and Somero 1993; LeBlanc et al. 2011), and acute exposure of 18 °C-acclimated green sturgeon to 26 °C has been shown to elevate HSP70 protein levels (Wang et al. 2013). After the 2-h temperature exposure, fish were abruptly transferred to identical plastic buckets in a holding tank supplied with 18–19.1 °C flow-through well water, where they were held for 2 h. Fish were then rapidly euthanized in buffered MS-222 before brain and mucus samples were collected. Mucus samples were scraped from the left side of the body between the dorsal fin and lateral scutes. Samples were immediately frozen and stored at −80 °C until relative HSC/HSP70 protein

levels were determined by western blot. Mucus and brain HSP70 protein levels are thermally responsive, with mucus showing the greatest response of all tested tissues in green sturgeon (Wang et al. 2013). Brain HSP70 protein levels are also informative because endpoints of CTmax tests (loss of equilibrium or cessation of ventilation) are potentially manifestations of central nervous system collapse (Friedlander et al. 1976; Pörtner et al. 1998).

Sample preparation for SDS-PAGE and protein quantification

Five mg tissue samples were sonicated (ca. 100 J to each sample, Model 120 Sonic Dismembrator, Fisher Scientific, Waltham, MA, USA) in 0.6 ml homogenization buffer (0.1 % SDS, [w/v], 190 μg ml^{-1} EDTA, 0.7 μg ml^{-1} pepstatin A, 0.5 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin, 20 μg ml^{-1} phenylmethanesulfonyl fluoride in 100 mM Trish-HCl buffer, pH 7.5) on ice, then denatured at 100 °C for 5 min and centrifuged at room temperature for 10 min at 30,000 g to collect the supernatant containing dissolved protein. Total protein concentration of the supernatant was determined using the bicinchoninic acid method (Smith et al. 1985). Aliquots of the supernatant were suspended in 5×Laemmli's buffer (50 % glycerol [v/v], 10 % SDS [w/v], 0.01 % bromophenol blue [w/v], 25 % β-mercaptoethanol [v/v] in 0.3 M Tris–HCl buffer, pH 6.8; Laemmli 1970), boiled at 100 °C for 3 min and then stored at −80 °C until SDS-PAGE.

SDS-PAGE and western blotting for relative HSC/HSP70

For each sample, an amount containing 20 μg (mucus) or 8 μg (brain) of total protein was resolved in a 4 % SDS-PAGE stacking gel and a 12.5 % SDS-PAGE separating gel. Each gel was also loaded with 5 μl of pre-stained molecular markers (Precision Plus Protein™ Dual Xtra Standards, Bio-Rad, Hercules, CA, USA) and 15 μl of heat-shocked HeLa cell lysate (ADI-LYC-HL101, Enzo Life Sciences, NY, USA) diluted 40× with 1× Laemmli's buffer as an internal standard among gels, and referred to as internal standard throughout the rest of this manuscript. Samples were stacked for 20 min at 75 V, then separated for 50 to 120 min at 175 V in a Mini-Protean® Tetra Cell with a PowerPac™ power supply (Bio-Rad).

After SDS-PAGE separation of proteins, gels were trimmed at the 60 kDa protein band of the ladder lane, then semi-dry transferred to nitrocellulose membranes in a Trans-Blot® Turbo™ (Bio-Rad). Transfer was performed in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375 % SDS [w/v], 20 % methanol [v/v], pH 9.0–9.4) for 30 min at 25 V and 1 A.

Following protein transfer, nitrocellulose membranes were blocked in 2 % skim milk [w/v] in Tween-20 Tris-buffered saline (TTBS; 20 mM Tris–HCl, 0.14 M NaCl, 0.1 % Tween-20 [v/v]) with 0.05 % [w/v] sodium azide for 1 h, and then stored overnight at room temperature before antibody binding and quantification. In the morning, membranes were briefly blocked again before antibody incubation. Primary antibody incubation was performed in rabbit IgG for rat HSC/HSP70 antibody (ADI-SPA-757, Enzo Life Sciences) diluted 500-fold with blocking solution for 90 min with light agitation. The primary antibody used detects both the constitutive (HSC70) and inducible (HSP70) isoforms of the 70-kDa heat shock protein. Membranes were then thoroughly washed with TTBS before incubation in a 1:4,000 dilution of horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody (31460, ThermoScientific, IL, USA) for 90 min. After antibody incubation, membranes were washed in TTBS followed by TBS to remove Tween-20, then submerged in the chemiluminescence reaction mixture (Clarity™ Western ECL Substrate, Bio-Rad) with gentle agitation for 10 min. Imaging was performed immediately on wet membranes placed directly on the image screen of a ChemiDoc XRS imager (Bio-Rad). Exposure time was standardized to within 10 s prior to over-exposure of the internal control.

Images were analyzed for determination of relative HSC/HSP70 protein quantity using ImageDoc software (Bio-Rad). HSC/HSP70 protein levels are presented relative to the internal standard band intensity. Some samples did not produce visible bands through chemiluminescence, or produced band configurations suggestive of degraded proteins (i.e. one large band in the >170 kDa region of the blot, with no bands corresponding to lower molecular weights), therefore, mucus and brain HSC/HSP70 sample sizes ranged from 6 to 12 samples per treatment.

Statistics

All statistical analyses were performed in R (R Core Team 2015) with a significance level of 0.05. The type of model applied to each variable is identified in Table 1. Variables with heterogeneous variance were analyzed

using a robust linear model using the 'rlm' command of the MASS package of R (Venables et al. 2002); p values were calculated from t values and degrees of freedom. Residuals were weighted following Huber's function. For variables with replicate estimates per tank (see variables in Table 1 with lmer indicated for test), mixed effects analyses using the 'lmer' function of the lme4 package in R (Bates et al. 2015) were performed with tank as a random effect. All other variables were analyzed using the 'lm' function in the base package of R. For all models, regardless of form (i.e. rlm, lm, lmer), the relationship was first tested with feed ration as a continuous variable for determination of effects of feed ration on dependent variables. For all variables, except those analyzed with tank as a random effect, when feed ration effects were significant in the regression, an ANOVA with feed ration as a categorical factor was performed followed by Tukey's multiple comparisons, using the 'tukeyHSD' function in R. For the mixed effects models that showed significant effects of feed ration, an ANOVA was performed on a new lmer model including feed ration as a categorical factor. Tukey's multiple comparisons were performed using the 'glht' function of the R multcomp package (Hothorn et al. 2008).

Some variables analyzed with mixed effects modeling had potential predictor variables other than feed ration (i.e. mass for CTmax and temperature for mucus and brain HSC/HSP70). In these cases, the best predictive model was chosen by comparing Akaike Information Criterion (AIC) of competing models, and retaining the model with the lowest AIC value (Burnham and Anderson 2002). The best predictive model was used for multiple comparisons when significant effects were observed. This meant that some non significant terms were maintained in the model; for example, feed ration and the interaction between feed ration and temperature were not significant in the model explaining mucus HSC/HSP70 expression. However, the model including these variables was a better fit than the model including the only significant variable, temperature, therefore all three variables were maintained in the model.

Results

Effects of feed restriction on growth

No mortalities occurred during the 2-week feed restriction growth study. The relationship between feed ration and mean body mass (MBM) was significant, producing profound and statistically significant differences in fish mass after 2 weeks of feed ration treatment (Tables 1 and 2). The mass of fish in the lowest ration group did not change significantly from the initial value of 57.0 (±0.1) g, and mass of the highest ration increased 1.6-fold from 56.9 (±0.1) to 89.8 (±0.4) g. These differences in final mass were due to a significant effect of feed ration treatment on SGR. The SGR ranged from −0.09 (±0.05) %BM day^{-1}, for the lowest feed ration tanks, and 3.25 (±0.17) %BM day^{-1}, for the highest ration tanks (Table 1).

Nutritional status: feed ration effects on energy substrates

Long-term energy supply

Whole body energy significantly increased with feed ration (Table 1). This relationship was primarily driven by the high whole body energy of 4.23(±0.13) mJ kg^{-1} BM^{-1} for the 2.0 %BM day^{-1} feed ration group, compared to energy levels ranging from 3.16 (±0.09) to 3.49(±0.09) mJ kg^{-1} BM^{-1} for the 0.25 to 1.0 %BM day^{-1} feed ration groups (Table 2).

The relationship between whole body energy and feed ration reflects the relationships between feed ration and whole body lipid, protein and moisture. Whole body lipid and protein increased significantly with feed ration (Table 1). Whole body lipid increased 2.3-fold from 1.60(±0.18)% to 3.74(±0.36)% for 0.25 to 2.0 %BM day^{-1} feed ration. Whole body protein increased 1.1-fold from 9.78(±0.12)% for 0.25 %BM day^{-1} feed ration to 11.03(±0.26)% for 2.0 %BM day^{-1} feed ration. Whole body moisture decreased significantly with feed ration (Table 1). This relationship resulted in nearly 3 % decrease in whole body moisture from 85.17(±0.36)% to 82.2(±0.26)% for 0.2 to 2.0 %BM day^{-1} feed ration.

Short term energy supply

Feed ration significantly affected short-term energy stores, as reflected in plasma TAG, glucose, and protein levels (Table 1). Plasma TAG was 2.5-fold greater for 2.0 %BM day^{-1} feed ration, relative to 0.25 %BM day^{-1} feed ration fish (Table 2). Plasma glucose increased 3.7-fold greater from 0.25 to 2.0 %BM day^{-1} feed ration (Table 2). Feed ration effect was less pronounced for plasma protein, which was 1.2-fold greater for the

Table 1 Statistical test results including degrees of freedom, t- (for regressions) or f-values (for anovas) and p-values for fish size and growth, plasma metabolite and proximate composition measurements

		df	Slope	*f/t* value	*p* value
MBM (g)					
Test: rlm	FR (cont)	1, 14	19.25	77.79	1.1×10^{-14}
Test: aov	FR (factor)	3,12		155.10	7.3×10^{-10}
SGR (%BM day^{-1})					
Test: rlm	FR (cont)	1, 14	0.10	18.80	2.5×10^{-11}
Test: aov	FR (factor)	3,12		228.47	7.6×10^{-11}
FE (%)					
Test: lm	FR (cont)	1, 14	96.95	12.91	2.9×10^{-3}
Test: aov	FR (factor)	3,12		73.12	5.6×10^{-8}
Mass (g) of individual fish sampled for plasma					
Test: lmer	FR (cont)		22.06	9.21	2.6×10^{-7}
Test: lmer	FR (factor)			40.68	1.5×10^{-6}
TL (cm) of individual fish sampled for plasma					
Test: lmer	FR (cont)		22.06	5.48	8.1×10^{-5}
Test: lmer	FR (factor)			16.65	6.36×10^{-5}
K of individual fish sampled for plasma					
Test: lmer	FR (cont)		0.035	7.13	$5.8\text{x}10^{-9}$
Test: lmer	FR (factor)			17.06	1.7×10^{-7}
Plasma Triglycerides (mg dl^{-1})					
Test: lm	FR (cont)	1,14	177.0	10.65	5.5×10^{-4}
Test: aov	FR (factor)	3,12		4.43	2.6×10^{-2}
Plasma glucose (mg dl^{-1})					
Test: lm	FR (cont)	2,13	22.2	3.55	4.0×10^{-3}
	FR^2 (cont)				4.9×10^{-2}
Test: aov	FR (factor)	3,12		17.81	1.0×10^{-4}
Plasma Protein (mg dl^{-1})					
Test: lm	FR (cont)	1,14	0.13	6.31	2.4×10^{-2}
Test: aov	FR (factor)	3,12		2.30	0.13
Whole body energy (mJ kg^{-1} BM^{-1})					
Test: lm	FR (cont)	1,14	0.07	63.15	1.5×10^{-6}
Test: aov	FR (factor)	3,12		21.52	4.1×10^{-5}
Whole body lipid (%)					
Test: lm	FR (cont)	1,14	0.15	67.88	9.8×10^{-7}
Test: aov	FR (factor)	3,12		19.72	6.3×10^{-5}
Whole body protein (%)					
Test: lm	FR (cont)	1,14	0.19	12.62	3.2×10^{-3}
Test: aov	FR (factor)	3,12		4.25	2.9×10^{-2}
Whole body moisture (%)					
Test: lm	FR (cont)	1,14	0.30	29.20	9.2×10^{-5}
Test: aov	FR (factor)	3,12		12.91	4.6×10^{-4}
CTmax (°C)					
Test: lmer (AIC=50)	FR (cont)		−0.191	−0.79	0.48
	Mass (cont)		−0.003	−0.33	0.79
*Test: lmer (AIC=41)	FR (cont)		−0.256	−2.20	3.3×10^{-2}

Table 1 (continued)

		df	Slope	*f/t* value	*p* value
Test: lmer (AIC=46)	FR (factor)			1.56	0.26
Brain HSP70					
Test: lmer (AIC=69)	FR (cont)		−0.015	−0.41	0.70
	Temp (cont)		0.009	1.73	9.1×10^{-2}
Test: lmer (AIC=77)	FR (cont)		0.038	−0.37	0.71
*Test: lmer (AIC=62)	FR (cont)		0.009	1.67	9.2×10^{-2}
Mucus HSP70					
*Test: lmer (AIC=−0.55)	FR (cont)		0.20	1.36	0.20
	Temp (cont)		0.04	4.79	6.6×10^{-6}
	FRxTemp		−0.01	−1.61	0.11
Test: lmer (AIC=−8.21)	FR (cont)		−0.05	−1.60	0.14
	Temp (cont)		0.03	5.84	4.9×10^{-8}
Test: lmer (AIC=10.50)	FR (cont)		−0.06	−2.00	5.1×10^{-2}
Test: lmer (AIC=−13.07)	Temp (cont)		0.03	5.99	2.8×10^{-8}
Test: lmer (AIC=−18.7)	FR (factor)		0.43	5.05	2.7×10^{-3}
	Temp (factor)		2.67	31.18	4.4×10^{-15}
	FRxTemp		1.03	4.01	1.6×10^{-4}

FR is feed ration; (cont) or (factor) indicate whether the variable was analyzed as a continuous variable or factor, respectively; MBM is mean body mass; SGR is specific growth rate; FE is feed efficiency; rlm is robust linear model; lm is linear model; aov is anova; Tukey is Tukey's multiple comparisons; lmer is mixed effects model with tank as a random effect; df is degrees of freedom for the main effect and residual (df are not included for mixed effects lmer tests), x signifies an interaction term; AIC is Akaike Information Criteria. SGR was calculated as 100 x [ln (final mean body mass per fish in a treatment tank / initial mean body mass per fish in a treatment tank)/14]. FE was calculated as 100 x (final mean body mass per fish in a treatment tank - initial mean body mass per fish in a treatment tank)/ total amount fed per fish in that tank]. For CTmax and HSP70 analyses, the best fit model (indicated with a *) was determined based on the lowest AIC value

2.0 %BM day^{-1}, relative to 0.25 %BM day^{-1} feed ration fish (Table 2).

Critical thermal maximum and HSC/HSP70 protein levels

CTmax significantly decreased with increasing feed ration (p=0.03) (Table 1). This relationship resulted in a small magnitude of change in CTmax from 34.5±0.19 to 34.1±0.14 °C for 0.25 to 2.0 %BM day^{-1} feed ration groups, but, when feed ration was treated as a categorical variable to perform multiple comparisons in the mixed effects model, no significant differences among feed rations were detected (Tables 1 and 2).

Levels of the HSC/HSP70 protein increased in magnitude between 18 °C (acclimation temperature) and 28 °C for both mucus and brain, but the temperature effect was only significant for mucus (p<0.001 for mucus; temperature was not included in the best fit model in brain) (Table 1, Fig. 1a and b). Though, the mixed effects model for mucus HSC/HSP70 protein levels included temperature, feed ration and their interaction, only temperature was significant. However, when the model was repeated with temperature and feed ration as categorical factors, all three terms became significant (Table 1). Multiple comparisons showed mucus HSC/HSP70 protein levels to significantly increase with temperature only for the two lower feed rations (0.25 and 0.5) and only at the highest test temperatures (28 °C; Fig. 1a). There was no effect of feed ration on HSC/HSP70 protein levels in brain tissue.

Discussion

This is the first assessment of nutritional effects on high temperature tolerance of any sturgeon species. We showed reduced growth and long- and short-term energy supplies associated with low feed ration, and increased upper temperature tolerance with feed restriction.

Table 2 Mean (±SEM) sizes, condition, proximate compositions and plasma metabolites of fish sampled after 2 weeks of feed ration treatment (N=12 per ration treatment for sizes and condition; for proximate composition and plasma metabolites, N=4 replicate tanks, with three fish sampled and pooled per tank)

	Feed ration (%BM day^{-1})			
	0.25	0.5	1	2
Tank biomass-based measurements				
MBM(g)*	56.2(±0.4)^{a}	62.1(±0.3)^{b}	72.8(±0.7)^{c}	89.7(±2.2)^{d}
SGR(%BM day^{-1})*	−0.09(±0.05)^{a}	0.64(±0.04)^{b}	1.76(±0.08)^{c}	3.25(±0.17)^{d}
FE(%)*	−37.1(±19.6)^{a}	132.0(±7.6)^{b}	187.9(±8.4)^{c}	183.2(±9.8)^{bc}
Size and condition of individual fish sampled for plasma				
BM(g)*	54.0(±2.2)^{a}	59.4(±1.9)^{a}	78.7(±3.3)^{b}	92.1(±2.7)^{c}
TL(cm)*	25.2(±0.4)^{a}	25.6(±0.3)^{a}	28.0(±0.3)^{b}	28.0(±0.3)^{b}
K*	0.34(±0.01)^{a}	0.35(±0.01)^{a}	0.36(±0.01)^{a}	0.40(±0.01)^{b}
Whole body proximate analyses				
Lipid(%)*	1.60(±0.18)^{a}	1.95(±0.05)^{a}	2.44(±0.11)^{a}	3.74(±0.36)^{b}
Moisture(%)*	85.17(±0.36)^{a}	84.58(±0.42)^{a}	84.73(±0.42)^{a}	82.2(±0.26)^{b}
Protein(%)*	9.78(±0.12)^{a}	10.06(±0.29)^{a}	10.06(±0.35)^{a}	11.03(±0.26)^{b}
Energy (mJ kg^{-1} BM^{-1})*	3.16(±0.09)^{a}	3.38(±0.09)^{a}	3.49(±0.09)^{a}	4.23(±0.13)^{b}
Plasma metabolites				
Triglycerides (mg dl^{-1})*	245.8(±35.4)^{a}	423.2(±54.5)^{ab}	503.2(±70.4)^{ab}	603.0(±106.9)^{b}
Glucose (mg dl^{-1})*	21.2(±2.5)^{a}	38.0(±4.6)^{ab}	61.1(±9.4)^{bc}	79(±5.3)^{c}
Protein (g dl^{-1})*	1.24(±0.03)	1.38(±0.13)	1.40(±0.04)	1.52(±0.06)
Temperature				
CTmax(°C)*	34.5(±0.2)	34.5(±0.2)	34.4(±0.1)	34.1(±0.1)

Asterisks at variable name indicate significant main effects of feed ration on the variable. Different letters within a row demarcate a significant difference between the feed rations based on Tukey's multiple comparisons. Whole body energy was calculated as 4.18 x [(5.65 x whole body protein x 100^{-1})+9.4 x (whole body lipid x 100^{-1})+4.23 x (whole body nitrogen free extract x 100^{-1})]. See Table 1 for explanation of variable abbreviations

Nutritional status

As expected, growth rate of 100 dph green sturgeon correlated with feed ration treatment over the 2-week growth experiment. Measured growth rates were comparable with previously reported growth rates for older juvenile green sturgeon. Green sturgeon at 144 dph, weighing 184 g and reared at 19 °C on a ration of 1.3 to 2.1 %BM day^{-1} grew at a rate of 1.1–2.3 %BM day^{-1} (Mayfield and Cech 2004). This is comparable to the SGRs of 1.7(±0.1) and 3.2(±0.2) %BM day^{-1} for slightly younger (104 to 118 dph) and smaller green sturgeon reared on feed rations of 1.0 and 2.0 %BM day^{-1} during the 2-week growth experiment here. Similarly, 222 dph green sturgeon beginning at 202 g and reared for 4 weeks on rations predicted to be 12.5 and 100 % of optimum feeding rate, grew at a rate of −0.4 and 1.89 %BM day^{-1}, respectively (Haller et al. 2015). Though here, growth rate under the lowest feed ration was also negative in magnitude (−0.09(±0.1) %BM day^{-1}), mean body mass of this treatment group did not significantly differ between initial and week two measurements. This suggests that the dietary energy provided with the 0.25 %BM day^{-1} feed ration was sufficient to maintain mass, but insufficient to fuel growth in juvenile green sturgeon. Poor growth for the lowest feed ration combined with a 5-fold increase in SGR from 0.5 to 2.0 %BM day^{-1} treatments suggest the four feed ration treatments achieved a gradient in nutritional status, which was further supported by long- and short-term energy supply indices.

Similar to growth rate, at the end of the 2-week tank growth study, feed ration significantly correlated with nutritional status as seen in long-term (whole body lipid and protein) and short-term (plasma TAG, protein and

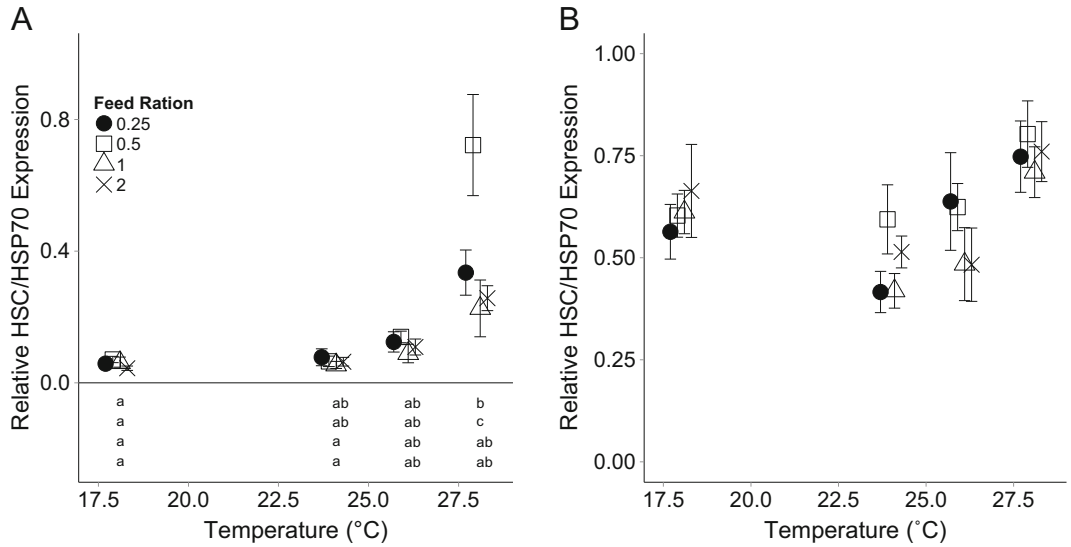

Fig. 1 Effect of feed ration (%BM day^{-1}) on relative HSC/HSP70 protein levels (mean±SEM) in mucus (**a**) and brain (**b**) of fish acclimated to 18 °C for 4 months and abruptly exposed to 18, 24, 26, and 28 °C for 2 h, followed by 2 h of recovery at 18 °C. Points within a temperature have been jittered for improved visibility. N values per point range from 6 to 12 (see methods for detailed explanation). Results of multiple comparisons of the interaction between temperature and feed ration for mucus are shown at the bottom of A. Different letters signify significant differences, at the temperature where letters are positioned, with each row representing each diet in the order: 0.25, 0.50, 1, 2

glucose) fuel substrates. For long-term storage indices, the feed ration effect was most profound on whole body lipid, which was 2.3-fold greater for 2.0 %BM day^{-1} than 0.25 %BM day^{-1} fish. Ration effects were more pronounced on short-term substrates, such that plasma TAG and glucose of 2.0 %BM day^{-1} fish were 2.5- and 3.7-fold greater for 0.25 %BM day^{-1} fish. The cumulative effect was a 1.3-fold lower total body energy for the 0.25 %BM day^{-1}, relative to 2.0 %BM day^{-1} feed ration fish.

Through limiting feed ration to 0.25 %BM day^{-1} compared to the expected optimum of 2.0 %BM day^{-1} for 2 weeks, fish acquired insufficient energy to support growth, and were left with a profound reduction in long- and short-term energy substrates resulting in reduced whole body energy. In other words, we were able to achieve a group of nutritionally deprived green sturgeon with the 0.25 %BM day^{-1} feed ration treatment.

Fish size

Through effects of feed ration on 2 weeks of growth, nutritionally deprived (0.25 %BM day^{-1}) green sturgeon were smaller than those fed higher rations (namely 2.0 %BM day^{-1}) in terms of mass and total length (TL). After the 2-week tank treatments, the lowest ration fish weighed only 59 % of the highest ration fish. Total length of fish was affected similarly to mass, with the lowest ration being only 89 % of the highest ration after 2 weeks of treatment. Due to the more rapid divergence of BM compared to TL across feed ration treatments, Fulton's condition factor (K) of the highest ration group was nearly 1.2-fold greater than for the lowest ration group at all assessed time points.

Whole body energy levels, size and condition are known to affect fish performance and survival in nature (Perrin and Rubin 1990; Biro et al. 2004; Cooke et al. 2006; Van Leeuwen et al. 2011, 2012). The only study investigating nutritional effects on green sturgeon performance found salinity tolerance was impaired with nutritional deprivation in larger and older juvenile green sturgeon (Haller et al. 2015) at the life stage expected to develop full strength seawater tolerance (Allen and Cech 2007; Allen et al. 2009, 2011). The degree of nutritional deprivation achieved in the older juvenile green sturgeon was similar to that achieved in this study. That is, the older sturgeon nearly doubled MBM and whole body energy increased 1.5-fold between the lowest and highest feed rations after 2 weeks of treatment. Though the influence of nutritional status on green sturgeon fitness-related performance is not

well studied, in the more thoroughly studied salmonids, effects of nutritional status appears to depend on the specific performance measurement. Whole body lipid stores limit juvenile rainbow trout winter survival in a Canadian lake (Biro et al. 2004). On the other hand, adult Pacific salmon (*Oncorhynchus* sp.) with high somatic energy stores and larger body size when sampled in the ocean were less likely to successfully enter rivers to migrate to their spawning grounds (Cooke et al. 2006). The combined effects of size and nutritional deprivation on the ability to endure environmental stressors like high temperature are difficult to predict.

High temperature tolerance

With downstream water conditions potentially driving fish upstream into warmer waters, high temperature tolerance can be an important performance index to the survival of juvenile green sturgeon in the SFE. Feed ration did have a small, but significant effect on critical thermal maximum (CTmax). Contrary to expectations, CTmax decreased with increasing ration. The effect was small, with a 0.4 °C decrease in CTmax from 34.5±0.2 to 34.1±0.1 °C for 0.25 to 2.0 %BM day^{-1} rations, respectively. These values are similar to a previously reported CTmax value of 34.2±0.1 °C for slightly smaller (58 g) green sturgeon acclimated to the same temperature (Sardella et al. 2008).

Though the biological significance of a 0.4 °C effect of feeding ration on CTmax is debatable, small differences in CTmax can reflect biologically significant performance differences at high temperatures. For example, northern and southern populations of the common killifish (*Fundulus heteroclitus* Linnaeus, 1766) experience high temperature extremes of 21 and 31 °C, respectively, but CTmax of the southern population is only 1.5 °C greater than that of the northern population (Fangue et al. 2006). According to aerobic scope measurements, on the other hand, the temperature of onset of declining performance may differ by as much as 3 °C between populations of killifish (Healy and Schulte 2013). Reduced high temperature tolerance for high feed ration fish may be a reflection of large metabolic demands. In juvenile coho salmon (*Oncorhynchus kisutch*, Walbum, 1792), higher diet rations were shown to elevate resting metabolic rate independently of specific dynamic action (SDA) (Van Leeuwen et al. 2012). Furthermore, after 2 weeks of starvation, the resting metabolic rate of place (*Pleuronectes platessa* Linnaeus, 1758) declined (Jobling 1980). These, non-SDA-related changes in metabolism with feed rations are likely due to reduced visceral mass in nutritionally deprived fish, as observed in fasted white sturgeon (Hung et al. 1997).

Levels of HSC/HSP70 protein in the brain after acute high temperature exposure were not significantly related to feed ration, but mucus HSC/HSP70 protein levels were significantly affected by the interaction between temperature and feed ration. Though effects of nutritional status on the cellular HSP70 protein response to warming had not previously been assessed for green sturgeon, the HSP70 response to warming has been measured in larval (Werner et al. 2007; Silvestre et al. 2010; Linares-Casenave et al. 2013), juvenile (Allen et al. 2006) and 2 kg (Wang et al. 2013) green sturgeon. Studies generally exposed sturgeon to temperatures of 24 to 28 °C, and all showed significant increases in HSP70 protein levels with high temperature. However, all but one study (Wang et al. 2013) chronically acclimated sturgeon to the high temperature before sampling for HSP70. The one methodologically comparable study to ours (Wang et al. 2013) found a 23-fold increase in mucus HSP70 protein levels after a 4 h exposure of 18 °C-acclimated 2 kg green sturgeon to 26 °C. Though the present study also showed a significant increase in mucus HSC/HSP70 protein levels with warming, an acute heat stress of raising temperature from 18 to 26 °C only invoked a 2-fold, and insignificant increase. These differences could be a reflection of differences in fish age and/or size, experimental protocol (i.e. heat shock duration of 4 h versus 2 h in the present study), antibody affinity to the HSC/HSP70 protein, and even inter-cohort variability in thermal tolerance (Linares-Casenave et al. 2013).

In terms of feed ration effects on HSC/HSP70 protein levels, Wang et al. (2013) found a significant elevation in mucus HSP70 protein levels with 7 days starvation at acclimation temperature. Here, on the other hand, there was no effect of feed ration treatment on mucus or brain HSC/HSP70 protein levels at acclimation temperature (18 °C) or most of the heat shock temperatures (24 to 28 °C for brain and 24 to 26 °C for mucus). Nutritional effects on the cellular HSP70 response to heat shock are better studied for the sympatric white sturgeon, as both starvation (Han et al. 2012) and low ration (Deng et al. 2009) were shown to reduce the HSP70 protein response in larval white sturgeon.

Conclusion

Rearing juvenile green sturgeon for 2 weeks on a ration of 0.25 %BM day^{-1} resulted in nutritionally deficient fish in terms of failure to gain mass, and low long-term and short-term energy substrates. High temperature tolerance, as assessed through measurements of CTmax, was slightly improved for low ration relative to high ration juvenile green sturgeon. Improved high temperature tolerance of low ration fish may be a reflection of physiological adjustments to reduce baseline metabolic demands in the face of low dietary energy supply. Indeed, increased mucus HSC/HSP70 protein levels in low feed ration treatments at high temperature are consistent with these physiological adjustments. Though this suggests that green sturgeon are tolerant of short periods of poor prey availability, other important performance variables, such as swimming capacity and the transition to full strength seawater tolerance, which onsets during this life stage (Allen and Cech 2007; Allen et al. 2009, 2011) should also be assessed.

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