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Effects of feed restriction on the upper temperature tolerance and heat shock response in juvenile green and white sturgeon



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ABSTRACT

The objective of the current study was to investigate the effects of feed restriction on whole-organism upper thermal tolerance and the heat shock response of green and white sturgeon to determine how changes in food amount might influence physiological performance of each species when faced with temperature stress. Two parallel feed restriction trials were carried out for juvenile green (202 g; 222-day post hatch: dph) and white sturgeon (205 g; 197-dph) to manipulate nutritional status at 12.5%, 25%, 50%, or 100% of optimum feeding rate (100% OFR were 1.6% and 1.8% body weight/day, respectively) for four weeks. Following the trials, the critical thermal maximum (CTMax, 0.3 °C/min) of sturgeon ($N = 12$ /treatment/species) was assessed as an indicator of whole-organism upper thermal tolerance. To assess temperature sensitivity, sturgeon ($N = 9$ /treatment/species) were acutely transferred to two temperature treatments (28 °C and 18 °C as a handling control) for 2 h followed by 2 h of recovery at 18 °C before being sacrificed, and gill, brain, and mucus sampled for measurements of 70-kDa heat shock protein levels (Hsc/Hsp70). Feeding rate had species-specific effects on CTMax in green and white sturgeon such that CTMax of green sturgeon decreased as the magnitude of feed restriction increased; whereas, CTMax of white sturgeon did not change with feed restriction. Elevated temperature (28 °C) and feed restriction increased Hsc/Hsp70 levels in the gill tissue of green sturgeon, while heat shock increased Hsc/Hsp70 levels in the mucus of white sturgeon. Our results suggest that green sturgeon may be more susceptible to temperature stress under food-limited conditions.

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1. Introduction

Global climate change (GCC) will profoundly change our aquatic ecosystems with projections forecasting increases in water temperature and sea level along with a multitude of changes in other physical, chemical and biological factors (IPCC, 2014). It has been well documented that increased temperature can shift geographic distributions and result in population collapses and/or local extinctions (Fields et al., 1993; Lubchenco et al., 1993; Hofmann and Todgham, 2010); however, recent studies have revealed that species' distribution and abundance will also be affected by changes in other factors such as ocean chemistry, ocean circulation, and food web dynamics (reviewed by Harley et al., 2006). To more accurately predict the vulnerability of aquatic organisms to

projected changes in local environments and GCC, an understanding of the physiological responses of aquatic organisms to concurrent changes in multiple environmental factors is critical (Todgham and Stillman, 2013).

The San Francisco Bay Delta (SFBD) has been highly modified through anthropogenic activities (e.g., water diversion, urban development, invasive species) driven by dense human settlement following the Gold Rush in California and is expected to experience continued environmental change due to GCC projections (Dasmann, 1999; Cloern and Jassby, 2012). The projected regional effects of GCC in the SFBD not only include increasing water temperature (ca. 0.3 °C per decade for the A2 scenario, Cloern et al., 2011) but also increasing salinity as a result of 1) precipitation shifts from snow to rain which result in a lower spring freshwater runoff from the Sierra Nevada mountains and 2) seawater intrusion into the SFBD associated with sea level rise (Knowles and Cayan, 2002, 2004; Cayan et al., 2008a, 2008b; Cloern et al., 2011). Modifications to local food web dynamics driven by GCC, such as declining phytoplankton production as well as disruption of trophic linkages between phytoplankton and zooplankton (Winder and Schindler, 2004; Auad et al., 2006; Boyce et al., 2010) have become

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important issues with respect to indirect effects of GCC on species' vulnerability to environmental change due to the potential for reduced energy flow among trophic levels. The SFBD is considered an estuary of low productivity in comparison to other estuaries worldwide (Cloern et al., 2014), due in large part to the introduction of the invasive Asian clam (*Corbula amurensis*) in the 1980s (Carlton et al., 1990; Cloern and Jassby, 2012). The high feeding efficiency of the Asian clam exceeds the local production of phytoplankton biomass (Thompson, 2005) and has contributed to substantial declines of fish population in the SFBD (Kimmerer, 2006; Moyle et al., 2012). Importantly, impacts to lower trophic levels and overall food availability on fishes are likely to be exacerbated by the effects of global and local climate change, including low freshwater inflow, salinity intrusion, and amplification of the drought effect by water diversions (Cloern and Jassby, 2012; Glibert et al., 2014). Thus, anticipated food web alterations driven by the anthropogenic activities occurring in the SFBD may add another stressor for aquatic organisms at higher trophic levels that manifests as a reduction in nutritional status.

Green (*Acipenser medirostris*) and white sturgeon (*Acipenser transmontanus*) are native to the SFBD and are of high ecological and economic value (Moyle, 2002). These species of sturgeon have received a lot of attention due to their vulnerability to anthropogenic activities such as overfishing, habitat alterations, and chemical contaminants (Kohlhorst, 1980; Linville et al., 2002; NMFS, 2006). The Southern Distinct Population Segment (DPS) of green sturgeon that inhabit in the SFBD (Adams et al., 2007) are currently listed as threatened under the Endangered Species Act (NMFS, 2006), and white sturgeon are listed as State S2 status (low abundance, restricted range, and potentially endangered species) in the California Natural Diversity Database (2009). Given the anticipated climate change impacts in the SFBD, environmental alterations such as decreasing food availability and increasing salinity have been recently spotlighted due to the implications for green and white sturgeon population resilience (Haller et al., 2015; Klimley et al., 2015; Lee et al., 2015; Vaz et al., 2015). Currently, there is limited information available on integrative physiological performance of green and white sturgeon when faced with increased water temperature and low food availability.

Maintenance of good nutritional status is critical because food-limited fish are more susceptible to predation (Metcalf et al., 1998; Metcalf and Steele, 2001), disease (Oliva-Teles, 2012), and other environmental factors (e.g., salinity; Haller et al., 2015; Lee et al., 2015). Energy allocation to different molecular and cellular pathways essential to the stress response can be limited in fish with reduced nutritional status (i.e., physiological trade-offs), resulting in decreased stress tolerance (Sokolova, 2013). Previous studies investigating the effects of feed restriction on salinity tolerance in green and white sturgeon revealed that osmoregulatory capacities in sturgeon are energy-dependent (Haller et al., 2015; Lee et al., 2015). Feed-restricted sturgeon that exhibited negative growth following a four-week feeding trial demonstrated a slower recovery from osmotic stress than non-feed-restricted sturgeon (Haller et al., 2015; Lee et al., 2015). Under conditions of low food availability and increasing water temperature, energy balance may be significantly shifted, potentially lowering upper thermal tolerance limits in green and white sturgeon (Verhille et al., 2015).

Since green and white sturgeon are closely related and white sturgeon have relatively large populations compared to the threatened green sturgeon, white sturgeon have been considered a surrogate model for green sturgeon for predicting possible biological responses of green sturgeon to environmental alterations. Comparative studies of white and green sturgeon from our group have shown that these species differ significantly in their sensitivity to chemical contaminants such as mercury and selenium (Lee et al., 2011, 2012; De Riu et al., 2014), to salinity challenges (Haller et al., 2015; Lee et al., 2015) and show behavioral differences to variable water velocities around water diversions (e.g., Poletto et al., 2014). Taken together, results from these studies demonstrate that green sturgeon in general are more

sensitive to environmental change than white sturgeon and therefore white sturgeon serve as a poor surrogate species for green sturgeon when it comes to understanding stress tolerance, effects of food limitation, and overall vulnerability to GCC.

The current study was conducted to test the following predictions: 1) food limitation would decrease the temperature tolerance of juvenile green and white sturgeon, and 2) food limitation would result in greater temperature sensitivity of green sturgeon. Two parallel feed restriction trials were carried out for four weeks to manipulate nutritional status of green and white sturgeon. Following the trials, feed-restricted and non-feed-restricted groups were subjected to temperature stress in order to measure critical thermal maximum (CTMax) as an indicator of whole-organism upper thermal tolerance and to measure relative 70-kDa heat shock protein levels (Hsc/Hsp70) in different tissues as an indicator of temperature sensitivity. Findings from the current study will enhance our predictive capacity on the impacts of the projected increases in water temperature and decreases in food availability derived from the global and local climate change impacts in the SFBD on performance of green and white sturgeon.

2. Materials and methods

2.1. Animal source and husbandry

Green sturgeon larvae were F2 offspring from captive F1 broodstock originating from wild-caught Klamath River Northern DPS sturgeon (Van Eenennaam et al., 2008, 2012). The captive F1 broodstock were maintained at an annual mean temperature of 18.5 ± 1.3 °C (mean \pm SD) and artificially spawned at 15 ± 0.5 °C at the Center for Aquatic Biology and Aquaculture (CABA) at the University of California, Davis, CA, USA. Fertilized eggs were incubated at 15 ± 0.5 °C until hatch and then green sturgeon larvae were transferred to stocking tanks (ca. 152 cm diameter, 45 cm height, 750 L water volume) supplied with 18.5 ± 0.5 °C flow-through degassed well water. White sturgeon larvae were acquired from domesticated broodstock at a local fish farm (Lazy Q Fish Ranch LLC, Dixon, CA, USA). White sturgeon broodstock were reared at a fish farm located near UC Davis (<25 km) at similar temperatures (ca. 18 °C) to the green sturgeon broodstock. When white sturgeon broodstock were matured, they were maintained and spawned at 13 – 15.5 °C. Fertilized eggs were incubated at 15.5 ± 0.5 °C on a recirculating hatchery system. After hatch, white sturgeon larvae were switched to a flow-through tank system with water at 18 – 19 °C for three days. Then, white sturgeon larvae (3 days post hatch; dph) were transported to CABA for rearing and experimentation.

Both green and white sturgeon larvae were reared under similar conditions until reaching a target size as juvenile fish (~170 g, approximately 6.5 to 7 months). Details of rearing practices for green and white sturgeon can be found in Haller et al. (2015) and Lee et al. (2015), respectively. All handling, care, and experimental procedures were approved by the UC Davis Institutional Animal Care and Use Committee (Protocol #16541).

2.2. Feed restriction trial

Two parallel feed restriction trials were carried out for juvenile green and white sturgeon. Juvenile green ($N = 840$, 174 ± 0.4 g (mean \pm SEM), 214-dph) and white ($N = 840$, 173.2 ± 0.6 g, 189-dph) sturgeon were randomly distributed into fiberglass tanks (ca. 750 L), resulting in 70 fish per tank and 12 tanks per species. During the acclimation period (8 days), fish were fed at an optimum feeding rate (OFR) estimated by an OFR prediction model. The OFR is defined as the rate (% body weight per day) at which growth is maximal. A prototype model developed for white sturgeon (Cui and Hung, 1995) was used to estimate an OFR for green sturgeon, since no feeding model exists for green sturgeon, and a modified prediction model (Lee et al., 2014) was applied to estimate OFR for white sturgeon. Although different models were used, the

estimated OFRs for both species were similar (1.6% and 1.8% for green and white sturgeon, respectively).

A commercial feed (Salmonid Commercial Diet 2.0 mm sinking pellet, Skretting, Tooele, UT, USA) was fed continuously throughout the day using a belt feeder (Zeigler Brothers Inc., Gardners, PA, USA) located on top of each tank. Because white sturgeon are a nibbler feeder unlike salmonids known as a meal feeder, continuous feeding is required for sturgeon to achieve optimal growth (Cui et al., 1997). Proximate composition of the feed (%), as determined through the Association of Official Analytical Chemists method (Jones, 1984), was 8.7 moisture, 42.0 crude protein, and 26.7 crude lipid. Once the feed loading was done for all tanks, water in each tank was drained quickly to ca. 50% of total volume to remove fecal matter once a day. After the 8-day acclimation period, the 12 tanks were randomly assigned to one of the four feed restriction groups (12.5%, 25%, 50%, 100% of OFR), resulting in three replicate tanks per feed restriction group. The feed restriction trial was carried out for four weeks. The average initial body weight of green and white sturgeon was 202 ± 1.5 g and 205 ± 0.9 g, respectively. After two weeks of feeding, all fish in each tank were batch weighed, and the amount of feed per tank was adjusted according to the weight change (Haller et al., 2015; Lee et al., 2015). Throughout the feeding trial, water quality was measured daily and was maintained at favorable conditions for growth (18.6 ± 0.8 °C (mean \pm SEM) and 18.3 ± 0.1 °C for green and white sturgeon, respectively; >6 mg O₂ /L for both species). Total ammonia nitrogen (NH₄⁺, NH₃) and pH were measured weekly, using a colorimetric method (Hach, Loveland, CO, USA) and a pH meter (YSI 60, YSI Life Sciences, Yellow Springs, OH, USA), respectively, and levels were maintained below 0.1 mg /L and at 7.6–8.0, respectively, for each trial. The experimental tanks were located outdoors, exposing the fish to a natural photoperiod throughout the feeding trials.

2.3. Temperature tolerance and sensitivity

2.3.1. Critical thermal maximum (CTMax)

The upper temperature tolerance of feed-restricted (12.5%, 25%, 50% OFR) and non-feed-restricted (100% OFR) green and white sturgeon juveniles were determined following standard CTMax methodology for teleost fishes (0.3 °C/min; e.g., Fanguie and Bennett, 2003) and used previously in sturgeon research (Sardella et al., 2008; Verhille et al., 2015). Following the four-week feed restriction trial, fish from each feed restriction group ($N = 12$, four fish per replicate tank for a given feed restriction group) were individually placed in one of four half-full, 57 L glass aquaria for 30 min (18.6 ± 0.5 °C, mean \pm SEM). Each aquarium was positioned parallel to each other in a water table connected to an external heating system. To enhance temperature conductivity while preventing water transfer between aquaria, each aquarium was supplemented with a heat exchanger attached to a supporting water pump situated in the water table. Aquarium temperatures were monitored using a certified alcohol thermometer (Thermo Fisher Scientific, Pittsburg, PA, USA). Following the 30-min adjustment period, temperature was increased at 0.3 °C/min and the rate in each chamber was recorded with temperature data loggers (iBCod 22L, Alpha Mach Inc. Ste.-Julie, QC, Canada). To maintain oxygen concentrations, airstones were placed in each aquarium (>5.8 mg O₂ /L at the highest temperature). Fish behaviors, including irregular swimming patterns as well as opercular movements, were monitored continuously throughout the trial. Experimental endpoint indicating CTMax was loss of equilibrium (LOE). At LOE, fish were gently moved with a probe to ensure that LOE was maintained, then immediately removed from treatment aquaria and placed in a flow-through recovery tank (18.6 ± 0.5 °C and 9 mg O₂ /L, respectively) for 2 h. Once fish regained their equilibrium they were transferred and kept in 30 L tanks individually (18.6 ± 0.5 °C and 9 mg O₂ /L, respectively) for a 24-h recovery period. Mortality was monitored throughout the recovery period, and survival was 100%.

2.3.2. 70-kDa Heat shock protein (Hsc/Hsp70) response to elevated temperature

At the end of the four-week feed restriction trial, nine fish from each feed restriction treatment ($N = 9$, three fish per feed restriction treatment per replicate tank) were transferred to one of four temperature treatments (24, 26, 28 and 18 °C as a handling control) for 2 h then recovered for 2 h (18.5 ± 0.6 °C, mean \pm SEM) to examine the effect of food restriction on the Hsc/Hsp70 response (Verhille et al., 2015). All tanks were equipped with aeration devices and monitored continuously for dissolved oxygen (>6 mg O₂ /L). Water temperature was monitored using certified alcohol thermometers (Fisher Scientific) and recorded with temperature data loggers (iBCod 22L). Following the recovery period, fish were euthanized with an overdose of buffered tricaine methanesulfonate (MS-222, 500 mg L⁻¹, Argent Inc., Redmond, WA, USA, 6 g L⁻¹ NaCl, and 420 mg L⁻¹ NaHCO₃). Fish were then weighed and measured for total length. Filaments of the second left gill arch and brain tissues were excised, blotted dry and placed in pre-labeled aluminum foil. Mucus was scraped with a scalpel blade from the left anterior side between lateral and dorsal scutes (covering epaxial muscles) and was placed in pre-labeled aluminum foil. All tissues were immediately frozen in liquid nitrogen and stored in -80 °C until subsequent analysis of Hsc/Hsp70. Preliminary Hsc/Hsp70 analysis of a subset of samples demonstrated that the two intermediate levels of feed restriction (25%, 50% OFR) and temperatures (24 °C, 26 °C) did not significantly affect Hsc/Hsp70 protein levels (data not shown) and therefore only tissue samples from sturgeon in the 12.5% and 100% OFR feed restriction groups exposed to 18 °C and 28 °C were analyzed in the present study.

Tissue samples (50–100 mg depending on tissue type) were sonicated in homogenization buffer (0.1% SDS (w/v), 190 µg/mL EDTA, 0.7 µg/mL pepstatin A, 0.5 µg/mL leupeptin, 1 µg/mL aprotinin, 20 µg/mL phenylmethanesulfonyl fluoride in 100 mM Tris-HCl buffer, pH 7.5) on ice at a ratio of 10 mg of tissue to 100 µL of buffer. Gill tissue was first homogenized (Polytron® PT 1200 E, Kinematic AG, Lucerne, Switzerland) to break up connective tissue and then sonicated. The sonicated sample was heated at 100 °C for 5 min and then centrifuged at room temperature for 5 min at 13,000g. Supernatant was transferred to a fresh tube and diluted with 5 × Laemmli's buffer (50% glycerol (v/v), 10% SDS (w/v), 0.005% bromophenol blue (w/v), 25% β-mercaptoethanol (v/v) in 0.3 M Tris-HCl buffer, pH 6.8; Laemmli, 1970). These samples were heated at 100 °C for 5 min and then stored at -80 °C before electrophoresis (samples were kept frozen for ca. 1–3 weeks). Protein concentration of the tissue homogenate was determined using the bicinchoninic acid method following manufacturer's instructions (Pierce™ BCA protein Assay kit, Thermo Scientific, Rockford, IL, USA).

Levels of Hsc/Hsp70 were measured using the discontinuous SDS-PAGE method of Laemmli (1970) followed by immunoblotting as detailed by Verhille et al. (2015) and modified from Todgham et al. (2005) and Deng et al. (2009). Amount of total protein used for SDS-PAGE varied depending on tissue type and sturgeon species: 7.5 and 13 µg (gill), 7.5 and 5 µg (brain), and 10 and 5 µg (mucus) for green and white sturgeon, respectively. A 4% stacking and a 12.5% separating gel were prepared. Prestained molecular weight makers (PageRuler™ Prestained Protein Ladder, Thermo Scientific) and a heat-shocked HeLa cell lysate (Enzo Life Sciences, Farmingdale, NY, USA), as an internal standard (referred to as 'standard'), were added to every gel to standardize between gels. Concentrations of total protein for each tissue and the internal standard were determined on the basis of standard curves developed for each tissue to ensure protein loaded fell within the linear range of detection. Proteins were separated by SDS-PAGE at 75 V for 20 min followed by 175 V for 45–60 min, using the Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA, USA). Following separation, proteins were transferred to nitrocellulose membranes (0.45 µm, Bio-Rad) for immunoblotting using a Trans-Blot Turbo™ Blotting system (Bio-Rad) at 25 V and 1 A for 35 min with transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS (w/v), 20% methanol (v/v), pH 8.9–9.0). After transfer,

membranes were blocked in blocking solution, containing 2% skim milk (w/v), 0.05% sodium azide (w/v), and Tween-20 Tris-buffered saline (TTBS; 20 mM Tris-HCl, 0.14 M NaCl, 0.1% Tween-20 (v/v)), for 30 min, dried and then stored overnight at room temperature. The following day, membranes were blocked in blocking solution for 30 min and then incubated with a primary antibody for 90 min with light agitation. The primary antibody was prepared using a rabbit antibody to rat Hsp70 (Hsc/Hsp70, ADI-SPA-757-F, Enzo Life Sciences) diluted 1:500 with blocking solution. Following three 5-min washes in TTBS, and membranes were incubated with a secondary antibody for 90 min with light agitation. The secondary antibody was prepared using a horseradish peroxidase-conjugated goat antirabbit IgG (Pierce® Antibody, 31460, Thermo Scientific) diluted 1:4000 with TTBS. Membranes were then washed twice with TTBS for 5 min and once with Tris-buffered saline for 5 min before being developed using chemiluminescence protein detection following manufacturer's instructions (Clarity Western ECL Substrate, Bio-Rad) and imaged (ChemiDoc™ XRS + System with Image Lab™ Software, Bio-Rad). Imaged protein band densities were expressed as Hsc/Hsp70 levels relative to standard. All chemicals, unless otherwise noted, were supplied by Thermo Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St. Louis, MO, USA) at >98% purity.

2.4. Statistical analysis

Statistical analyses were performed using a statistical software package (SAS, version 9.4, SAS Institute, Cary, NC, USA). Data evaluation for assumptions including normality and homogeneity of variation, using Shapiro–Wilks and Levene's tests, respectively, did not show violations of these assumptions ($p > 0.05$).

Before conducting subsequent statistical analyses using two-way analysis of variance (ANOVA) on CTMax and Hsc/Hsp70 data, the tank effect on growth data was tested and showed no significant difference ($p > 0.05$) among the replicated tanks within the same treatment. This supports the assumption that the responses of different fish in given tanks are statistically independent and the data from different tanks were pooled for two-way ANOVA. CTMax data were analyzed using two-way ANOVA to assess the main effects of feed restriction (four levels: 12.5%, 25%, 50%, 100% OFR) and species (two levels: green sturgeon, white sturgeon), and their interaction. Two-way ANOVAs were used to examine the main effects of feed restriction (two levels: 12.5%, 100% OFR) and temperature (two levels: 18 °C, 28 °C), and their interaction on Hsc/Hsp70 levels for each species. Multiple comparisons were performed using Tukey's honest significant difference (HSD) test with the SAS macro "pdglm800" (Saxton, 1998). Significance was tested at $p < 0.05$.

3. Results

3.1. CTMax

A significant interaction between feed restriction and species on CTMax was detected (Fig. 1). Although there was no main effect of feed restriction or species on CTMax in the more feed-restricted groups (12.5%, 25% OFR) of either species, the less feed-restricted (50% OFR) and non-feed-restricted (100% OFR) groups of green sturgeon showed a significantly higher CTMax compared to the same feed-restricted groups of white sturgeon. A linear regression fit of CTMax of green sturgeon in response to feed restriction showed that CTMax of green sturgeon in the 100% OFR group was higher than the 12.5% OFR group (p -value of the slope = 0.011; $R^2 = 0.1323$); whereas in white sturgeon, CTMax of the 100% OFR group was slightly lower than the 12.5% OFR group, although not statistically significant (p -value of the slope was greater than 0.05).

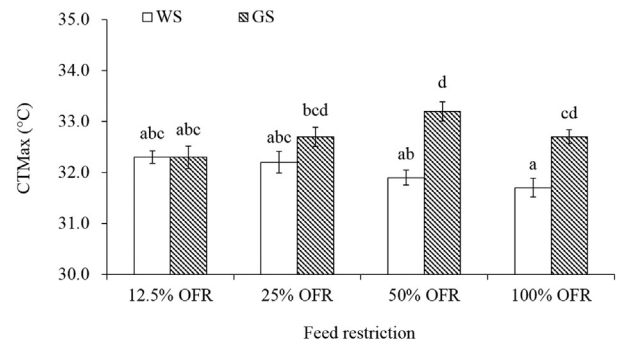


Fig. 1. Critical thermal maximum (CTMax; mean \pm SEM; $N = 12$ /treatment/species) of white (open bar; WS) and green (hatched bar; GS) sturgeon following a four-week feed restriction trial. Fish were fed at 12.5%, 25%, 50%, or 100% of optimum feeding rate (OFR). Differences in letters represent statistically significant ($p < 0.05$) differences in CTMax between feed restriction treatments and sturgeon species.

3.2. Hsc/Hsp70 protein levels

Significant main effects of feed restriction ($p = 0.0242$) and temperature ($p = 0.0015$) on Hsc/Hsp70 levels in gill tissue of green sturgeon were detected; however, there was no significant interaction ($p = 0.1425$) between temperature and feed restriction (Table 1). Overall, exposure to 28 °C significantly increased gill Hsc/Hsp70 levels and feed-restriction significantly increased Hsc/Hsp70 levels (Fig. 2A) in green sturgeon. When exposed to a 28 °C heat shock, Hsc/Hsp70 levels were increased to a greater degree in the 12.5% OFR compared to the 100% OFR feed restriction group (Fig. 2A). There was no significant effect of temperature ($p = 0.1305$), feed restriction ($p = 0.6240$), or an interaction ($p = 0.5155$) between main effects on the gill Hsc/Hsp70 levels of white sturgeon (Table 1; Fig. 2B).

There was no significant effect of temperature ($p = 0.8550$ and 0.2094 in green and white sturgeon, respectively), feed restriction ($p = 0.1577$ and 0.3865 in green and white sturgeon, respectively), or an interaction ($p = 0.8662$ and 0.8450 in green and white sturgeon, respectively) between temperature and feed restriction on Hsc/Hsp70 levels in brain tissue of either species (Table 1; Fig. 3A and B for green and white sturgeon, respectively).

There was no significant main effect of feed restriction ($p = 0.3800$) or temperature ($p = 0.6737$) on Hsc/Hsp70 levels in mucus tissue of green sturgeon, nor was there a significant interaction ($p = 0.9709$) between temperature and feed restriction (Table 1; Fig. 4A). A significant main effect of temperature ($p = 0.0086$) on mucus Hsc/Hsp70 levels was detected in white sturgeon such that increased temperature significantly increased Hsc/Hsp70 levels (Table 1, Fig. 4B).

Table 1

Two-way analysis of variance of relative levels of 70-kDa heat shock protein (Hsc/Hsp70) in gill, brain, and mucus tissues of green and white sturgeon following a four-week feed restriction trial and acute temperature exposure. P -values in bold are statistically significant ($p < 0.05$).

Species	Sources	p -Value		
		Gill	Brain	Mucus
Green sturgeon	FR ^a	0.0242	0.1577	0.3800
	Temperature ^b	0.0015	0.8550	0.6737
	FR \times Temperature	0.1425	0.8662	0.9709
White sturgeon	FR	0.6240	0.3865	0.2849
	Temperature	0.1305	0.2094	0.0086
	FR \times Temperature	0.5155	0.8450	0.2011

^a Feed restriction at 12.5% or 100% of optimum feeding rate.

^b Acute temperature exposure to 18 °C or 28 °C.

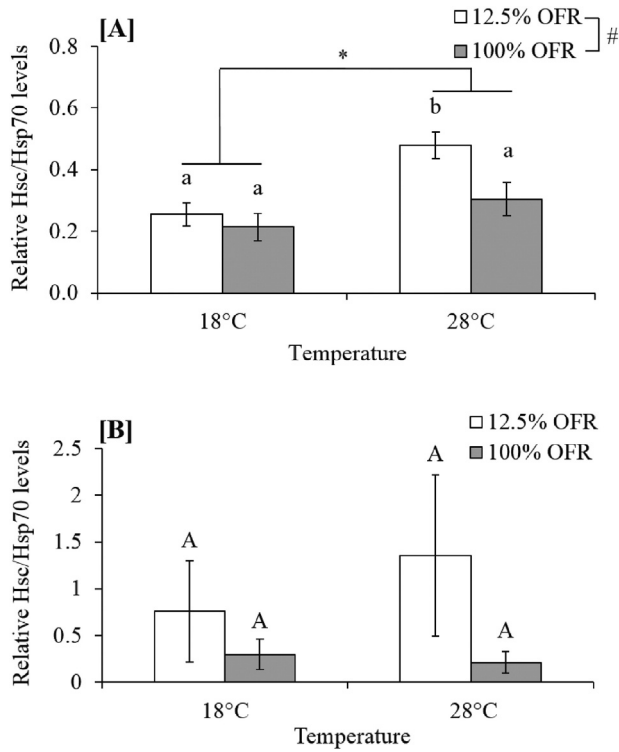


Fig. 2. Relative levels of 70-kDa heat shock protein (Hsc/Hsp70; mean \pm SEM; $N = 9$ /treatment/species) in gill tissue of green (A) and white (B) sturgeon following a four-week feed restriction trial. Hsc/Hsp70 levels were measured in the 12.5% (open bar) and 100% (filled bar) optimum feeding rate (OFR) groups following a 2 h recovery from a 2 h exposure to 18 °C or 28 °C. Differences in letters represent a statistically significant ($p < 0.05$) difference in Hsc/Hsp70 levels. Lower and upper case letters indicate significant differences within green and white sturgeon, respectively. Hash (#) and asterisk (*) symbols represent a significant main effect of feed restriction and of temperature, respectively. Note different scales on the y-axis between A and B. Different protein concentrations were used for each species, therefore, Hsc/Hsp70 levels between two species cannot be compared.

4. Discussion

The effects of feed restriction on whole-organism upper thermal tolerance and the heat shock response of green and white sturgeon were investigated to determine how changes in food availability might influence physiological performance of each sturgeon species when faced with increased temperature. In previously published studies from the same larger experiment, feed restriction impacted growth of both green (Haller et al., 2015) and white (Lee et al., 2015) sturgeon. In the current study, food availability affected whole-organism upper thermal tolerance (measured as CTMax) and the heat shock response (measured as Hsc/Hsp70 protein levels) of green and white sturgeon, with species-specific differences in the responses. CTMax of green sturgeon was significantly influenced by food availability; whereas, little effect of feed restriction on CTMax of white sturgeon was observed. In the less feed-restricted (50% OFR) and non-feed-restricted (100% OFR) groups, CTMax of green sturgeon was significantly higher than that of white sturgeon. Overall, elevated temperature (28 °C) and feed restriction increased Hsc/Hsp70 levels in the gill tissues of green sturgeon, while the heat shock increased Hsc/Hsp70 levels in the mucus of white sturgeon. These are the first comparative findings on the integrative physiological responses of green and white sturgeon to variations in food availability and increased temperature.

4.1. CTMax

The species-specific effects of feed restriction on CTMax in the two sturgeon species provide evidence that changes in food availability

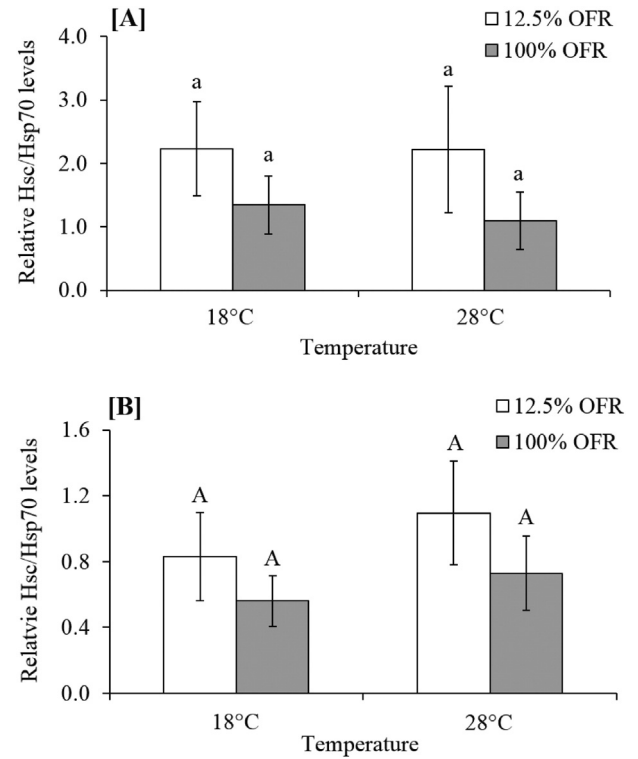


Fig. 3. Relative levels of 70-kDa heat shock protein (Hsc/Hsp70; mean \pm SEM; $N = 9$ /treatment/species) in brain tissue of green (A) and white (B) sturgeon following a four-week feed restriction trial. Hsc/Hsp70 levels were measured in the 12.5% (open bar) and 100% (filled bar) optimum feeding rate (OFR) groups following a 2 h recovery from a 2 h exposure to 18 °C or 28 °C. Differences in letters represent a statistically significant ($p < 0.05$) difference in Hsc/Hsp70 levels. Lower and upper case letters indicate significant differences within green and white sturgeon, respectively. Note different scales on the y-axis between A and B. Different protein concentrations were used for each species, therefore, Hsc/Hsp70 levels between two species cannot be compared.

may impact the upper temperature tolerance of green sturgeon. A previous study on hybrid striped bass (female striped bass, *Morone saxatilis* \times male white bass, *Morone chrysops*) demonstrated that starved fish had a lower CTMax than did satiated fish (Woiwode and Adelman, 1992). Food limitation has also been shown to affect the thermal physiology of other ectotherms such as crabs (Curtis and McGaw, 2012; McLean and Todgham, 2015). Our results are in contrast to smaller and younger green sturgeon (ca. 70 g; 121 dph) that showed a decrease in CTMax as food availability was increased (Verhille et al., 2015). Reasons for the variation in effects of food limitation on the upper temperature tolerance of green sturgeon are unknown but may be a result of the differences in the length of the feed restriction trials (2-week versus 4-week feed restriction trial for Verhille et al., 2015 study and the current study, respectively) or ontogenic changes in thermal tolerance that has been documented in other fishes due to different energy demands of different developmental stages (Pörtner and Farrell, 2008; Komoroske et al., 2014).

In contrast to green sturgeon, CTMax of white sturgeon was relatively insensitive to changes in food availability. The variation in the effects of food restriction on upper temperature tolerance of these sturgeon may be due to the fact that similar levels of food restriction led to different impacts to nutritional status of these sturgeon (Haller et al., 2015; Lee et al., 2015). Body composition changes associated with food availability can be a good indicator of nutritional status, and Bar and Volkoff (2012) provide a summary of sequential compositional changes in response to starvation as follows: “Phase I. A short transient stage where both protein tissues and fat reserves are mobilized”, “Phase II. A longer steady state with mobilization of fat as the main source of energy, that lasts until fat sources reach a critical threshold”, and “Phase III. A state

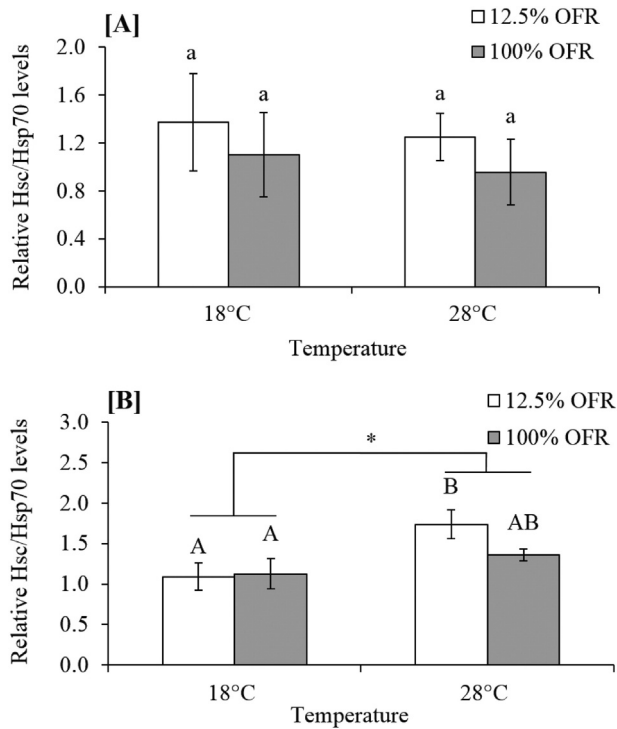


Fig. 4. Relative levels of 70-kDa heat shock protein (Hsc/Hsp70; mean \pm SEM; $N = 9$ /treatment/species) in mucus of green (A) and white (B) sturgeon following a four-week feed restriction trial. Hsc/Hsp70 levels were measured in the 12.5% (open bar) and 100% (filled bar) optimum feeding rate (OFR) groups following a 2 h recovery from a 2 h exposure to 18 °C or 28 °C. Differences in letters represent a statistically ($p < 0.05$) significant difference in Hsc/Hsp70 levels. Lower and upper case letters indicate significant differences within green and white sturgeon, respectively. An asterisk (*) symbol represents a significant main effect of feed restriction. Note different scales on the y-axis between A and B. Different protein concentrations were used for each species, therefore, Hsc/Hsp70 levels between two species cannot be compared.

in which the mobilization of protein tissue is largely increased as this becomes the main source of energy". In the current study, the most feed-restricted white sturgeon (12.5% OFR group) showed no mortality and no change in body protein content but did experience a reduction in plasma protein levels (e.g., from $24.0 \pm 1.6 \text{ g L}^{-1}$ in 100% OFR group to $17.6 \pm 0.4 \text{ g L}^{-1}$ in 12.5% OFR group) and body lipids (e.g., from $11.8 \pm 0.2\%$ in 100% OFR group to $9.0 \pm 0.3\%$ in 12.5% OFR group) following the four-week feed restriction trial (Lee et al., 2015). In contrast, green sturgeon exposed to the same feed restriction treatments exhibited some mortality and a significant loss of body protein (e.g., from $12.1 \pm 0.4\%$ in 100% OFR group to $9.5 \pm 0.1\%$ in 12.5% OFR group) accompanied with a substantial decrease in plasma protein (e.g., from $16.1 \pm 1.2 \text{ g L}^{-1}$ to $8.8 \pm 0.1 \text{ g L}^{-1}$ in 12.5% OFR group) and body lipids (e.g., from $3.6 \pm 0.4\%$ in 100% OFR group to $1.4 \pm 0.1\%$ in 12.5% OFR group) (Haller et al., 2015). Therefore green sturgeon may have transitioned into 'Phase III' while white sturgeon were transitioning into 'Phase II' as a result of feed restriction. In addition, the most feed-restricted green sturgeon lost approximately 10% of their initial body weight following the feed restriction trial; whereas, the most feed-restricted white sturgeon lost only 3% of their initial body weight (Haller et al., 2015; Lee et al., 2015). The variation between these sturgeon species in response to feed restriction indicates that white sturgeon appear better able to maintain body condition during feed restriction and therefore likely had the energy reserves available to cope with increased temperatures. A previous study using a different CTMax methodology (e.g., $0.1 \text{ }^\circ\text{C/min}$) and feeding regime (fed twice a day to satiation) on shortnose sturgeon (*Acipenser brevirostrum*; Zhang and Kieffer, 2014) showed an effect of body size on the upper thermal tolerance (i.e., CTMax increased as body size increased). Such the relationship was not detected in either green sturgeon or white

sturgeon (data not shown). Lastly, as a truly anadromous species, green sturgeon may have greater energy demands during juvenile development than the semi-anadromous white sturgeon (Doroshev, 1985; Moyle, 2002). Green sturgeon used in the current study were at the size previously shown to undergo the necessary physiological transformations to prepare for migration to full-strength seawater (Allen et al., 2011), and this likely leads to relatively high energy expenditures for basal maintenance (i.e., reduction in the aerobic scope) compared to white sturgeon (Haller et al., 2015; Lee et al., 2015). Further investigations are needed to examine whether the magnitude of changes in the aerobic scope would differ between the two sturgeon species at a similar stage of development with acclimation to elevated temperature, in association with feed restriction. Previous studies in green sturgeon have demonstrated that metabolic rate increases with temperature (Sardella et al., 2008) and it would be useful to better understand whether green sturgeon become oxygen limited at a lower temperature than white sturgeon when food restricted (Pörtner and Farrell, 2008). This could be particularly important for the juvenile life stage undergoing transitions from freshwater to seawater on their outward migration out of the SFBD and into the coastal ocean.

In the current study, we observed that when feed was not restricted (100% OFR) or restricted by half (50% OFR) green sturgeon had a significantly higher CTMax than white sturgeon. It has been well documented that congeners of fish (e.g., cyprinid *Danio* species: *Danio rerio*, *Danio albolineatus*, *Danio choprae*; Sidhu et al., 2014) and different fish populations within a species (e.g., common killifish: *Fundulus heteroclitus macrolepidotus*, *Fundulus heteroclitus heteroclitus*; Fangue et al., 2006; Healy and Schulte, 2012) can show variation in upper thermal tolerance. Acclimation to different temperatures is also known to affect thermal tolerance in fishes (e.g., Ziegeweid et al., 2008; Fangue et al., 2011); however, acclimation temperature was similar for both sturgeon and therefore unlikely to be the cause of differences in upper temperature tolerance.

4.2. Hsc/Hsp70 protein levels

Underlying biochemical (e.g., heat shock proteins; Sørensen, 2010) factors known to influence upper temperature tolerance may account for the difference in CTMax observed between green and white sturgeon. Changes in Hsc/Hsp70 levels in the various tissues of green and white sturgeon in response to food availability and increased temperature were determined in order to examine the relationship between upper thermal tolerance (CTMax) and temperature sensitivity of the heat shock response (Hsc/Hsp70 levels) to a sublethal increase in temperature. In the current study, the effects of feed restriction and elevated temperature on Hsc/Hsp70 levels were tissue- and species-specific. A more pronounced increase in Hsc/Hsp70 levels in response to a 28 °C heat shock was demonstrated in response to feed restriction in the gills of green sturgeon compared to white sturgeon. A significant effect of elevated temperature on Hsc/Hsp70 levels was detected in mucus of white sturgeon only. Similar to the CTMax results where green sturgeon's upper temperature tolerance was reduced (i.e., more temperature sensitive) with increasing restriction of food; feed restriction increased the temperature sensitivity of the gills of green sturgeon to heat stress. Similarly, CTMax was insensitive to changes in food availability in white sturgeon as was Hsc/Hsp70 levels in all tissues measured.

A previous study conducted on green and white sturgeon investigating the response of Hsp70 to either elevated temperature or food deprivation demonstrated that Hsp70 levels in gills of both sturgeon species were highly sensitive to increased temperature (e.g., 7- and 13-fold increases in green and white sturgeon, respectively; Wang et al., 2013). Highly induced Hsp70 levels in response to heat shock in the same tissues have also been reported in other fishes (e.g., fathead minnow, *Pimephales promelas* (Dyer et al., 1991); mummichog, *Fundulus heteroclitus* (Koban et al., 1991)). The discrepancy in Hsp70 responses to increased temperature between the current study and Wang et al.

(2013) may be a result of differences in age, size, and heat shock experimental protocol (i.e., 8-h exposure to increasing temperature from 18 to 26 °C at a rate of 4 °C/h in the previous study versus 2-h acute exposure to 28 °C in the current study). There is little information available on the interactive effects of food availability and temperature on changes in gill Hsp70 levels in other fishes; however, Cara et al. (2005) reported the synergistic effect of starvation and heat stress on whole-body Hsp70 levels in rainbow trout (*Oncorhynchus mykiss*). These results may suggest that food limitation may increase sensitivity of Hsp70 to heat stress, which can be associated with alterations in protein homeostasis. A significantly reduced whole-body protein content was detected in the most feed-restricted green sturgeon (12.5% OFR group) compared to that of the non-feed-restricted green sturgeon (100% OFR group); whereas, no such trend was detected in white sturgeon (Haller et al., 2015; Lee et al., 2015). Because we only examined the whole-body composition changes responding to different levels of feed restriction, further investigation on changes in nutrient compositions and metabolism in gills of green and white sturgeon in association with the combined stressors may enhance our understanding of species-specific responses.

The effect of feed restriction in the absence of temperature change on Hsc70 and Hsp70 levels in fishes have been variable. The small change in Hsc/Hsp70 levels in the gills of green and white sturgeon associated with food availability at ambient temperatures was similar to what has been reported in food-deprived sturgeon (Wang et al., 2013). This is in contrast to previous studies that have shown that food deprivation elicited increases in Hsp70 levels in the whole body of gilthead sea bream (*Sparus aurata*) and rainbow trout (Cara et al., 2005) and in the liver of Indian major carp (*Labeo rohita*; Yengkokpam et al., 2008). These findings can be explained by proteotoxicity associated with protein denaturation and breakdown that can occur under a highly food-limited state, which serve as cues to increase the concentrations of inducible Hsp70 levels (Cara et al., 2005). Increased protein catabolism in response to food limitation is closely related to gluconeogenesis, the process to utilize gluconeogenic substrates such as amino acids to maintain circulating glucose levels (McCue, 2010). There have also been studies that have shown a positive correlation between increased Hsp70 levels and increased food availability (Deng et al., 2009; Han et al., 2012). The energy-dependence of the Hsp70 response can be attributable to increased protein synthesis, alterations in protein metabolism, and/or several ATP-dependent reactions that involve Hsp70-like proteins (Pelham, 1986) and therefore changes in food availability can affect how energy is allocated to protein homeostasis.

In the current study, we observed no effect of heat shock on Hsc/Hsp70 levels in brain of juvenile green and white sturgeon, similar to findings reported in green sturgeon fingerlings (Verhille et al., 2015). This is in contrast to previous studies showing heat-shock-induced increases in Hsp70 levels in brain tissue of other fishes, including killifish (Koban et al., 1991), fathead minnow (Dyer et al., 1991), buffalo sculpin (*Enophrys bison*), speckled sanddab (*Citharichthys stigmaeus*), and English sole (*Parophrys vetulus*) (Dietz and Somero, 1993). Similar to our previous studies on green sturgeon fingerlings (Verhille et al., 2015), there was a high degree of variability in brain Hsc/Hsp70 levels of the most feed restricted green and white sturgeon, regardless of temperature. This variability may be attributed to subtle differences in nutritional condition of feed restricted sturgeon; however, this will need to be addressed in future experiments as nutritional condition of sturgeon used in heat shock experiments was not assessed in the current study. Lastly, Hsc/Hsp70 levels in mucus of white sturgeon were significantly influenced by heat shock, while no change in Hsc/Hsp70 levels in mucus were observed in green sturgeon. Mucus is known to play an important role in body protection (Shephard, 1994). A previous study on green and white sturgeon showed that the Hsp70 response in mucus was sensitive to a variety of stressors, including heat shock, air exposure, cold shock, and feed deprivation (Wang et al., 2013). The

mechanistic underpinnings of the interspecific variation in the response of mucus Hsc/Hsp70 levels in response to heat shock and feed restriction are unknown and remain to be elucidated in white and green sturgeon.

Due to the role of molecular chaperones in general and Hsps in particular in repairing the heat-shock-induced denaturation of proteins, sensitivity of Hsps to thermal stress is believed to contribute to setting thermal tolerance limits (Somero, 2004). Numerous studies have shown a strong relationship between Hsps and CTMax in fishes (e.g., Nakano and Iwama, 2002; Fanguie et al., 2011); however, there have also been many studies documenting a lack of relationship between Hsp levels and upper temperature tolerance in fishes (e.g., Fanguie et al., 2006; Healy and Schulte, 2012). In the current study, the Hsc/Hsp70 response to feed restriction, heat shock, or a combination of the two factors were highly variable among the measured tissues of green and white sturgeon. There was a stronger relationship between the Hsc/Hsp70 response and CTMax levels in green sturgeon than white sturgeon; however, this relationship was still relatively weak. The relationship between Hsc/Hsp70 levels and CTMax in association with nutritional status is not straightforward and additional studies are needed to understand the complex interactions between feed restriction and elevated temperature in sturgeon.

5. Conclusion

Understanding physiological performance of organisms in response to concurrent changes in multiple environmental factors, driven by global and local climate change, is necessary to make informed conservation and management decisions of our aquatic resources (Todgham and Stillman, 2013). Due to current and projected food web alterations and temperature increase in the SFB, investigating the effects of feed restriction on whole-organism upper thermal tolerance (measured as CTMax) and the temperature sensitivity of the heat shock response (measured as Hsc/Hsp70 protein levels) of green and white sturgeon is necessary to determine how changes in food availability might influence physiological performance of each sturgeon species when faced with increased temperature. Similar to previous studies reporting species-specific differences in sensitivity to chemical contaminants (Lee et al., 2011, 2012; De Riu et al., 2014) and changes in water velocities near water-diversion fish screens (Poletto et al., 2014), green sturgeon may also be less tolerant than white sturgeon to temperature stress when food is limited. As a result, concurrent environmental alterations including increasing temperature and reduced food availability driven by anthropogenic activities may have a relatively larger impact on resilience of the threatened green sturgeon.

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