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Temperature stress induces notochord abnormalities and heat shock proteins expression in larval green sturgeon (*Acipenser medirostris* Ayres 1854)

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Summary

The effects of thermal stress on survival, development and heat shock protein (hsp) expression of green sturgeon (GS) yolk-sac larvae, from hatching through yolk depletion were investigated to provide insight into effects of highly altered natural river hydrographs. Hatched GS larvae were reared at constant water temperatures 18°C (control) through 28°C at 2°C increments. Larval survival significantly decreased at 26–28°C, with 28°C being lethal. Significant proportions of deformed larvae were found at sub-lethal (20–26°C) and lethal 28°C rearing temperatures, with kyphosis (i.e. backward flexion of notochord) accounting for >99% of morphological deformities. Histological analysis of larvae preparations indicate that elevated water temperature affects notochord cell function and physiology. At rearing temperatures 20–28°C, thermal stress elicited a quick (24 h) and long lasting (yolk-sac absorption) significant over-expression of measured heat shock proteins (hsps), all of which are known components of intracellular protein repair and stabilization mechanism. Thermal sensitivity, as indicated by the incidence of abnormalities and expression of different hsps, varied significantly between crosses. Thermally tolerant progeny exhibited a short but rapid hsp72 (size in kDa) over-expression, and more pronounced hsp60 and hsp90 over-expression, than less tolerant progeny which exhibited a prolonged hsp72 and hsp78 over-expression. At environmentally relevant water temperatures bent larvae exhibited spiral swimming, which in the wild would compromise the ability of emerging larvae to forage, avoid predators, and migrate downstream, ultimately compromising survival and recruitment. Before larvae hsp content can be used as a thermal-stress biomarker for GS, field validation studies are needed.

Introduction

The green sturgeon (*Acipenser medirostris*) is distributed along the Pacific coast of North America from the Bering Sea to Baja California (Moyle, 2002; Colway and Stevenson, 2007). The closely related Sakhalin sturgeon (*Acipenser mikadoi)* inhabits the Asian Pacific coast. Both species are similar in morphology (North et al., 2002; Artyukhin et al., 2007), life history (Artyukhin and Andronov, 1990), and chromosome number (~250) in karyotypes (Vasil'ev et al., 2009; Vishnyakova et al., 2009). Historically, green and Sakhalin sturgeon were present in low numbers, and continuing fishery further reduced their abundance (Artyukhin and Andronov, 1990; Moyle, 2002; Omoto et al., 2004). Sakhalin sturgeon is listed in the Red Data books of Russia (Artyukhin and Andronov, 1990) and Japan (Omoto et al., 2004), and the southern Distinct Population Segment of green sturgeon is listed as threatened under the Endangered Species Act (Israel et al., 2004; NOAA, 2006).

Most of the information on life history, environmental physiology and reproduction of green sturgeon became available only recently (Klimley et al., 2007). Physiological studies indicate that green sturgeon is 'one of the most anadromous' species among acipenserids, capable of moving into full-strength seawater at a young age (Allen and Cech, 2007; Allen et al., 2009a,b; Sardella and Kültz, 2009). The adults migrate long distances along the coast, foraging in the bays and estuaries (Erickson and Hightower, 2007; Lindley et al., 2008). Sexually mature 15–20 year old adults return to rivers to spawn and broadcast demersal eggs (Van Eenennaam et al., 2006). The ovulated eggs of green sturgeon average 4.33 mm in diameter and have considerably more yolk compared to eggs of the other species (Van Eenennaam et al., 2006). The newly hatched larvae have a large yolk sac (yolk-rich endoderm), limited mobility, scarce pigmentation, and photonegative behaviour (Deng et al., 2002; Kynard et al., 2005). In wild Gulf sturgeon (*A. oxyrinchus desotoi*) the hatchlings hide within the spawning site gravel substrate for several days while the yolk is absorbed (Kynard and Parker, 2004). Hatchery observations of Sakhalin and green sturgeon larvae behaviour suggest that newly hatched green sturgeon may also hide within the spawning site substrate until most of the yolk is absorbed (Artyukhin and Andronov, 1990; Deng et al., 2002).

Green sturgeon are known to reproduce in the Rogue, Klamath and Sacramento rivers (Moyle, 2002). All three rivers were impounded between 1920–1960, therefore the historic spawning grounds of green sturgeon remain largely unknown. Currently, they reproduce in the lower reaches of Klamath and Rogue rivers within approximately 100 km of the river mouth (Van Eenennaam et al., 2006; Erickson and Webb, 2007). However, in the Sacramento River green sturgeon swim upstream and spawn above (RK 426) and below (RK 366) the Red Bluff Diversion Dam (Brown, 2007; Heublein et al., 2009). The Klamath River has the largest spawning run of green sturgeon, occurring from April through June (Moyle, 2002; Van Eenennaam et al., 2006). Spawning success of *A. medirostris* in the Klamath River appears to depend on regulation of river flow and the thermograph of many hydropower and irrigation dams (Van Eenennaam et al., 2005, 2006). As of the early 1960s, the average annual river temperature in the main stem of the lower Klamath River has increased at a rate 0.5°C·decade^{-1} (Bartholow, 2005). During 'dry' years, the June temperature

may exceed the critical upper level for the green sturgeon embryos (Van Eenennaam et al., 2005). Summer daily maxima may reach 26.6°C for up to 10 days per year (Bartholow, 2005), which may affect the viability of the larvae (Werner et al., 2007).

The reproductive cycle and spawning migration of green sturgeon have evolved to ensure that the seasonal timing of spawning coincides with favorable river conditions, including temperature. The effects of dams and altered river flow on sturgeon reproduction vary from complete reproductive failure (Paragamian et al., 2005) to an intermittent reproduction during the 'bonanza' years with high precipitation (Schaffer and Kohlhorst, 1999). One of the common consequences of regulated river flow is unseasonably elevated river temperature, particularly in southern habitats with an arid climate. The environmental temperature profoundly affects sturgeon gamete maturation, spawning, and early development (Dettlaff et al., 1993). Temperature optima for spawning and early stages of embryos (cleavage and gastrulation) lay within the range 10–20°C for most acipenserids (Wang et al., 1985; Dettlaff et al., 1993; Bardi and Chapman, 1998; Cech and Doroshov, 2004; Kynard and Parker, 2004). Incubation of green sturgeon eggs in constant temperatures ranging from 11 to 26°C revealed high survival and normal development to hatching at 11–17°C, but the percentage of abnormalities increased and hatching success decreased at 17.5–22°C, with the higher temperatures being lethal at gastrulation (Van Eenennaam et al., 2005). The effect of temperature on development of yolk-sac larvae has been evaluated in only a few species of sturgeon (Wang et al., 1987; Hardy and Litvak, 2004), in spite of the critical importance of this vulnerable stage for stock recruitment (Blaxter, 1992). Previous studies with *A. medirostris* revealed an acute effect of 26°C on axial skeleton deformities concomitant with up and down regulation of synthesis of several proteins, including high expression levels of hsp72 and hsp78 (Werner et al., 2007), Collagen II and hsp 90 (Silvestre et al., 2010) in yolk-sac larvae.

Adaptive cellular responses to thermal stress include hsp induction and expression (Lindquist, 1986). Over-expressed hsps act as chaperons, rescuing and refolding damaged proteins to restore normal cellular function (Bosch et al., 1988; Iwama et al., 1998; Feder and Hofmann, 1999). Adaptive significance of hsps to environmental temperature is evident in closely related species (and individuals within a species) along the temperature gradients (Dietz and Somero, 1992; Somero, 2002). In fish, the ability of hsp *de novo* over-expression in response to thermal stress is acquired during embryo development, before hatching (Werner et al., 2001).

The scarcity of information on hsps in sturgeons, together with the adaptive significance of the hsp response to cope with thermal stress prompted us to include hsp responses in the present study.

In this study we exposed the yolk-sac larvae of two progenies, from hatching through yolk depletion, to a range of temperatures up to 28°C, in order to compare effects of different thermal stresses on survival, development, and hsp expression. We also provide histological evidence of larval notochord deformities in green sturgeon caused by thermal stress.

Materials and methods

Spawning and egg incubation

Newly hatched green sturgeon *A. medirostris* larvae (developmental Stage 36; Dettlaff et al., 1993) were obtained from two single full-sib crosses of wild-caught Klamath River GS broodstock provided by the Yurok Tribe gillnet fisheries. Larval staging was done using external morphological characteristics described for developmental staging of Russian sturgeon (*A. gueldenstadtii*) and green sturgeon (*A. medirostris*) (Dettlaff et al., 1993; Deng et al., 2002). Broodfish were caught during the 2003 spawning run on April 23–24 (Cross 1) and May 14–15 (Cross 2). Captured broodfish were transported (278 miles) to the Center for Aquatic Biology and Aquaculture facilities (University of California, Davis) in a 1,000-L insulated transport tank supplied with pure oxygen, 3 ppt sea salt (Instant Ocean, Spectrum Brands, Inc.) and zeolite, and placed in 4 m diameter flow-through spawning tanks. A temperature data logger probe (Onset Computer, Bourne MA) was used to monitor water temperature at one hr intervals in the river, transport tank, and spawning tanks. River water temperature on capture days remained between 9.3–10.5°C and 12.8–13.4°C for Cross 1 and Cross 2, respectively. Water temperature in the spawning tank was gradually increased to 14.5–15.0°C (at ovulation and spermiation), and in incubation jars from 15.0 ± 0.5°C at fertilization to 16.5 ± 0.5°C at hatching.

Broodstock ovulation and spermiation were induced by injection of 20 μg kg^{-1} and 10 μg kg^{-1} GnRHa [(Des-Gly^{10}, D-Ala^{6}, Pro-NHEt^{9})-LHRH; Peninsula Laboratories, Belmont, CA] respectively, and the gamete collection, artificial fertilization and egg incubation procedures followed protocols described in Van Eenennaam et al. (2008). Both males produced milt with >90% and 50% sperm progressive motility, at activation and at 3 min post-activation, respectively. Embryos were incubated at a density of 800 ml·jar^{-1} at 16.0 ± 0.5°C. When approx. 75% embryos had hatched, jar contents were released into 3 ft diameter flow-through tanks. Only newly hatched larvae (Stage 36) with normal external morphology were selected for experimentation.

Experimental design and procedures

Experimental water temperature exposures (constant 18, 20, 22, 24, 26 and 28°C) were applied to newly hatched larvae (Stage 36) and continued through yolk-sac depletion (Stage 45). Most defining characteristics of larvae that had reached the yolk depletion stage included well defined rudimentary dorsal scutes, a well-developed mouth, elongated barbels, well differentiated stomach and intestine, two-lobed and large liver anterior to the stomach, and well differentiated ventral fins. Most larvae reaching stage 45 had released the pigment plug from the spiral valve.

For each cross, Stage 36 larvae were randomly selected (after >80% larvae had hatched) and assigned to water temperature treatments (100 larvae·tank^{-1}). Water temperature exposures were carried out indoors, using closed-systems with 10–20 circular fiberglass tanks each of 15-L and having a biological filter, aeration, and a temperature controller (YSI-Model 74, OH). Water temperatures were set using a certified calibrated thermometer (National Institute of Standards and Technology), and tank flow rates kept at 1.5–2.0 l min^{-1}. Natural photoperiod was artificially maintained (Time Switch, Dayton, Chicago, IL), and 75% of the tank surface was covered with shade-cloth.

Larvae acclimation to experimental temperatures (constant 18, 20, 22, 24, 26 and 28°C) was accomplished by stepwise floating individual sealed bags (100 larvae·bag^{-1} filled with pure oxygen) on the water surface of individual tanks (from

18 to 28°C), at an acclimation rate of 2°C·h^{-1}. We used four replicate tanks and one sampling tank per temperature treatment. After acclimation, water temperature was maintained within ± 0.1°C of the target temperature. Oxygen was maintained at near saturation (>98%) and pH ranged from 8.4 to 8.9 (DANR Analytical Laboratory, Cooperative Extension, University of California Davis).

Larvae weight (±0.001 g) and length (±0.1 mm) at stocking were determined from 30 randomly selected Stage 36 larvae. We recorded daily tank larvae mortality and larvae behavior. At the experimental end point (stage 45), larvae were counted (% survival) and incidence of larvae exhibiting external morphological abnormalities recorded. The main (>99%) morphological abnormality observed was kyphosis or backward bending of the notochord, which was recorded as% larvae with mild (bent <45°), medium (bent 45–90°), and high (90–360°) degree of kyphosis (Fig. 1, top). Larvae from survival tanks were then pooled by temperature treatment for each cross, euthanized (100 ppm MS-222), rinsed, and randomly sampled (n = 30) for weight (±0.1 mg) and length (±0.1 mm). Either three (Cross 1) or two (Cross 2) samples (ca. 40 larvae) were randomly selected from pooled larvae for each temperature treatment, lyophilized, and used to determine energy content (micro-bomb calorimetry, Parr Isoperobol Calorimeter). Remaining larvae were fixed in 10% buffered formalin for photography and histology. Fixed whole larvae were dehydrated, paraffin infiltrated and embedded, sectioned at 5 μm, and stained using Gomori's trichrome stain. For hsps analysis larvae from all treatments (18, 20, 22, 24, 26, and 28°C) were sampled at 24 h and 96 h post-acclimation and at experimental end point. At each sampling, eight yolk-sac larvae from sampling tanks were netted, briefly blotted on paper towels, placed in 4 ml cryotubes and snap frozen in liquid nitrogen (−196°C). Frozen larvae were stored at −80°C until tissue extraction and subsequent hsps analyses were performed on individual larvae.

Heat shock proteins

Hsp60, 70 and 89 were analyzed using western blotting techniques as described by Werner et al. (2001, 2007). Individual larvae were homogenized on ice in a hypotonic solution containing protease inhibitors, pelleted by centrifugation at 4000 g (30 min), and relative protein concentration in supernatant determined by colorimetric assay (DC Protein Assay; Bio-Rad #500-0116) based on the Lowry method (1951). Sample proteins (25 μg·sample^{-1}) were separated by SDS-PAGE (Laemmli, 1970; Blattler et al., 1972). Standards included hsp60, 70 and 90 antigens (StressGen Biotechnologies Corp, Victoria, BC, Canada). Electrophoresed proteins were electroblotted onto Immobilon-P membranes, stained with Ponceau S solution, and complete transfer verified by Coomassie blue staining of gels. Proteins in membrane were probed for hsp60, 70 and 90 with 1:1000 SPA-804 (StressGen

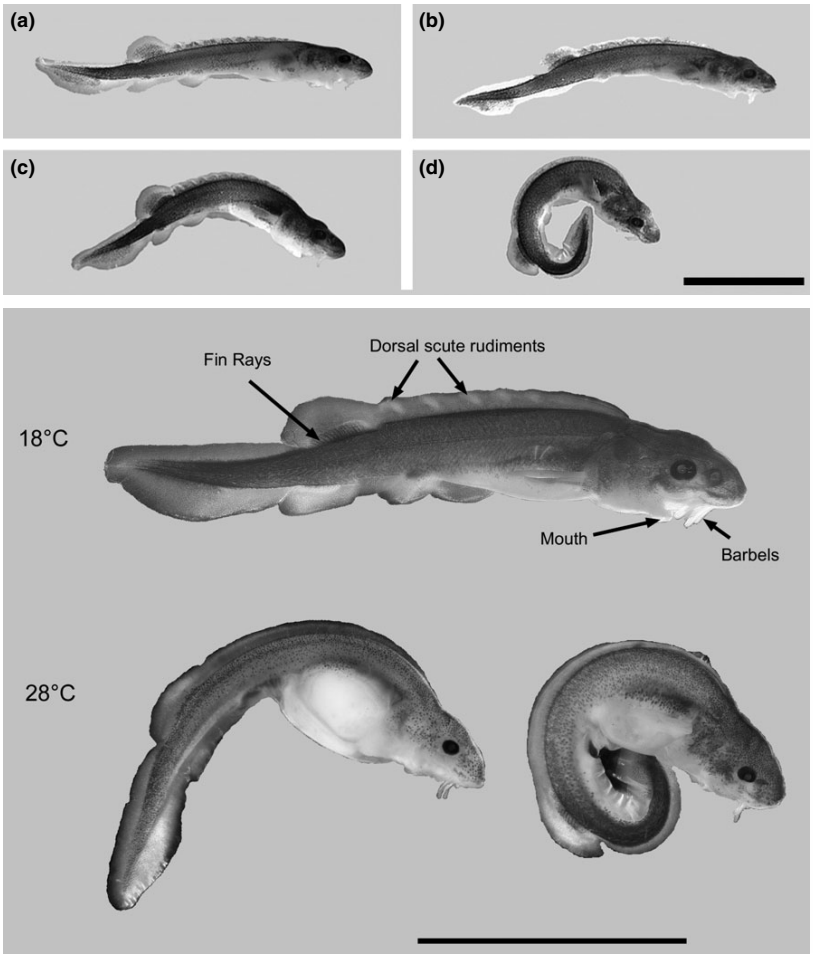

Fig. 1. Top: Photographs of *Acipenser medirostris* larvae at Stage 45 (experimental end point). (a) normal larvae; (b–d): larvae exhibiting different degrees of kyphosis: (b) mild, (c) medium, (d) severe. Bottom Plate: Photograph of Cross 2 larvae at experimental end point reared at 18°C (12 dph) and 28°C (7 dph). Scale bar = 1 cm

Biotechnologies Corp.), 1 : 500 MA3-001 (Affinity Bioreagents Inc., Golden, CO) and 1 : 500 SPA-830 (StressGen Biotechnologies Corp.), respectively. The hsp70 antibody recognized two hsp70 isoforms of 72 and 78 kDa. Hsp-antibody complexes were visualized by alkaline phosphatase conjugated to goat-anti-rabbit IgG (BioRad), and goat-anti-rat and goat-anti-mouse IgG (Sigma) for hsp60, 70 or 90 probes respectively, and a chemiluminescent enzyme substrate (CDP-Star; Tropix, Bedford, MA). Relative protein content was determined by densitometry (Biorad GS710).

Statistical analysis

All data were tested for normality (Shapiro-Wilk W test) and equality of variance (Levene test). Differences in ova diameter and hatched larvae length and weight between the two progeny were tested by *t*-test. End point data from temperature treatment 28°C was excluded from the statistical analysis since larvae development was arrested and never reached stage 45 (experiment end-point). Percent survival and proportion of survivors with abnormal morphology data were arcsine transformed prior to analysis of variance (ANOVA), whereas larvae weight (W), dry weight (DW), energy content (EC), and length (L) data were untransformed. All ANOVA were performed using the general linear model. Temperature effect on end-point survival and incidence of abnormalities was tested by a completely randomized block factorial (CRBF) design. Effect of temperature on end-point larvae DW, EC, W, L was determined by ANOVA using a completely randomized block (CRB) design with sub-samples. When significant effects of temperature were detected, pair-wise comparisons between temperature treatment means were performed using Dunnett's test with 18°C as control.

Due to limitations at the experimental facility, a single tank was available for each cross:temperature combination to sample larvae for hsp analysis. At each time-point (24 h, 96 h and YSD), the average hsp content in larvae exposed to control temperature (18°C) was used as a reference. All other hsp data were expressed as the percentage of average hsp in control treatment, and normalized by log-transformation. For each time-point, hsp data (as% of control) from eight individual larvae were used to determine the average hsp level for each cross:temperature combination. Four three-way ANOVA with time-point as repeated measure were performed to determine the effect of temperature, cross and time-point on hsp60, hsp72, hsp78, and hsp89 expression. In addition, average hsp data for each individual cross was analyzed by two-way full factorial ANCOVA with time-point as covariate. To further investigate effect of temperature on hsp expression for each time-point:cross combination, one-way ANOVAs, followed by Dunnett's test with 18°C as control were carried out using individual larvae as the experimental unit. The accepted significance level was $P < 0.05$. Data were analyzed using JMP 5.0.1 (S.A.S. Institute Inc., Cary, NC).

Results

Larval morphology and survival

At elevated temperatures (22–26°C), larvae exhibited gross abnormalities, the majority of which (>99%) were backward-bending along the rostro-caudal body axis (kyphosis), ranging from mild (>45°) to severe (360°) kyphosis as compared to straight longitudinal axis of normal larvae (Fig. 1, top plate). At yolk sac depletion, larvae reared at 18–26°C exhibited normal external morphological characteristics: dorsal scute rudiments present, well developed mouth and barbels, fin rays, and completely reabsorbed yolk sac (Fig. 1, top plate A and bottom plate 18°C). Rate of larval development after hatching was accelerated by temperature with larvae reaching stage 45 at 11.15, 9.92, 9.42, 9.00, and 8.25 days post-acclimation at 18, 20, 22, 24, and 26°C, respectively. At yolk sac depletion, survival was nearly 100% at temperatures 18–24°C, but significantly ($P < 0.01$) decreased at 26°C as compared to control 18°C (Table 1). Overall, Cross 1 larvae exhibited significantly greater thermal sensitivity than Cross 2 larvae ($P = 0.013$; block effect), data not shown. Nonetheless, survival trends in the 18–26°C range were similar for larvae from both progenies ($P = 0.775$; interaction effect). Although thermal stress negatively impacted larval survival at temperatures above 24°C (Table 1), it also significantly ($P < 0.001$) affected the incidence (Fig. 2, bar graph) and severity of kyphosis (Fig. 2, pie graph) at temperatures above 20°C. ANOVA revealed significant block effect ($P < 0.001$) and interaction, which we attribute to greater thermal sensitivity of Cross 1 larvae compared to Cross 2 larvae at 22–26°C (Fig. 3).

Abnormalities were first observed on the second day after initiation of temperature treatment at 26 and 28°C, concomitant with dramatic increases in mortalities at 28°C. By day 3, maximum proportions of abnormal larvae were observed in treatment 26°C (direct observation, data not shown). Temperature treatments 18–24°C exhibited a maximum number of abnormal larvae after four days; thereafter, a portion of larvae recovered by yolk sac depletion (direct observation, data not shown). In a follow-up experiment (Werner et al., 2007), newly hatched GS larvae were reared at constant water temperatures of 17 (control) or 26°C for 3 days then maintained at 17°C until completion of yolk-sac absorption, and the daily proportion of larvae exhibiting kyphosis recorded. After 3 days at 26°C exposure, 33% of the larvae exhibited some degree of kyphosis. After transfer to 17°C, 16.5% showed kyphosis at completion of yolk-sac absorption, which suggested a significant number of larvae had recovered.

Table 1
A. medirostris larvae mean ± SD weight (g) and length (mm) of larvae reared at 18–26°C (n = 30 larvae)

	18	20	22	24	26
Cross 1 (°C)					
Weight (g)	0.095 ± 0.003	0.100 ± 0.008	0.099 ± 0.004	0.094 ± 0.005	0.100 ± 0.006
Length (mm)	25.9 ± 0.6	26.2 ± 0.6	25.8 ± 1.0	26.0 ± 0.8	27.1 ± 0.8
Cross 2 (°C)					
Weight (g)	0.112 ± 0.006	0.117 ± 0.006	0.119 ± 0.007	0.118 ± 0.006	0.114 ± 0.007
Length (mm)	26.2 ± 0.6	26.9 ± 0.7	26.8 ± 0.7	27.3 ± 0.7	26.7 ± 0.8

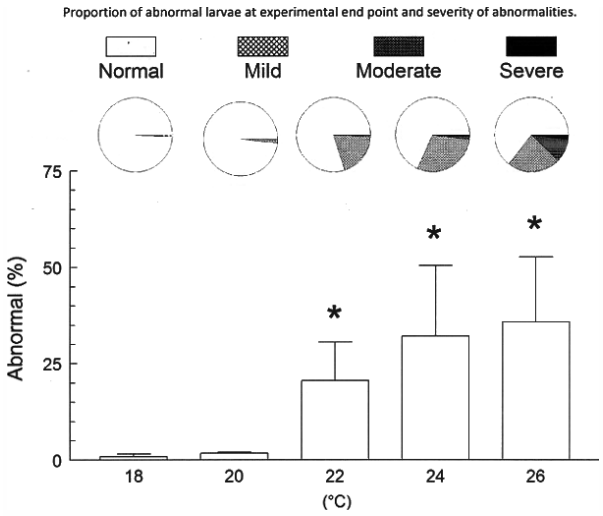

Fig. 2. Proportion of *A. medirostris* larvae (n = 2 crosses) exhibiting normal morphology and different degrees of kyphosis relative to total survivors (pie graphs). Abnormal larvae (mean ± SEM; n = 2 crosses) at yolk resorption stage (bar graph). Asterisk = significant difference ($P \leq 0.05$) from control (18°C)

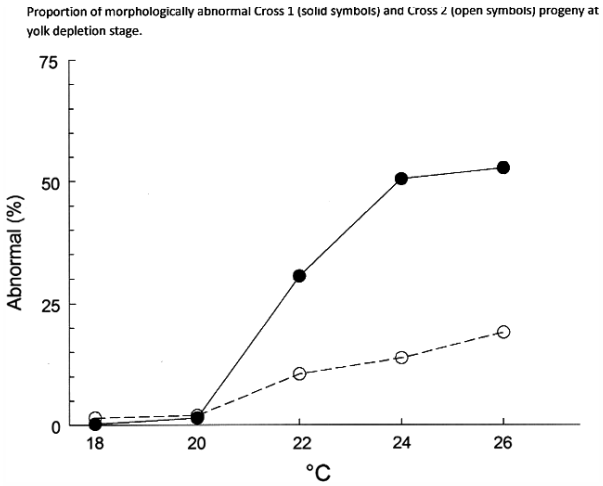

Fig. 3. Proportion of Stage 45 Cross 1 (solid symbols) and Cross 2 (open symbols) *A. medirostris* larvae exhibiting gross morphological abnormalities (Kyphosis)

A temperature of 28°C was lethal for both crosses, with highest mortalities experienced after 2–4 days of treatment. Cumulative mortalities for Cross 1 at 28°C were 0, 8, 54, 87, 97, 98, and 100% for days 1–7 post-acclimation, and for Cross 2 were 0, 30, 57, 69, 72, 80, 86 for days 1–7 post-acclimation. At 7 days post-hatch (dph), the remaining Cross 2 larvae were moribund and had arrested development indicated by lack of any stage 45 larvae characteristics and large yolk-sac (Fig. 1, Bottom plate, 28°C).

Histological preparations stained with Gomori's trichrome (Figs 4a-b) show notochord morphology of normal larvae. The notochord is a cylindrical structure composed of an outermost elastica externa (stained dark magenta), and an outer fibrous sheath (stained green) filled with notochord cells. Two distinct types of notochord cells can be distinguished, vacuolated and non-vacuolated cells. Non-vacuolated cells are small with a prominent nucleus, and form a single cell layer notochord epithelium adjacent to the fibrous sheath. The vacuolated notochord cells posses a nucleus and a large cytoplasm completely occupied by a compartmentalized vacuole. Histological sections showed abnormal constriction of the notochord in deformed larvae (Figs 4 c-d). Constricted areas contained notochord cells with a marked reduction in size, due to a dramatic reduction in the cytoplasmic vacuole, and large quantities of extra-cellular material forming strands.

Temperature (18–26°C) had no significant effects on Stage 45 larvae weight ($P = 0.335$) or length ($P = 0.502$), Table 1. Effect of temperature on Stage 45 dry weight (DW) and energy content (EC) of larvae are shown in Table 2. Temperatures 22–26°C significantly decreased DW larvae as compared to larvae reared at 20°C and 18°C (control), suggesting higher metabolism at higher water temperatures. Similar trends were observed when we measured EC, but there were no significant effects of temperature on EC. The fact that DW was a more powerful variable than live

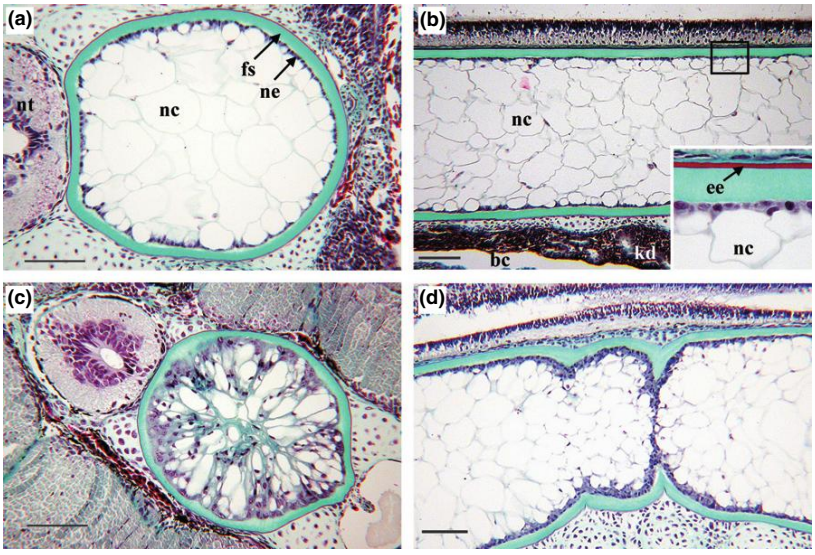

Fig. 4. Photomicrographs of 45-day-old green sturgeon (*A. medirostris*) larvae notochords. a, b: normal; c, d: abnormal. bc = body cavity; ee = elastica externa; fs = fibrous sheath; kd = kidney; ne = notochord epithelium; nc = notochord cell; nt = neural tube. Scale bar = 100 μm

Table 2
A. medirostris larvae survival, dry weight and energy content (mean ± SEM, n = 2 crosses) at experimental end point (yolk sac depletion). Larvae from survival tanks were pooled and two-three sub-samples (30–40 larvae each) randomly taken for dry weight and calorimetry

	Survival (%)	Dry weight (mg)	Energy content (kJ/larvae)
18°C	98.9 ± 0.63	12.29 ± 2.15	0.24 ± 0.04
20°C	98.9 ± 0.63	12.20 ± 2.26	0.25 ± 0.05
22°C	98.4 ± 0.9	11.76 ± 1.97*	0.23 ± 0.04
24°C	98.2 ± 0.2	11.61 ± 2.00*	0.23 ± 0.03
26°C	94.4 ± 0.4*	11.75 ± 2.10*	0.24 ± 0.05

*Asterisk = significantly different ($P \leq 0.05$) treatment means from control (18°C).

weight to detect differences between the temperature treatments reflects a greater sampling error when using live larvae.

Stage 45 Cross 2 larvae were significantly larger than Cross 1 larvae (block effect $P < 0.001$ for W, DW and EC), although this difference was not significantly ($P = 0.117$) reflected in larvae length (overall mean length 26.2 and 26.8 for Cross 1 and Cross 2, respectively). Fig. 5 shows Stage 45 larvae EC (circles) and DW (triangles) for Cross 1 (solid symbols) and Cross 2 (open symbols). Differences in size of Stage 45 larvae can be attributed to significant larger Cross 2 hatched larvae (Cross 2 larvae averaged 43.0 mg W, 18.78 mg DW, 14.7 mm L, and 0.514 kJ EC, whereas Cross 1 larvae averaged 37.1 mg W, 15.26 DW, 14.6 mm L, and 0.409 kJ) and unfertilized eggs (Cross 2 ova averaged 21.04 mg DW, 4.49 mm diameter, and 0.566 kJ EC, whereas Cross 1 ova averaged 16.62 mg DW, 4.35 mm diameter, and 0.435 kJ).

Larvae reared at constant 28°C for 7 days were moribund, and had a higher EC (87.5 ± 2.88 kJ) and DW (15.02 ± 0.02 mg) compared to larvae from the other treatments. The higher EC and DW are attributed to unutilized yolk and an underdeveloped body (Fig. 1, Bottom plate 28°C) resulting from developmental arrest.

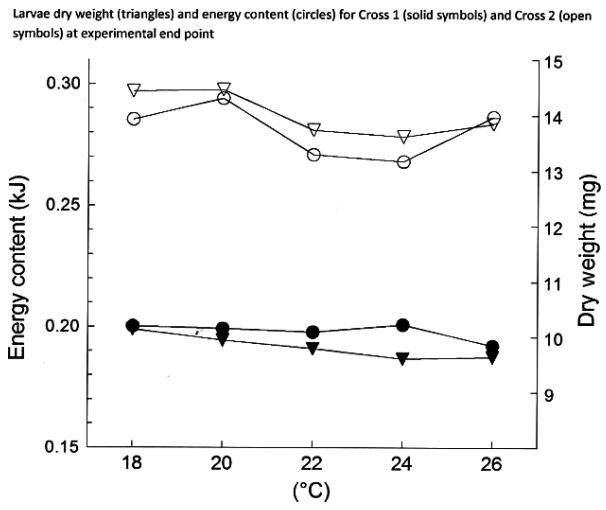

Fig. 5. Average (n = 3 sub-samples) *A. medirostris* larvae dry weight (triangles) and energy content (circles), Cross 1 (solid symbols) and Cross 2 (open symbols) at experimental end point

Hsp Expression

Analysis results of hsp expression data within each cross are shown in Table 3 and Fig. 6. Table 3 shows results of two-way full factorial ANCOVA for each progeny with time-point as covariate. For both progenies, temperature had a very strong inducing effect on hsp expression. Over time, both crosses exhibited a significant decline in hsp 60 and 89; in addition, Cross 2 also exhibited a significant decline in hsp 72. There were significant differences between crosses with respect to the nature and intensity of hsp over-expression (Fig. 6). Larvae of both crosses rapidly over-expressed hsp after exposure to all temperature treatments at and above 20°C (Fig. 6). After 96 h and at YSD, expression levels were

Table 3
Test effect (P-values) of two-way full factorial ANCOVA examining effect of temperature and time-point on hsp expression for each cross

	60	72	78	89
Cross 1				
Temperature	< 0.001	< 0.001	< 0.001	< 0.001
Time-point	< 0.001	0.315	0.250	< 0.001
Temperature × Time-point	0.297	0.810	0.732	0.003
Cross 2				
Temperature	< 0.001	< 0.001	< 0.001	< 0.001
Time-point	< 0.001	< 0.001	0.838	0.017
Temperature × Time-point	0.074	0.017	0.983	0.070

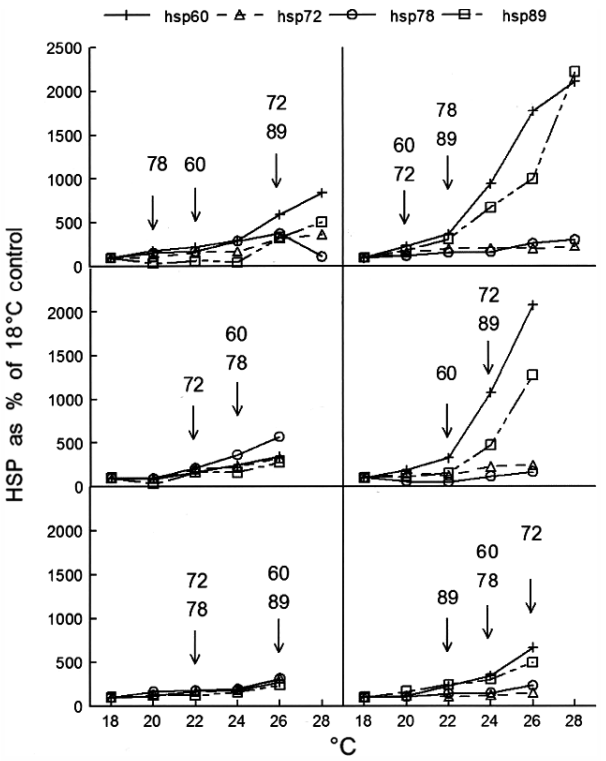

Fig. 6. Mean *A. medirostris* larvae hsp content (n = 8), 18–28°C temp. at 24 h (top) and 96 h (middle) post-acclimation, and at yolk depletion stage (bottom), Cross 1 and Cross 2 (left and right graph columns, respectively). Arrows within each cross = threshold temp. at which hsp expression is significantly different from 18°C control for each sampling time (one-way ANOVA followed by Dunnett's test, $P \leq 0.05$)

back to normal in the 20°C treatment, but hsp remained elevated at 22°C and above. Overall, Cross 2 larvae over-expressed hsp60 and hsp89 far more strongly than Cross 1 larvae at all experimental time-points. At 96 h and YSD, hsp72 and 78 levels were over-expressed at lower temperatures ($\geq$22°C) in Cross 1 than in Cross 2. Surviving Cross 2 larvae exposed to 28°C expressed all hsps at higher levels than larvae exposed to lower temperatures (data not shown).

Discussion

We present the first experimental evidence on the effect of constant temperatures (18–28°C) on survival, development and hsp response of newly-hatched green sturgeon larvae through Stage 45. Larvae survival to yolk-sac depletion was optimal at 18–20°C, sub-optimal at 24–26°C, and lethal at 28°C. Developmental rate, indicated by the time to yolk-sac depletion, was accelerated by increasing water temperature to 18–26°C, although at 28°C, larvae arrested yolk absorption and exhibited underdeveloped bodies (Fig. 1, bottom). We observed a low incidence of kyphotic larvae at 18–20°C and a significant gradual increase at 22–28°C, reaching 100% abnormal larvae at 28°C. At 24 h post-acclimation, larvae exhibited a significant over-expression of hsps at 20–28°C, which was long lasting at temperatures above 20°C (Fig. 6). Larvae from the two crosses exhibited a significantly different water temperature threshold for survival, incidence of kyphosis, and hsp expression. Cross 2 larvae exhibited significantly better survival, less incidence of axial abnormalities, and had relatively greater over expression of hsp72 at 24 h and of hsp60 and 89 at all sampling times, compared to Cross 1 larvae. At the end point, Cross 1 larvae hsp 70s remained significantly elevated above 20°C, whereas Cross 2 larvae hsp 70s remained elevated above 22 (hsp78) and 24°C (hsp72).

Acipenserids exhibit a narrow upper environmental temperature tolerance range for normal development and physiologic function, and, within their natural temperature range, their developmental rate is accelerated by an increase in the temperature environment (Dettlaff et al., 1993). In our study environmental temperature accelerated GS larvae development rate was indicated by the time required to reach yolk-sac depletion (11.2, 9.9, 9.4, 9.0, and 8.3 days at 18, 20, 22, 24, and 26°C, respectively) and resulted in survival rates greater than 95% at 18–26°C (Fig. 1). Hardy and Litvak (2004) reported increasing yolk-sac utilization and development rate in shortnose (*A. brevirostrum*) and Atlantic (*A. oxyrincus*) sturgeon yolk-sac larvae within 13–21°C. White sturgeon (WS) embryos and yolk-sac larvae originating from San Francisco Bay stock also exhibited accelerated developmental and yolk depletion rates in response to increasing water temperature (Wang et al., 1985, 1987). Thermal sensitivities of GS and WS yolk-sac larvae are indicated by the narrow (2°C) upper temperature tolerance range for normal development (GS, this study; Wang et al., 1985). Thermal sensitivity in GS and other sturgeon species decreases during ontogeny (Dettlaff et al., 1993). Green sturgeon embryos exhibited an optimal and sub-optimal thermal tolerance of 17°C and 17.5–22°C, respectively (Van Eenennaam et al., 2005), whereas GS yolk-sac larvae in our study exhibited greater optimal (20°C) and sub-optima (22–26°C) temperature tolerance. Similarity in temperature requirements for development of embryos and yolk-sac larvae of GS and WS is not surprising, since the spawning runs of these species in the Sacramento and Klamath rivers overlap.

In our studies abnormal larval morphology as a result of temperature stress was limited exclusively to kyphosis, i.e. backward-bending of the long axis (notochord or axial flexure). In sturgeons, as in paddlefish, lampreys, hagfishes, and ratfish, the notochord remains present throughout life (un-constricted notochord). The notochord is the first organ to fully differentiate during embryogenesis and is essential for the patterning of surrounding ectodermal, mesodermal and endodermal tissue (Scott and Stemple, 2005). In sturgeon the development of the notochord is initiated before neurulation and is completed by stages 20–25 (neurulation), before hatching (Dettlaff et al., 1993). In this study the notochord in GS yolk-sac larvae experienced significant growth concomitant with an overall increase in larvae length from hatching (12 mm) to the yolk sac depletion stage (23 mm). We found that thermal stress can lead to a significant dose-dependent increase in incidence of kyphosis. Tissue sections of whole lordotic GS yolk-sac larvae showed areas of constriction/narrowing in the notochord that were associated with a marked reduction in vacuolated notochordal cells as well as an increase in the number of peripheral un-vacuolated notochordal cells. It appears that temperature affected the functioning of the notochord cells, which lead to morphologic changes and a non-functional notochord. Sub-optimal environmental temperature as well as exposure to xenobiotic compounds during embryo and larvae development can result in axial deformities in amphibians, fish and other taxa. Deformation of larvae in several species of sturgeon has been related to pathological processes initiated by oxidative damage during embryogenesis (Chernyshov and Isuev, 1980). Similarly, Siberian (*A. baerii*) sturgeon larvae fed a diet containing high levels of oxidized lipid showed a substantial accumulation of lipid peroxidation products in tissue and concomitant axial malformations (Fontagne et al., 2006). Although little is known about the physiology and function of sturgeon notochord cells, we hypothesize that temperature-induced kyphosis in GS yolk-sac larvae is a result of oxidative stress, which precludes notochord cells of normal function to maintain osmotic pressure and structural integrity of the notochord sheath. Overall temperature-induced cellular damage in our study was indicated by hsp expression patterns.

The significant differences in the hsp response between crosses, and the expression patterns of individual hsp families measured in this study were reflective of larval tolerance of high water temperatures, and offer insights into the diagnostic potential of hsp measurements. Cross 2 larvae, which exhibited a lower incidence of abnormalities and better survival at high temperatures, showed a more pronounced over-expression of hsp60 and hsp89 than Cross 1 larvae. This appears to corroborate findings of a related study in green sturgeon larvae (Werner et al., 2007), where the ability of larvae to readily over-express hsp60 was shown to be associated with better protection from thermal injury. In the Werner et al. (2007) study, larvae that were unable to maintain or recover their normal morphology had lower hsp60 levels than controls and heat-resistant larvae. Attenuation of the inducibility of hsps, in particular the mitochondrial hsp60, may account for an impaired ability of cells to respond to environmental stress, and thus result in accumulation and aggregation of damaged cellular proteins, ultimately leading to cellular dysfunction. Thus, we hypothesize that a low level of hsp60 and possibly hsp89 expression in larval green sturgeon is associated with increased vulnerability to

thermal stress during development. Werner et al. (2007) also found that prolonged hsp72/78 expression was associated with reduced survival and higher incidence of abnormalities, supporting the conclusion that hsp72 and to a lesser extent hsp78 are indicative of thermal damage rather than thermal tolerance. Increased levels of cytoplasmic hsp70 may indicate an attempt of the cell to counteract the increase in levels of damaged proteins when activity of other chaperones such as hsp60 and hsp89 is insufficient (Ivanina et al., 2008). This is apparent at experimental time-points 96 h and YSD, where hsp72/78 over-expression occurs at $\geq$22°C in the more sensitive Cross 1 and at $\geq$24°C in the less sensitive Cross 2 larvae. As we observed peak incidence of kyphosis after 2–3 days of temperature treatment, similar to observation made by Werner et al. (2007), future studies should determine hsp concentration and concomitant developmental changes at short intervals during the first 3 days of temperature treatments.

Significant difference in thermal sensitivity between the two crosses was shown in this study. Gamete quality, as measured by hatching success, was similar (44.3 and 45.9% for Cross 1 and Cross 2, respectively), but Cross 2 had larger eggs/larvae that were significantly more thermo-tolerant than Cross 1 larvae, as indicated by the lower incidence of axial deformities and difference in pattern of hsp expression in response to temperature treatment. Van Eenennaam et al. (2005) attributed thermal tolerance differences between GS embryos from two single crosses to the egg quality of progeny, since brood fish were obtained the same day (i.e. same river conditions), had ova in a similar stage of maturity as indicated by oocyte polarization index (PI), and were caught in the Klamath River at ca. rkm 70. In our study, females contained ova with a similar degree of maturational competence, as indicated by identical PI (0.05) and brood fish were also caught near Klamath River rkm 70, but water temperatures experienced by Cross 1 and Cross 2 were strikingly different. Cross 2 broodstock was caught later in the season at higher river temperatures as compared to Cross 1 (8.1–9.4°C and 11.7–12.7°C for Cross 1 and Cross 2, respectively). Although controlled studies are needed to establish causality, our data indicate that the difference in temperature experienced by broodstock (and gametes) could have contributed to the thermal sensitivity differences (incidence of kyphosis and hsp expression) observed between the two crosses. Sytina and Shagaeva (1987) reported similar observations in stellate sturgeon (*A. stellatus*) embryos after finding a positive correlation between thermal tolerance of embryos and time broodstock was caught in the Volga River. In addition, studies on the hsp response have established the importance of an organism's recent thermal history in determining the temperature at which hsps accumulate in the cell (Feder and Hofmann, 1999; Werner et al., 2006). In an investigation into the mechanisms underlying the plasticity in threshold temperature for hsp induction in the eurythermal goby *Gillichthys mirabilis*, thermal acclimation changed the temperature range over which heat shock transcription factor 1 (HSF1) was activated, a key step in the regulation of hsp genes (Buckley and Hofmann, 2002).

In conclusion, we have provided experimental evidence on the effect of chronic thermal stress on survival, morphology and hsp expression of green sturgeon, *Acipenser medirostris*, yolk-sac larvae. It is concluded that elevated temperatures experienced by yolk-sac larvae can lead to death and significantly reduced growth (28°C), and deformities (22–28°C). Deleterious effects of thermal stress were closely associated with long term over-expression of hsp72 and hsp78, while the intensity of hsp60 and to some extent hsp89 over-expression appeared to be indicative of the degree of thermal protection. Notochord flexures (severity and incidence) were directly proportional to environmental temperature (22–26°C), and elevated temperatures hindered ability of notochord cells to maintain adequate osmotic pressure and collagenous sheath integrity (i.e. a functional notochord). Even mild kyphosis precluded GS yolk-sac larvae from straightforward swimming and greatly diminished their ability to swim up the water column. In the wild, impaired swimming of deformed larvae would undoubtedly compromise larvae capacity for predator avoidance, foraging, and migration to nursing grounds in lower reaches of the river system, and thus affect annual recruitment. Our results also show that hsp72/78 levels in wild yolk-sac larvae could be useful sublethal biomarkers of thermal stress and predictive of damage at the organism level; however, field studies are needed to validate this approach.

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