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The effect of temperature stress on development and heat-shock protein expression in larval green sturgeon (*Acipenser medirostris*)

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Abstract Water temperature is an important environmental variable influencing the distribution and health of coldwater fishes such as the green sturgeon, *Acipenser medirostris*. In this study, we investigated if larval sturgeon were able to tolerate or recover from acute, non-lethal temperature stress that commonly causes deformed notochords, and sought to identify the role of heat-shock proteins (hsp) in stress tolerance. The hsp response is one of the most important cellular mechanisms to prevent the damaging effects of thermal cellular stress, and differences in the ability to over-express hsps during stressful conditions may be associated with an organism's vulnerability and the extent of thermal injury. In this study, newly hatched larvae were maintained at 17°C (control), or exposed to (a) 26°C for 3 d then maintained at 17°C until yolk-sac absorption or (b) 26°C until yolk-sac absorption. Individuals with deformed notochords were counted, and hsp60, 72, 78 and 89 were analyzed in both normal and deformed larvae by western blotting. Approximately 33% of fish developed curved notochords within the first 3 d of exposure to 26°C. After transfer to cool water 16.5% showed deformities at stage 45, suggesting a significant number of larvae had recovered. Hsp levels remained elevated for at least 9 days after termination of heat-exposure. Overall, percentage of deformed larvae, and hsp72/hsp78 levels were highest in fish continuously exposed to 26°C until yolk-sac absorption. Deformed individuals had significantly higher expression levels of hsp72 and hsp78, and lower hsp60 levels than normal larvae. We conclude that expression of hsp72 and hsp78 and potentially hsp60 are linked to phenotypic variation in the response and vulnerability of green sturgeon larvae to thermal stress.

Keywords Heat-shock proteins · Thermal stress · Green sturgeon · Larval development

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Introduction

The green sturgeon (GS), *Acipenser medirostris*, is an anadromous species that inhabits the North American Pacific Ocean, from the Aleutian Islands to California. It is considered a rare or

vulnerable species in the United States and Canada (Birstein 1993; Moyle 2002; Campbell 1997), an endangered species in Russia (Artyukhin and Andronov 1990) and a candidate species by the National Marine Fisheries Service (NMFS 2003) under the U.S. Endangered Species Act. Most recently, the National Oceanic & Atmospheric Administration (NOAA) has proposed the listing of the southern population of North American green sturgeon as threatened (NOAA 2005). The southern distinct population segment encompasses the region south of the Eel River and contains the Sacramento River spawning population (Israel et al. 2004).

Water temperature is among the most important environmental variables influencing the distribution and reproductive success of fish populations (Baltz et al. 1987). In many streams of the Pacific Coast, structural modifications of the channels and changes in land use patterns have led to altered flow and thermographs with associated increases in sedimentation and water temperatures. For example, in the Klamath River, a major site of green sturgeon reproduction, seven hydroelectric dams and numerous irrigation diversions affect the river flow and anadromous fish migration. Since the early 1960s water temperatures in the Klamath River have increased by approximately 0.5°C per decade (Bartholow 2005). They range from 10 to 23°C during the spawning run of green sturgeon, which typically starts in April and extends through June (Van Eenennaam et al. 2005). Mayfield and Cech (2004) recently determined that bioenergetic performance of juvenile green sturgeon was optimal between 15 and 19°C. The preferred temperatures of age-0 sturgeon acclimated to 11 and 19°C were 15.9 and 15.7°C, respectively. In a study on the effects of incubation temperature on green sturgeon embryos, abnormal development (defined as notochord curvature at hatch) was seen at 17.5°C and above, and hatching success decreased at 20.5°C and above. All embryos died before hatch at temperatures at and above 23°C (Van Eenennaam et al. 2005).

The heat-shock protein (hsp) response is one of the most important cellular mechanisms to repair damaged proteins and prevent the damaging effects of thermal cellular stress (Feige et al. 1996; Somero 2002). Among the hsp protein families, hsp60, hsp70 and hsp90 are the most prominent groups, present in all cell types and highly conserved across phyla. These protein families can be comprised of multiple constitutively expressed and inducible isoforms. Cellular functions of these proteins include the correct folding, repair and translocation of intracellular proteins, suppressing protein aggregation and reactivating denatured proteins (Parsell and Lindquist 1993; Feige et al. 1996). They are essential for the activation of nuclear hormone receptors and components of the immune system, and interact with signaling molecules of cell cycle and cell death pathways. Altered cellular levels of hsps in response to stress can change the overall response of an organism to physiological signals (Zuegel and Kaufmann 1999; Nollen and Morimoto 2002). Increased synthesis of hsps in response to thermal cellular stress has been reported for many species of teleosts (Iwama et al. 1998) and other organisms ranging from bacteria to humans (Morimoto et al. 1990). Modifications of hsp induction and expression have also been associated with adaptive responses to environmental conditions in closely related species (Dietz and Somero 1992; Somero 2002).

There is a scarcity of information on links between hsp expression and ''higher-level'' biological (e.g. developmental) effects of environmental stressors, and no information on how geno- or phenotypic plasticity affects hsp expression and temperature tolerance within a single progeny. It has been the subject of scientific discussion whether over-expression of hsps is indicative of deleterious consequences for a given organism, or whether it signals the activation of the cellular protective response and therefore containment of and recovery from the damaging effects of stressors (e.g. Werner et al. 2005). To our knowledge, the role of hsps in thermal stress and development have not been previously investigated in acipenserids, however, in a recent study on green sturgeon (Linares-Casenave et al., unpublished data), we found that embryos and larvae from different progenies showed distinctly different hsp expression patterns in response to temperature stress within the range 22–28°C. The difference in their hsp response was accompanied by a

difference in temperature tolerance measured as the percentage of abnormally developed larvae. It was also observed that some larvae with curved notochords (Fig. 1) were able to recover after removal of the thermal stress, while others remained deformed. While this response was observed at temperatures 22–26°C, the temperature 28°C arrested development and was lethal for green sturgeon larvae.

This study was designed to test the hypotheses, that (1) larval green sturgeon are able to recover from notochord deformations caused by acute temperature stress, and (2) expression levels of hsps differ between controls, heat-vulnerable, ''abnormal'', and heat-resistant, ''normal'' larvae. Abnormal larvae were defined by persistent notochord bending following exposure to thermal stress; normal larvae were defined as those who developed normally under stressful conditions, or were able to recover from a deformed notochord after the stressor was removed. While increased expression of hsps in normal larvae would emphasize the protective effect of hsps and thus their primary role in stress tolerance, the opposite (i.e. enhanced hsp expression in abnormal individuals) result would enhance their value as sublethal indicators—or biomarkers—for the deleterious effects of thermal stress.

Fig. 1 Lateral view of stage 45 (yolk-sac absorption; Dettlaff et al. 1993) green sturgeon larvae (experimental end point) showing normal larvae (top) and larvae exhibiting different degrees of forward bending of the notochord (lordosis)

Materials and methods

Animals

One pair of mature green sturgeon adults were obtained from the Yurok Tribe gillnet fishery on 28 April 2004 (female: 52.0 kg weight, 191.5 cm fork length) and 1 May 2004 (male, 34.7 kg weight, 162.0 cm fork length). Fish were brought to the University of California Davis for artificial reproduction and subsequent incubation of embryos to hatching as described in Van Eenennaam et al. (2001). On 9 May 2004 at peak hatch, 1,500 larvae exhibiting normal morphology were randomly chosen from 0.9 m diameter tanks (16.5°C) at the UCD Putah Creek Aquaculture Facility and distributed into 15 plastic bags containing 1 l of water. Bags were filled with pure oxygen, sealed with rubber bands, placed in coolers (16°C) and transported 10 min to the UCD Center for Aquatic Biology and Aquaculture (CABA) for stocking in re-circulating systems and exposure experiments.

Exposure experiments

We used indoor water recirculating systems with biological filter, aeration, YSI thermostat, chiller, and heater. Each system had either 10 or 20 circular fiberglass tanks (28 cm diameter, 35 cm deep, flow rate 1.5–2.0 l min^{-1}). During the course of the experiment, a natural photoperiod was artificially maintained, and tanks were covered with shade-cloth (80% light blocked) because green sturgeon larvae are sensitive to light (Kynard et al. 2005). We held newly hatched green sturgeon larvae (100 larvae/tank) at constant water temperatures of 17°C (5 replicate

tanks) and 26°C (10 replicate tanks). For short-term acclimation, control animals (5 bags) were floated in tanks with water temperature at 17°C (from 16°C), while treatment groups (10 bags) were floated in a water bath where temperature was gradually changed from 17 to 26°C at a rate of 1.5°C h^{-1}. After acclimation, bags were opened, and larvae released into the tanks. Three days later on 12 May 2004, when incidence of lordosis (the forward bending of the notochord) in 26°C treatment tanks was estimated at approximately 40% (for details see below), larvae from all tanks were placed in plastic bags containing 1 l of water, filled with oxygen, and floated in their respective tanks. Larvae were not separated into normal and abnormal groups after day 3. Five randomly chosen bags from the 26°C treatment were acclimated to 17°C (1.5°C h^{-1}), and released into five tanks at 17°C (''26/17°C''), whereas the control and the ''26/26°C'' treatment group were released back into their respective tanks and maintained at 17 and 26°C until termination of the experiment. Each experimental group (17°C, 26/17°C, and 26/26°C) had four replicate tanks for quantifying mortalities and abnormalities, and one tank for sampling fish for hsp analysis. We continued rearing larvae to stage 45 (yolk-sac absorption; Dettlaff et al. 1993). Diagnostic characters for stage 45 in green sturgeon were differentiation of dorsal scute placodes, unpaired fins, and the width of mouth (Deng et al. 2002).

Temperature in each system was monitored hourly by a temperature probe with data logger (Onset Computer Corporation, Massachusetts, USA), and kept within ±0.1°C. Oxygen concentration, measured daily, was maintained above 95% saturation throughout the experiment. Water pH and ammonia concentrations measured at initiation and at completion of the experiment ranged from 8.4 to 8.8, and 0.006 to 0.048 mg l^{-1}, respectively. Estimated percentages of abnormal larvae were determined using live video recordings on day 3 of the experiment. Counts of deformed larvae were done on recorded film on eight randomly selected tanks (4 at 17°C, 4 at 26°C). At stage 45, larvae from four survival tanks in each treatment were anesthetized and euthanized with MS-222, counted, fixed in 10% buffered formalin, and examined to determine percent abnormalities (notochord bending). For hsp analysis, larvae were sub-sampled from sampling tanks of control (3 d/17°C, $n = 8$) and heat-treated (3 d/26°C) groups after 3 d (8 abnormal, 8 normal larvae), and after yolk-sac absorption from control ($n = 8$) and from 26/17°C and 26/26°C treatment groups (8 abnormal, 8 normal larvae per treatment) groups. Based on previous work (Van Eenennaam et al. 2005) few abnormal larvae were expected to develop under control conditions (17°C), therefore only normal larvae were sampled from the control tank. Samples were snap-frozen in liquid nitrogen and stored at –80°C until analysis.

Hsp analysis: Hsp60, 70 and 90 were analyzed using western blotting techniques described by Viant et al. (2003) (Fig. 2). Briefly, whole larvae (1 individual/sample) were homogenized on ice in a hypotonic solution containing 66 mM Tris–HCl (pH 7.5), 0.1% Nonidet, 10 mM EDTA, 10 mM DTT and protease inhibitors, i.e., 10 mM benzamidine, 5 mM pepstatin, 0.001% aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), then centrifuged (30 min, 4000*g*). The supernatant was collected, sample buffer (Laemmli 1970) was immediately added, and samples were heated to 95 °C for 2 min. Total protein concentration in each fraction was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, California. USA). Subsamples of equal total protein content (25 μg) were separated by SDS-PAGE on 12.5% polyacrylamide gels with 5% stacking gels (Blattler et al. 1972). Hsp60, 70 and 90 antigens (StressGen Biotechnologies Corp, Victoria, British Columbia, Canada) were applied to one lane per gel to serve as an internal standard for blotting efficiency. Proteins were separated at 25 mA per gel for approximately 1.5 h then electroblotted onto Immobilon-P membranes at constant voltage (40 V) overnight. Blotted proteins were stained with Ponceau S solution to check for transfer efficiency and consistent blotting results. Gels were stained with Coomassie blue to ensure that complete protein transfer had occurred. Membranes were blocked with 5% skim milk in 20 mM tris buffer and 0.4 M NaCl (pH 7.5) with 0.05% Tween-20 for 30 min. A polyclonal antibody for hsp60 proteins

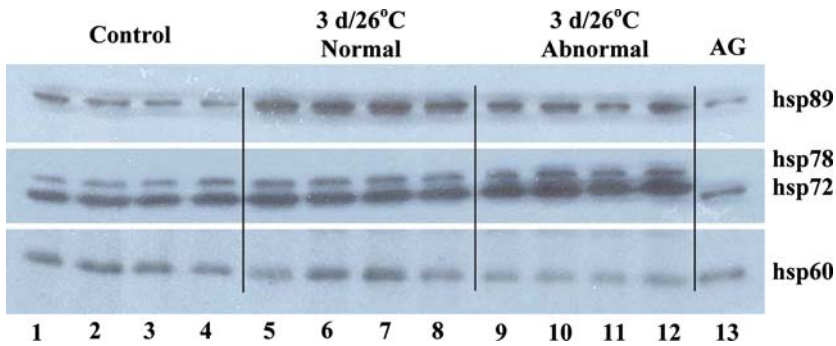

Fig. 2 Representative western blot of hsp 60, 72, 78 and 89 in individual green sturgeon larvae exposed for 3 d to 17°C (lanes 1–4), and 26°C (lanes 5–12). Lanes 5–8 are samples of normal larvae, lanes 9–12 are samples of abnormal larvae. Hsp antigens (AG; 50 ng each) were applied to lane 13 to serve as internal markers for blotting efficiency

(1:1000; SPA-804, StressGen Biotechnologies Corp.), and monoclonal antibodies for hsp70 (1:500; MA3-001, Affinity Bioreagents Inc., Golden, Colorado, USA) and hsp90 (1:500; SPA-830, StressGen Biotechnologies Corp.) were used as probes. The hsp60 antibody is directed against moth, *Heliothis verescens*, hsp60/63, and the hsp70 and hsp90 antibodies were raised against mammalian hsps. Blots were incubated for 90 min with primary antibody then washed three times for 30 min in tris-buffered saline solution containing 0.05% Tween-20. Goat-anti-rabbit (1:3000; Bio-Rad), alkaline phosphatase-conjugated goat-anti-rat IgG (1:30,000; Sigma, St. Louis, Missouri, USA), and goat-anti-mouse IgG (1:30,000; Sigma) were used to detect hsp60, 70 or 90 probes, respectively. Bound antibody was visualized by a chemiluminescent substrate (CDP-Star; Tropix, Bedford, Massachusetts, USA), and protein bands were quantified by densitometry (Bio-Rad GS710). The hsp70 antibody recognized two hsp70 isoforms of MW 72 and 78. Density measurements were then normalized to the arithmetic mean of the respective control values and expressed as percent control.

Statistical analysis

Data of temperature effect on percent survival and abnormalities at stage 45 were analyzed for four replicate tanks by one-way ANOVA followed by Tukey–Kramer HSD. Angular transformation was used to equalize variances. The accepted significance level was $P < 0.05$. Due to limitations at the flow-through facility, only one tank was available per treatment (3 d/26°C, 26/17°C, 26/26°C) to sample fish for hsp analysis, and thus fish from each treatment and the respective normal and abnormal groups were not truly statistically independent. For our ANOVA analysis, we therefore considered individual fish as the experimental unit. The normality and the homogeneity of variances of hsp expression levels were examined by treatment using Shapiro-Wilks and Bartlett's tests. To evaluate differences in hsp expression levels, data were analyzed with two-way full factorial ANOVA using fish type (control, normal and abnormal) and time point (day 4, stage 45) as factors. Tukey's HSD test was used to detect significant differences between pairs of treatments. Four ANOVAs were performed, one for each hsp (hsp60, hsp72, hsp78, and hsp89). In addition, we obtained independent data points for hsp expression by calculating the mean level of each hsp protein expressed by the eight fish sampled from each experimental chamber: three for each type (abnormal and normal) of fish, and one data point per experimental condition (3 d/26°C, 26/17°C, and 26/26°C). We examined the difference in hsp expression between normal and abnormal fish by performing general linear models with two factors: fish type and experimental condition. Two models were examined for each hsp, one treating experimental condition as a categorical variable, and another treating experimental conditions as a continuous variable with units of (days x°C). Data were analyzed using JMP 5.0.1 for Microsoft Windows.

Results

Time to stage 45 (yolk sac absorption) was 14 d in control (17°C), 12 d in the 26/17°C, and 10 d in the 26/26°C treatment groups. There was only a slight reduction in survival of temperature-stressed green sturgeon larvae with no significant difference between treatment groups. Survival was 99.5 ± 0.5% (mean ± SE, $n = 4$) in the control group (17°C), and 97.3 ± 1.1% and 97.5 ± 1.0% in the 26/17°C and 26/26°C treatment groups, respectively.

An estimated 5.5 ± 0.6% (mean ± SE, $n = 4$) of larvae in the control group showed slight deformities of the notochord after 3 d (0% on day 1 and 2), while 33 ± 1.5% (mean ± SE, $n = 4$) of larvae exposed to 26°C exhibited notochord bending. At termination of the experiment, the percentage of fish with abnormally curved notochords was 7.3 ± 1.2% (mean ± SE, $n = 4$) in controls, 16.5 ± 0.3% in the 26/17°C treatment, and 25.2 ± 0.7% in the 26/26°C treatment (Fig. 3). There were significant differences among all treatment groups in the percentage of larvae with abnormal morphology. Of fish that had developed curved notochords within the first 3 d of exposure to 26°C (33.0 ± 1.5%) approximately one half were able to recover after transfer to cool water, while the remainder had bent notochords at stage 45.

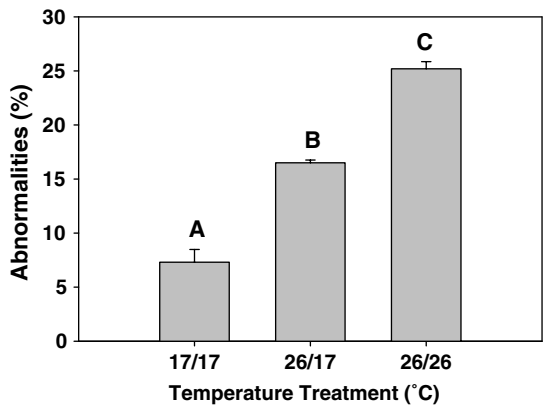

Fig. 3 Percentage of green sturgeon larvae with abnormal notochords at stage 45. Data is shown as means ± SE ($n = 4$). T-17 = control group (17°C), T-26/17 = larvae exposed to 26°C for 3 days then transferred to 17°C until yolk-sac absorption, T-26/26 = larvae exposed to 26°C until yolk-sac absorption. A, B, C indicate significantly different groups ($P < 0.05$)

Expression levels of hsps differed between control and treatment groups, as well as between abnormally developed larvae (curved notochords) and those with normal morphology (Fig. 4). After 3 d of exposure to 26°C, fish with abnormal morphology showed higher hsp72, hsp78 and hsp89 levels than control animals,

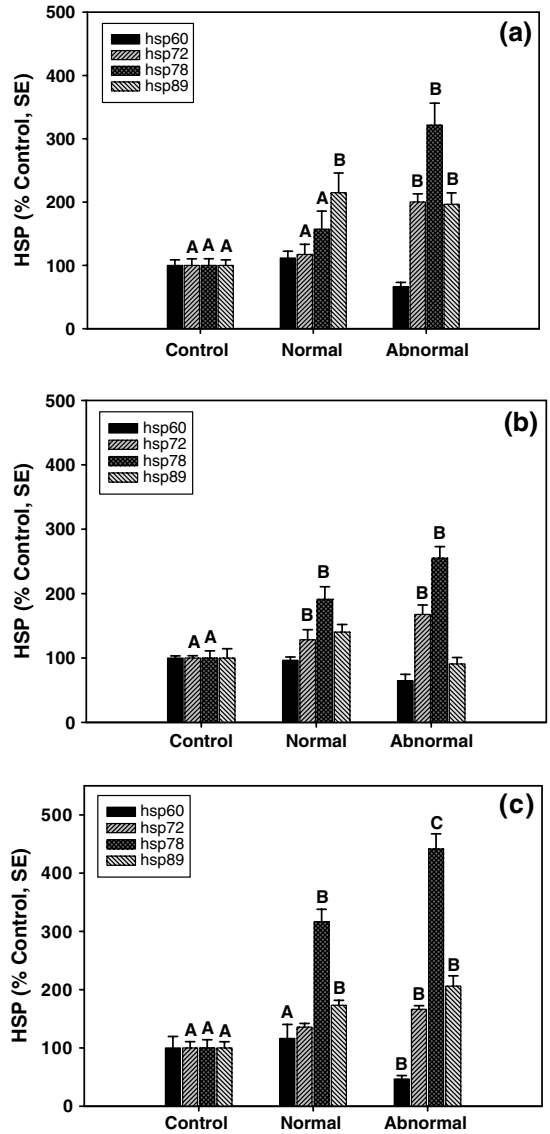

Fig. 4 Hsp protein levels shown as % control values (means ± SE, $n = 8$) in green sturgeon larvae with normal and abnormal notochords after (**a**) 3 d exposure to 26°C; (**b**) exposed to 26°C for 3 d then maintained at 17°C until yolk-sac absorption; (**c**) exposed to 26°C until yolk-sac absorption. Control animals were maintained at 17°C. Within each hsp class and graph different letters (A, B, C) indicate significantly different groups ($P < 0.05$)

while in larvae with normal morphology only hsp89 was above control levels (Fig. 4a). Both members of the hsp70 family, hsp72 and hsp78, were significantly higher in abnormal than in normal larvae. A slightly different pattern of hsp expression was seen in larvae that were returned to 17°C to recover from the 3-d heat exposure and sampled 9 days after transfer at stage 45 (26/17°C, Fig. 4b). Both normal and abnormal groups had higher hsp72 and hsp78 levels than controls, but were not different from each other. Larvae from the 26/26°C exposure group had the highest levels of hsp78 overall. Similar to the expression pattern seen after only 3 d of heat exposure, abnormal larvae had higher hsp72, hsp78 and hsp89 levels than controls (Fig. 4c), while only hsp78 and hsp89 were above control levels in normal larvae. Hsp78 was higher in abnormal than in normal larvae, and hsp60 was significantly lower than in normal larvae. Across heat treatment groups, our results show a trend for higher hsp72 and hsp78, as well as lower hsp60 expression in abnormal larvae than in normal larvae. Results obtained from a two-factorial linear model analysis (fish type, experimental condition) confirmed that hsp72 ($P = 0.023$) and hsp78 ($P = 0.027$) levels were significantly higher in abnormal than in normal larvae.

Discussion

There was considerable variation in the response and vulnerability to temperature stress of green sturgeon larvae originating from a single progeny. Notochord curvature in larvae was induced by the 3-d exposure to 26°C in only 33 ± 1.5% of exposed fish, and was reversed in 50% deformed individuals which were returned to cool (17°C) water. Thus, about half of deformed larvae were unable to recover after removal of the thermal stress. The notochord is a transient hydrostatic axial skeleton in most vertebrates, but in chondrosteans (sturgeons and paddlefish), it is retained throughout life. During embryo development the notochord is also involved in patterning and differentiation of adjacent tissues (Dettlaff et al. 1993; Cleaver et al. 2000). In sturgeon and amphibians, the notochord becomes functional before the onset of heart beat (Mookerjee et al. 1953; Dettlaff et al. 1993). The sturgeon notochord is rod-shaped and is comprised of an inner core of vacuolated notochord cells surrounded by an outer fibrous sheath (Schmitz 1998). The turgor pressure against the sheath, generated by notochord cells, provides most of the body stiffness in sturgeon and some other primitive fish (Long 1995; Long et al. 2002). A curved notochord precludes larvae from swimming straight, which is likely to increase their vulnerability to predation as well as their ability to initiate exogenous feeding. Such developmental abnormalities can result in a decrease in recruitment of larvae into wild populations.

To investigate if the observed differences in the larvae's response to temperature were reflected in the expression of hsp proteins, we compared hsp expression levels between normal and abnormal larvae exhibiting notochord curvature after exposure to high (26°C) temperature. The overall pattern of hsp72, hsp78 and hsp89 expression reflected the extent of apparent thermal stress the fish larvae were experiencing during the course of the experiment. Although both, normal and abnormal, larvae groups over-expressed these hsps in response to heat exposure, expression levels of hsp72 and hsp78 were consistently higher in abnormal than in normal larvae. Furthermore, it is interesting to note that hsp72 and hsp78 levels remained elevated for at least 9 days after heat-exposed larvae were returned to cool water (Fig. 4b), particularly in abnormal larvae. It would seem that these hsps are either relatively stable or continue to be over-expressed due to continued cellular damage. Our findings suggest that members of the hsp70 protein family are indicative of thermal injury, and may in some cases signal irreversible damage to the organism. Since hsp70 proteins were also elevated—to a lesser extent—in heat-exposed larvae with normal morphology, it is likely that a threshold value exists, above which cellular damage can no longer be contained and injury occurs. Such a threshold is difficult to quantify due to the species, tissue, and organ specific nature of hsp expression (Sanders 1993, Lele et al. 1997). In addition, individuals acclimated to warm temperatures tend to have higher hsp induction temperatures than

individuals acclimated to cooler temperatures (Buckley and Hofmann 2004).

To our knowledge, little information is currently available on a link between vulnerability to temperature stress and hsp60 expression levels in fish. In this study, larvae that were unable to maintain or recover their normal morphology had lower hsp60 levels than controls and heat-resistant normal larvae with treatment means ranging from 47 to 66% of control values. Although the statistical power of our experimental data is low and results have to be considered preliminary, the observed trend was consistent and unexpected. Hsp60 is known to be induced by exposure to high temperatures in many species, and tends to behave similarly to hsp70 in response to environmental stressors (Werner et al. 2001; Martin et al. 2002). Studies examining hsps and their role in fish development generally confirmed the protective role of hsp60 proteins (Krone et al. 1997; Werner et al. 2001; Hallare et al. 2005), and a study on their role in development of *Drosophila melanogaster* demonstrated their importance for normal embryonic development (Sarkar and Lakhotia 2005). Although we are presently unable to offer a mechanistic explanation, the results presented here are in line with previous observations on the hsp response to thermal stress in two different progenies of green sturgeon larvae. The group that showed a more intense hsp60 response was more resistant to temperature stress (Linares-Casenave et al., unpublished data). It appears that the level of hsp60 expression in larval green sturgeon may be associated with their vulnerability to thermal stress during development, but further study is needed before this hypothesis can be substantiated and confirmed.

Summary

This study demonstrated the considerable variation that exists in the vulnerability to temperature stress of green sturgeon larvae originating from a single progeny, and provided initial evidence that heat-vulnerable larvae differ from heat-resistant larvae in their expression of several groups of hsps. Approximately 33% of larvae exposed to sublethal thermal stress developed abnormally. The percentage of abnormal larvae declined to 16.5% when returned to cool water, suggesting about half of them had recovered. Exposure to 26°C resulted in the induction of two members of the hsp70 protein family, hsp72 and hsp78, as well as hsp89, but hsp60 expression was not different from or lower than control values. Comparison between hsp expression in abnormal, heat-vulnerable, and normal, heat-resistant larvae demonstrated a consistent trend for higher hsp72 and hsp78 levels in abnormal than in normal larvae in response to temperature stress, while hsp60 expression in abnormal larvae was lower than in normal larvae. Our findings suggest that members of the hsp70 protein family are indicative of thermal injury, and may in some cases signal irreversible damage to the organism. We presently do not understand the mechanism of how hsp60 expression may be associated with temperature vulnerability of green sturgeon larvae, and further study is needed before conclusions can be drawn. Results of this study will assist in the interpretation of future studies on thermal stress involving hsp measurements.

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