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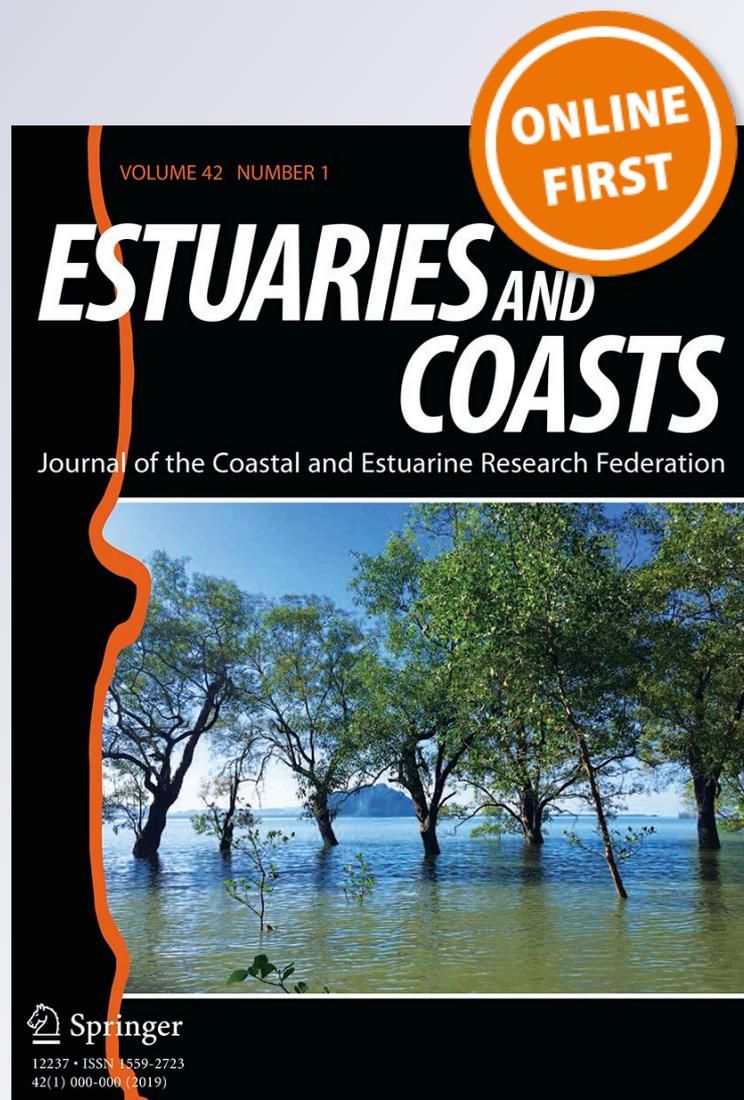
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# Upper Thermal Tolerance and Heat Shock Protein Response of Juvenile American Shad (*Alosa sapidissima*)

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## Abstract

Juvenile American shad (*Alosa sapidissima*) experience a wide range of temperatures in rivers before migrating to the ocean. Temperatures in these freshwater environments can vary greatly spatially, seasonally, year-to-year, and can be impacted by anthropogenic factors such as power plant discharge or climate change. Currently, there is uncertainty concerning juvenile American shad thermal tolerance due to a lack of a well-controlled study. Here, we report results of laboratory experiments to establish the upper thermal tolerance and heat shock protein response of juvenile American shad exposed to gradually increasing temperatures. Upper thermal tolerance was determined to be 35 °C (median; range = 34–36 °C) when fish were acclimated to 25 °C and temperatures were raised 1 °C day<sup>-1</sup>. Heat shock protein response was indicated by changes in branchial mRNA abundance of the inducible heat shock protein 90 alpha (*hsp90α*), which was significantly elevated (more than 5-fold increase) at 30 °C, and highest in fish that had reached their upper thermal maximum between 34 and 36 °C. Our findings indicate a higher upper thermal tolerance than previously reported for juvenile American shad, and an onset temperature of *hsp90α* induction at 30 °C, a temperature juvenile American shad commonly experience during summer months.

**Keywords** American shad · Temperature · Chronic lethal maximum · HSP · Climate change

## Introduction

Temperature has a profound influence on physiological processes in fish including growth, metabolic rate, swimming capacity, and survival (Brett 1971). For juvenile anadromous species, the interplay between growth and metabolic rate with temperature can influence many important life history traits, including determining the timing of seaward migration, predator-prey

dynamics, growth, and directly affecting survival if their upper thermal tolerance is reached (McCormick et al. 1997). It is important to know a species' upper thermal tolerance (defined by survival at a given temperature (Beitinger and Lutterschmidt 2011)) relative to the temperatures that species experiences in the wild, because not just exposure to temperature is important but also magnitude and rate of change (Beitinger and Lutterschmidt 2011). This knowledge is particularly important when environmental temperatures exhibit large seasonal and year-to-year variations and considering the general trend in water warming associated with climate change (IPCC 2019).

American shad (*Alosa sapidissima*) is an anadromous species native to eastern North America that spawns in rivers from Florida to Canada (Greene et al. 2009). Juvenile American shad larvae transform to juveniles 3 to 5 weeks after hatching (Leim 1924) and usually spend the summer in the river before emigrating to the ocean in the fall as temperature decreases (O'Leary and Kynard 1986). Thus, for most American shad populations, their early life history includes rearing in rivers during the summer, which exposes them to the highest temperatures reached each year.

Previous studies on the upper thermal tolerance of juvenile American shad have had conflicting results. Marcy et al.

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(1972) suggested that the upper thermal tolerance for juvenile American shad is at about 30 °C. This study drifted juvenile American shad in a live box through a temperature gradient created by dam effluent of 32.2 and 32.9 °C from an ambient of 22.7 and 19.0 °C, respectively. The first group experienced a 12.5% mortality rate (1 of 9 fish died) and a change in temperature of 10.2 °C, and the second group had a 100.0% mortality rate ( $n = 68$ ) with a change in temperature of 13.2 °C. Additionally, Moss (1970) cited unpublished data by Moss et al. describing rapid mortality when juvenile American shad acclimated to 24–28 °C experienced an acute temperature increase to 32.5 °C. These studies have been used to assign the upper thermal tolerance of juvenile American shad (see Greene et al. 2009) and have been added to modeling studies to predict how temperature affects a variety of American shad life traits and behaviors including growth, movement, and survival (Stier and Crance 1985; Limburg 1996).

Cellular indicators of thermal stress, such as the heat shock protein (HSP) response, have been utilized extensively to evaluate sub-lethal thermal stress in animals. Heat shock proteins are a highly conserved family of proteins that act as molecular chaperones in a variety of roles related to protein stability including protecting against the protein misfolding and aggregation resulting from thermal stress (Parsell and Lindquist 1993; Vargan-Chacoff et al. 2018). Two families of HSPs, HSP70 and HSP90, have isoforms that have been well established as being induced in response to heat stress. Similar to what has been reported for HSP70, HSP90 alpha (*hsp90 $\alpha$* ) is strongly induced in response to thermal stress (Basu et al. 2002). Heat shock proteins are considered to have an important role in thermal tolerance and adaptation, and expression of HSPs has been shown to reflect the recent thermal history of fish (Iwama et al. 1999; Chadwick et al. 2015). This makes HSPs an evolutionarily and ecologically interesting molecular biomarker with which to study thermal tolerance, particularly in ectotherms. Indeed, both upper thermal tolerance and the temperature at which the onset of a HSP response occurs can determine biogeographical distribution in aquatic species (Tomanek 2010). Relatively few studies have related whole organism thermal tolerance to the onset of an HSP response (Fangue et al. 2006, 2011), indicating the need for more studies in this area.

Our first objective was to use a modern, controlled laboratory setting to determine the upper thermal tolerance of juvenile American shad using the chronic lethal method (CLM). This method slowly increases temperature, allowing fish to acclimate to the increasing temperature and provides an accurate tolerance measurement (Beitinger et al. 2000; Widmer et al. 2006; Beitinger and Lutterschmidt 2011). This method differs from the often-used critical thermal maximum method (CT<sub>max</sub>; Becker and Genoway 1979), which rapidly raises temperature until endpoint. The CT<sub>max</sub> method prevents fish

from reacclimating during the study, which then leads to an overshoot of the endpoint. Thus, the CT<sub>max</sub> method is typically used as a relative or comparative measurement (Beitinger et al. 2000). Here, we used the CLM method, which allows limited acclimation, and a more accurate estimation of upper thermal tolerance (Beitinger et al. 2000). There is currently no literature reporting the temperature at which an HSP response is induced in American shad or how HSP induction is associated with thermal tolerance. Our second objective was to determine the onset temperature for induction of mRNA transcription of a known heat-inducible HSP, *hsp90 $\alpha$* , as an indicator of thermal stress during rising temperatures.

## Materials and Methods

### Fish Collection

Juvenile American shad were collected at the Cabot Station (Turners Falls, MA, USA) downstream bypass on 15 September 2015 via a fish trap. Fish were then immediately transferred to S.O. Conte Anadromous Fish Research Laboratory (Turners Falls, MA, USA) and placed in 1.5-m-diameter tanks equipped with flow-through river water (1 L m<sup>-1</sup>). Fish were maintained at a natural photoperiod with daily 50% water changes and fed ad libitum twice a day (BioPro 2 #2, BioTrout, Bio-Oregon, Westbrook, ME, USA). After a 16-day acclimation period at ambient temperatures (21.6 ± 1.1 °C; mean ± SD), water temperatures were slowly raised, over 3 days at 1 °C day<sup>-1</sup> with submersible heaters (Eheim Jager 300 W), set to 25 °C (24.1 ± 1.1 °C) and held at this temperature for 15 days.

### Temperature Exposure

Fish were transferred to five identical 0.9-m-diameter tanks with flow-through dechlorinated municipal water at 1 L min<sup>-1</sup> and fed up to 1% body weight day<sup>-1</sup>. Supplemental aeration was provided for each tank with oxygen levels remaining near saturation (90–100%). Tanks were divided up for each study as follows: 1 tank of 12 fish for upper thermal tolerance determination, 2 tanks of 24 and 25 fish for evaluation of HSP induction, and 1 tank of 12 fish as a control. Fish were acclimated to the experimental tanks for 3 days prior to initiation of temperature increases.

Temperatures were maintained for upper thermal tolerance and HSP induction tanks via a temperature control system (Omega cn7500, Omega Engineering, Inc., Stamford, CT, USA) that controlled water flow from header tanks through solenoid valves (Granzow, Inc., Charlotte, NC, USA) with temperatures monitored by RTD sensor probes (Omega Flexible Sealed PFA RTD Sensor, Omega Engineering, Inc.,

Stamford, CT, USA). The control tank was maintained at  $25.0 \pm 0.3$  °C with a submersible heater.

### Upper Thermal Tolerance

Upper thermal tolerance was determined by CLM (Beitinger et al. 2000; Widmer et al. 2006; Beitinger and Lutterschmidt 2011), where temperatures are slowly increased by  $1$  °C  $\text{day}^{-1}$  to allow fish to acclimate to the rising temperatures during the experiment. The endpoint is the lethal temperature (Beitinger et al. 2000; Widmer et al. 2006) and referred to here as the “upper thermal tolerance.” The endpoint is death for the CLM method due to the increased exposure time at thermally stressful temperatures (Beitinger et al. 2000). After 3 days acclimating at  $25.1 \pm 0.2$  °C, temperatures in each tank were raised  $1$  °C  $\text{day}^{-1}$  and mortalities were recorded twice a day and continued until all fish died. Dead fish were measured for fork length and mass.

### Sampling Protocol

Gills were sampled from fish throughout the temperature series at 25, 27, 29, 30, and 31 °C and from fish in a control tank held at a constant 25 °C. Fish were euthanized in a lethal dose of MS-222 ( $100$  mg  $\text{L}^{-1}$  buffered with  $\text{NaHCO}_3$ , pH 7.0), measured for fork length and mass, and sampled for gill tissue that was immediately frozen and stored at  $-80$  °C for mRNA analysis. Live fish were sampled at intervals (1–2 days) to quantify the gradual increase in HSP expression before thermal tolerance endpoint. Additionally, recently deceased fish that had reached their upper thermal tolerance were sampled in the same manner. We chose to use gill tissue for HSP mRNA analysis because the gill is a multifunctional, protein-rich organ for which a HSP response has been characterized in many fishes.

### RNA Isolation and Quantitative Real-time PCR

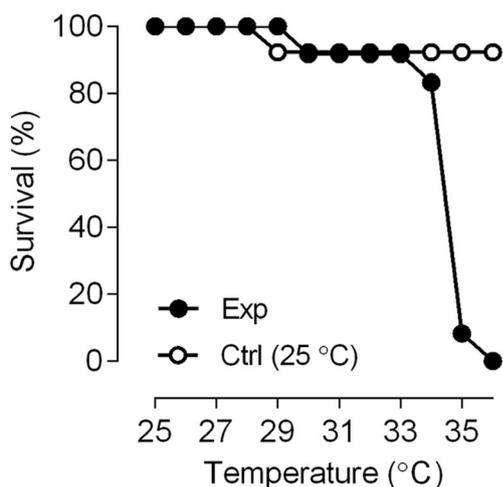
RNA was extracted from the gill using TRIzol reagent (Molecular Research Center Inc., Cincinnati, OH, USA) following the manufacturer's protocol. Total RNA concentration and purity of each sample were determined spectrophotometrically using a Take3 micro-volume plate (BioTek Instruments, Inc., Winooski, VT, USA). Quality of RNA was also assessed using gel electrophoresis. Only high-quality and high-purity RNA samples ( $A_{260}/A_{280} > 1.9$ ) were used for cDNA synthesis. First-strand cDNA synthesis was accomplished using a High-Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's protocol. Quantitative real-time PCR was carried out in  $10$   $\mu\text{L}$  reactions containing  $1$  ng cDNA template,  $150$  nM forward and reverse primers, and PowerUp SYBR master

mix (ThermoFisher, Waltham, MA, USA). Primers were designed on fully conserved sites based on a multiple alignment of known and predicted *hsp90 $\alpha$*  sequences for Atlantic herring (*Clupea harengus*), zebrafish (*Danio rerio*), and alewife (*Alosa pseudoharengus*): *ef1 $\alpha$*  F, 5'-AAGATCGGCTACAACCCTGC-3'; *ef1 $\alpha$*  R, 5'-TTACCCTCCTTGCGCTCA-3'; *hsp90 $\alpha$*  F, 5'-CCGAGGACAAGGACAACACTACAA-3'; *hsp90 $\alpha$*  R, 5'-ATGATGAAGACTCTGCGCAC-3'. Analysis of *hsp90 $\alpha$*  was chosen because transcriptome-derived sequence data for *hsp90 $\alpha$*  in Alewife, a close relative of American shad, was previously provided to us. The mRNA levels of the housekeeping gene *ef1 $\alpha$*  were stable in the temperature series from 25 to 31 °C and so *hsp90 $\alpha$*  mRNA level of each sample in the temperature series was calculated using the Comparative  $C_T$  Method ( $\Delta\Delta C_T$ ) and analyzed using one-way ANOVA (with temperature as factor of variation) with a statistical significance considered at an  $\alpha$  of 0.05. The mRNA levels of *ef1 $\alpha$*  were significantly lower in experimental fish that had reached their upper thermal tolerance (Exp) when compared with control fish (Ctrl), and thus would tend to exaggerate a finding of increased *hsp90 $\alpha$*  mRNA levels using the  $\Delta\Delta C_T$  method, so relative *hsp90 $\alpha$*  mRNA level in each sample in the Ctrl-Exp comparison was calculated based on a *hsp90 $\alpha$*  standard curve using a 10-fold serial dilution (efficiency = 0.98) and analyzed using Student's *T* test. The reactions were performed in optical 96-well reaction plates in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA) using the following thermal profile: holding at 50 °C (2 min) then 95 °C (2 min); cycling (40 cycles) from 95 °C (15 s) to 60 °C (1 min) to 72 °C (30 s). A dissociation step (melt curve analysis) increasing from 60 to 95 °C was used to confirm a single product resulted from each reaction. Additionally, a single reaction product was also confirmed by electrophoresis and sequencing (GENEWIZ Inc, South Plainfield, NJ, USA).

## Results

### Upper Thermal Tolerance

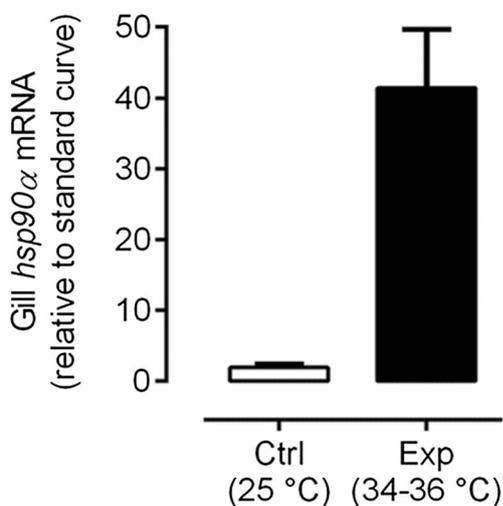
The twelve fish used for the upper thermal tolerance experiment had a mean fork length of  $8.3 \pm 0.4$  cm and a mass of  $6.4 \pm 1.0$  g. Death occurred at a median temperature of 35 °C (range 33–36 °C) with 83.3% of mortalities occurring at temperatures  $\geq 35.0$  °C and one fish surviving to 36.0 °C (Fig. 1). The control tank ( $n = 12$ ) had a mean fork length of  $8.3 \pm 0.3$  cm and a mass of  $6.5 \pm 1.0$  g. One mortality was observed in the control tank over the 13-day experiment, 7 days after placement into the experimental tank.



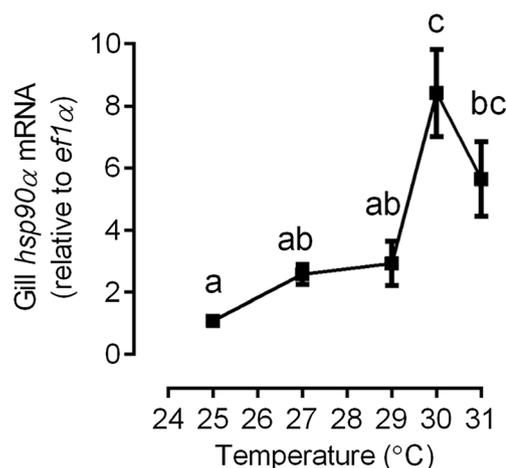
**Fig. 1** Percent survival of juvenile American shad exposed to increasing temperature: experimental fish (Exp; filled circles;  $n = 12$ ) experienced a  $1\text{ }^{\circ}\text{C day}^{-1}$  increase in temperature and control fish (Ctrl; open circles;  $n = 12$ ) were maintained at  $25\text{ }^{\circ}\text{C}$

### Heat Shock Protein Transcription

The thirty-four fish sampled for HSP analysis had a mean fork length of  $8.5 \pm 0.4$  cm and a body mass of  $6.4 \pm 1.1$  g. Highest levels of *hsp90α* mRNA abundance were observed in fish that had reached their upper thermal tolerance, approximately 20-fold higher *hsp90α* levels than the  $25\text{ }^{\circ}\text{C}$  control (Fig. 2; Student's *T* test:  $p < 0.001$ ). In the temperature series, branchial *hsp90α* mRNA abundance at  $30\text{ }^{\circ}\text{C}$  was significantly elevated approximately 8-fold above the  $25\text{ }^{\circ}\text{C}$  control (Fig. 3; one-way ANOVA:  $F_{5,29} = 7.48$ ,  $p < 0.001$ ).



**Fig. 2** Relative gill *hsp90α* mRNA abundance in juvenile American shad as a comparison of experimental fish (Exp;  $n = 8$ ) sampled after reaching their upper thermal tolerance ( $34\text{--}36\text{ }^{\circ}\text{C}$ ) and control fish (Ctrl;  $n = 10$ ) maintained at  $25\text{ }^{\circ}\text{C}$ ; all data are presented as mean  $\pm$  standard error of the mean



**Fig. 3** Relative gill *hsp90α* mRNA abundance in juvenile American shad sampled at increasing temperatures ( $1\text{ }^{\circ}\text{C day}^{-1}$ ) beginning at  $25\text{ }^{\circ}\text{C}$  (left to right:  $n = 7, 8, 7, 8, 4$ ; different letters indicate significant differences at  $p < 0.05$ ); all data are presented as mean  $\pm$  standard error of the mean

### Discussion

Juvenile American shad were shown to have a median upper thermal tolerance of  $35\text{ }^{\circ}\text{C}$  (nearly a  $5\text{ }^{\circ}\text{C}$  increase from prior studies) and to upregulate *hsp90α* transcriptional response at  $30\text{ }^{\circ}\text{C}$ . Previous work attempting to establish the upper temperature tolerance of juvenile American shad used wide temperature differences between acclimation and temperature treatments for either circumstantial conditions (dam effluent) or direct transfers. In the present study, we used a controlled, laboratory experiment that gradually exposed juvenile American shad to increasing temperatures to more clearly establish their upper thermal tolerance. Additionally, this is the first description of an HSP response in American shad, and one of relatively few studies to directly relate whole organism upper thermal tolerance to the HSP response (Fangue et al. 2006, 2011). These results are informative to managers and modelers to indicate what temperatures juvenile American shad can survive and what can be expected as temperatures rise from anthropogenic causes such as power plant discharge or climate change.

The southernmost population of American shad spawns in the St. Johns River in Florida, where juveniles are exposed to temperatures of up to  $35\text{ }^{\circ}\text{C}$  during summer months (Kroening 2004), the observed maximum of this study. These maximum temperatures occur from August to September overlapping with the mid-point of juvenile American shad emigration (June–December; Williams and Bruger 1972; Trippel et al. 2007). Fish in our study were acclimated to  $25\text{ }^{\circ}\text{C}$ , which represents a much larger temperature differential than American shad in the St. Johns River would typically experience during the warmest months; Trippel et al. (2007) indicated a more gradual increase in temperature as maximum temperatures were reached. Thus, it is unclear how well juvenile

American shad would tolerate higher temperatures if acclimated at temperatures above 25 °C, or if these differences are driven by separate phenotypes between northern and southern populations.

Given American shad's wide latitudinal range, variations among populations in thermal tolerance are possible. The majority of American shad return to their natal rivers to spawn, with approximately 3% straying to other rivers (Greene et al. 2009). However, Walther et al. (2008) found straying as high as 6% in the York River system in Virginia, but the strays were not far from their natal river and differences were typically between tributaries. Given that American shad populations generally home to specific regions, it can be expected that American shad thermal tolerance over the range of the species would correlate to latitude (Payne et al. 2016). Other biologic variations between American shad over its range include juvenile growth rates, fecundity, and iteroparity (Limburg et al. 2003). Thus, it can be expected that the absolute thermal tolerance results we obtained may be most relevant to American shad populations found in the region near the Connecticut River, with rivers that have a similar temperature profile and American shad emigration timing. Additionally, American shad may be expected to have higher thermal tolerances in the southern extremes of their range (i.e., Florida), and lower thermal tolerances in the northern extremes (i.e., Canada).

The observed upper temperature tolerance for juvenile American shad was similar to other laboratory studies of related species. Juvenile alewife (*Alosa pseudoharengus*) and adult threadfin shad (*Dorosoma petenense*) acclimated at 25 °C were observed to have a  $CT_{max}$  of 34.0 and 33.3 °C, respectively, when water temperatures were increased at 0.3 °C  $min^{-1}$  until fish experienced a loss of equilibrium (endpoint) (Otto et al. 1976; Monirian et al. 2010). Additionally, allis shad (*Alosa alosa*) embryos and larvae were observed to have similar maximal thermal thresholds as juvenile American shad (Jatteau et al. 2017). Beyond differences between species, these studies used a different method,  $CT_{max}$ , which rapidly raises the temperature until endpoint and typically has a higher endpoint temperature than methods such as the present CLM approach that allow for limited acclimation (Beitinger et al. 2000). The present study is the only laboratory-based upper thermal tolerance reported for American shad and, because of the prior acclimation to laboratory conditions, may represent a less stressful approach than previous field approaches. This, along with the slower rates of increase in temperature used in the present study, has likely resulted in the higher (and likely more accurate) estimates of upper thermal tolerance in American shad.

Although the presence of an HSP response to thermal stress is likely ubiquitous among fishes, the nature of heat shock response is highly variable across species (Iwama et al. 1999) and even within species (Fangue et al. 2006, 2011; Anttila et al. 2013; Stitt et al. 2014). The thermal history of a

fish may affect its HSP response to temperature. Unlike  $CT_{max}$  studies, for which the effects of acclimation temperature are well established (Lutterschmidt and Hutchison 1997; Beitinger et al. 2000), there remains some dispute regarding whether HSP induction has a single threshold temperature, or whether prior thermal history affects the threshold temperature (Basu et al. 2002; Tomanek 2010). In the present study, juvenile American shad acclimated to 25 °C significantly upregulated *hsp90 $\alpha$*  transcription at 30 °C. The literature on Clupeid thermal tolerance is limited, making it difficult to contextualize the present HSP results on a family level. However, there is an abundance of work in other teleost fishes comparing organismal and molecular responses with temperature (Fangue et al. 2006, 2011).

The range between the temperature at which an HSP response is induced and the upper thermal tolerance has not been widely examined, but has been reported in common killifish (*Fundulus heteroclitus*) to be between 5 and 10 °C (Fangue et al. 2006). In this context, juvenile American shad appear to exhibit a typical transcriptional HSP response to temperature in that the onset of *hsp90 $\alpha$*  induction is below their upper thermal tolerance. In addition to HSP90, there are other heat shock proteins that are important in cellular protection and recovery from thermal stress, such as the classically studied HSP70. Although a comprehensive profiling of the many HSP members and isoforms involved in the HSP response in American shad was outside the scope of the present study, such an analysis would certainly provide greater detail to the thermal sensitivity of this managed species. In addition, physiological factors that may determine upper lethal temperatures such as oxygen delivery (Pörtner and Knust 2007) may be distinct from the thermal thresholds for HSPs. Nonetheless, the onset of a HSP response is widespread among fishes and, in addition to upper thermal tolerance, appears to be important in determining biogeographical distribution (Tomanek 2010). Identifying the temperatures at which American shad induce a HSP response in relation to their upper thermal tolerance, as presented in this study, is a necessary step in understanding biogeographical distribution of this species or whether a limit exists on the long-term ability of American shad populations to tolerate warmer temperatures.

In summary, our results provide further clarity on the upper thermal tolerance of juvenile American shad, on which past research has been inadequate due to imprecise methods and acclimation temperatures. We also report an HSP response in American shad for the first time and find that the induction of *hsp90 $\alpha$*  occurs at 30 °C, which is 5 °C below their upper thermal tolerance. Future work is needed to assess acclimation effects on the upper thermal tolerance and the HSP response of juvenile American shad, and to determine whether there are population-specific differences (e.g., in southern populations) in this latitudinally widespread anadromous species.

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## Compliance with Ethical Standards

All experiments were carried out under US Geological Survey Institutional Animal Care and Use Committee Guidelines under protocol no. C09076.

**Disclaimer** Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

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