THERMAL RESPONSE OF BEHAVIOR AND GENE EXPRESSION
OF HEAT SHOCK PROTEINS IN THE LINED
SEAHORSE, *HIPPOCAMPUS ERECTUS*

by

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A THESIS

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ABSTRACT

THERMAL RESPONSE OF BEHAVIOR AND GENE EXPRESSION OF HEAT SHOCK PROTEINS IN THE LINED SEAHORSE, *HIPPOCAMPUS ERECTUS*

By Cara Johnson, B.S., University of New Hampshire

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The lined seahorse, *Hippocampus erectus*, is a dispersal-limited fish residing in the coastal and estuarine habitats of the Western Atlantic. With sea surface temperatures expected to rise +2°C in the next 85 years, these dispersal-limited fish will experience varying degrees of short-term (acute) and long-term (chronic) heat stress events. Previous studies have indicated that these different durations of increased temperatures will elicit distinctive behavioral changes in seahorses, but the molecular mechanisms underlying these changes are yet unknown. Three-hundred captive-bred *Hippocampus erectus* were exposed to control, mild, severe, and lethal temperatures for different durations to assess how these animals respond on a molecular and physiological level to heat stress events. Expression of the genes coding for heat shock proteins (HSPs) HSP60 and HSP70 were quantified to represent the molecular stress response of seahorses exposed to 22°C (the acclimation temperature of these animals, and therefore the control), 26°C, 30°C, and 32°C for acute (2, 6, and 10 hours) and chronic (4
weeks) periods of time. Respiratory rates, measured as breaths per minute, and activity levels were recorded daily to show the physiological response of seahorses to increased temperature for chronic durations. The relative expression of HSP60 and HSP70 increased in seahorses exposed to higher temperatures for acute durations as expected, and also increased through the 26°C treatment temperature for the chronic duration. There was, however, a noticeable lack of the heat shock response at 30°C for the chronic duration, coinciding with a higher mortality rate at this temperature and indicating an inefficiency of the molecular protein-repair mechanisms at this temperature. Respiratory rates also increased with exposure to higher temperature. Seahorses are often used as indicator species of the health of an ecosystem. This research indicates that the relative expression of HSPs are an efficient marker of temperature stress in seahorses only when a large proportion of a population can be sampled.
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Chronic treatment mysis eaten for 6 animals per treatment temperature, measured twice per day. Average mysis eaten weekly for each treatment temperature with SEM. Those marked with a * are significantly different (p<0.05) from 22°C during the same week.

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Relative expression of \textit{hsp}70 at chronic treatment duration of 4 weeks as box and whisker plots showing quartile 1, median, and quartile 3 on a log scale.
DEDICATION

This thesis is dedicated to my family for their unrelenting support. To my parents, Neal and Donna Johnson, for never denying me any opportunity or dream. To my sister and brother-in-law, Nicole and Mike Harrison, for their senses of humor. To my niece, Cadence, for reminding me of life’s simpler things. To my better half, Christopher Baril, for his patience, support, and understanding through a decade of indecision. And finally, to Dr. Junda Lin, for his inspirational career that led to so many dedicated and wonderful aquatic scientists across the globe, who, in turn, led me to his lab, and then on to labs across the world.
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INTRODUCTION

The Syngnathidae family is comprised of some of the world’s most enigmatic and recognizable fishes. Syngnathidae includes seadragons, pipefishes, and over 40 species of seahorses in the genus *Hippocampus* (Vincent 1999; Lourie et al. 2004). The unique life histories and intriguing morphologies found in the *Hippocampus* make these animals desirable for display in aquaria, and many seahorse species are also valued for their use in Traditional Chinese Medicine (TCM) (Lourie et al. 2004; Baum and Vincent 2005).

Several seahorse life history attributes make them more susceptible to population decline caused by degrading habitats, incidental capture (known as by-catch), and direct capture. These animals are also negatively impacted by invasive species, climate change, poor water quality, and noise pollution (Baum et al. 2003; Lourie et al. 2004; Vincent et al. 2011). Seahorses are known to occur in low-density subpopulations distributed in patches, and typically show high site fidelity, and limited dispersal abilities due to their small fin morphology (Vincent and Sadler 1995; Vincent 1999; Perante et al. 2002; Baum et al. 2003; Lourie et al. 2004). Seahorses are also monogamous, forming pair bonds for a breeding season, suggesting that disruption of social interactions by loss of one member of the pair would affect an entire reproductive season (Vincent 1999; Lourie et al. 2004).

Another major threat to seahorse populations is the shrimp trawl industry, as seahorse habitat (seagrass beds, mangroves, sandy bottom areas) typically
coincides with that of shrimp (Baum et al. 2003; Baum and Vincent 2005). Shrimp trawls damage seahorse habitat where the nets are dragged along the sea floor. Considering that the shrimp trawl industry accounts for 35% of the world’s total by-catch, trawling likely leads to a significant proportion of seahorse indirect capture. Baum et al. (2003) found that 72,000 seahorses were incidentally captured in a year by one shrimp trawl fleet in Florida, however the total population size was unknown. Globally, 95% of all traded Hippocampus spp. are sourced as by-catch, equating to tens of millions of seahorses per year (Lourie et al. 2004; Vincent et al. 2011; Lawson et al. 2015). The trade of seahorses is also increasing beyond the borders of Asia. Estimates for the number of countries involved in the trade of syngnathids increased from 80 (Vincent 1999; Lourie et al. 2004) to 175 (Vincent et al. 2011) over a seven-year period, with demand for seahorses increasing in places such as Latin America (Baum and Vincent 2005). The increased trade in seahorses suggests that seahorse populations will not be able to sustain future demands.

In 2004, the Convention on International Trade in Endangered Species (CITES) listed all seahorses, members of the genus Hippocampus, on Appendix II, in acknowledgment of declining seahorse populations. This provision allows seahorse populations to be monitored so that adjustments can be made to export limits for the countries that currently trade in seahorses in an effort to maintain sustainable populations (Lourie et al. 2004; Baum and Vincent 2005; Vincent et al. 2011; FWS.gov). Since this listing, 3 separate “Review of Significant Trade”
reports have been compiled, separating the trade of seahorses into dried and live categories. The majority of dried specimens originate from Thailand, Guinea, Chile, and compose the largest portion of the seahorse trade. The trade in live seahorses is smaller, and originates mainly from Vietnam, Indonesia, and Brazil (Fisheries Centre Research Reports 2016). At this rate, seahorse populations are not sustainable into the future.

Seahorses (*Hippocampus* spp.), like most fishes, are affected on a physiological level by increases in temperature due to their ectothermic physiology. It is well documented that fish are by nature poikilothermic: external environmental temperature shapes the internal temperature of the organism (Clarke and Johnston 1999; Roessig et al. 2004; Biro et al. 2007; Aurelio et al. 2013). On a population level, temperature affects fish distribution and reproductive success (Baltz et al. 1987). On the organismal level, external temperature affects metabolism, growth, and behavior. Generally, increased temperatures have beneficial effects on these phenotypes until a threshold temperature is reached, at which point these processes become negatively affected (Byrne 2011; Faleiro et al. 2015). When considering the lower and upper critical temperatures for a species, the fitness of the organism at measured body temperatures typically creates a parabolic distribution. The body temperatures where fitness (the capacity of an organism to survive and reproduce) approaches 0 are the lower and upper limits, and the peak is considered the organism’s optimal temperature. Within this parabola is the organism’s tolerance range of body temperatures (Elder and Seibel 2015).
Elevated temperatures often increase the aerobic scope up to a thermal maximum (Holt and Jorgensen 2015), alter the depth preference (Freitas et al. 2015), affect energy distribution (Biro et al. 2007; Munday et al. 2012; Kiorboe and Hirst 2014), alter survivorship of juveniles (Lin et al. 2008; Lin et al. 2009; Aurelio et al. 2013), and affect general behavior (Baltz et al. 1987; Biro et al. 2010; Nowicki et al. 2012; Planas et al. 2012; Freitas et al. 2015) of fishes like seahorses. The metabolic rate and activity levels of fishes increase proportionally as temperature increases until a thermal optimum for the species is exceeded, at which point behaviors like feeding decrease (Jobling 1997; Biro et al. 2007). It has been found that some fishes are able to compensate for these effects, and the ability of a species to become acclimatized to a new temperature regime requires energy at the expense of other life process, like reproduction (Silbermann and Tatar 2000; Somero 2002; Turingan and Sloan 2016).

The ability to compensate for the effects of rising temperatures will determine how a species is affected on a population level by our changing climate, but this acclimation competence, known as phenotypic plasticity, is only beneficial up to certain thermal maximums, above which the energy required to sustain normal biological system function is no longer available (Munday et al. 2012; Mascaro et al. 2016). The compensatory mechanisms working on a molecular level in fish species, like seahorses, that are restricted to small home ranges and have mobility limitations, remain poorly defined.
A secondary effect of increased temperatures on aquatic organisms occurs due to the decreasing solubility of oxygen. There is an inverse relationship on the solubility of oxygen and temperature, meaning that as temperatures increase, less oxygen is available in the water column (Perlman 2017). In aquatic organisms, the effects of oxygen limitations at higher temperatures often have repercussions on aerobically driven behaviors like feeding and swimming. In short, increases in temperature decrease the aerobic scope of an aquatic organism (Portner and Farrell 2008). The aerobic scope is defined as the “difference between the maximum metabolic rate and standard metabolic rate”, and becomes limiting when temperatures rise above the organism’s thermal optimum, where growth and reproduction are at their highest rates (Holt and Jorgensen 2015). This is particularly important for poikilothermic animals such as fish, as their metabolic rate depends on the surrounding water temperature (Clarke and Johnston 1999). Once above this thermal optimum temperature, the oxygen demands of the body system create a deficit, leading to less available energy for behaviors like feeding, swimming, and reproduction (Munday et al. 2012; Holt and Jorgensen 2015). Even for resting metabolic rates, tropical fish require as much as six times the amount of oxygen as fish living in low temperature water (Clarke and Johnston 1999).

Many species of fish compensate for the changes in metabolic rate caused by temperature changes by migrating to a habitat that fits within its tolerance range. This change in behavior is possible for fish that can efficiently cover long distances, like cod, but not possible for seahorses and other fish with small home
ranges and inefficient swimming modes (Freitas et al. 2015). Changes in surrounding water temperatures create an inescapable situation for seahorses. Therefore, their biological systems must change at the cellular level to counterbalance. These cellular changes can have lasting impacts on the energy available for normal behaviors. As more energy is being used by seahorses at higher temperatures to maintain normal behaviors and cellular interactions, it would be expected that other body systems may suffer.

Seahorses inhabit shallow coastal areas that will likely be more affected by warming temperatures than open ocean habitats (Lourie et al. 2004; Philippart et al. 2011). Increasing temperature has been shown to impact seahorse growth (Lin et al. 2008; Planas et al. 2012), reproduction (Lin et al. 2006, 2007, 2008), feeding (Lin et al. 2006; Koldeway and Martin-Smith 2010; Faleiro et al. 2015), and coloration (Lin et al. 2009), but studies on the overall effect of temperature on seahorse metabolism have shown mixed results. In one study examining the growth and survivorship of juvenile lined seahorses (*Hippocampus erectus*) at 24°C to 33°C, it was found that individual weight and length increased to a threshold temperature of 28°C to 29°C, and then declined at further increasing temperatures (Lin et al. 2008). In a separate study, Long-snouted seahorses (*Hippocampus guttulatus*) exposed to projected summer heat wave temperatures of +4°C in Portugal, showed an increase in metabolism, but the degree of increase remained within energetically sustainable levels (Faleiro et al. 2015). Aurelio et al. (2013) also found that ventilation rates in long-snouted seahorses were affected by the lower oxygen
content of warmer water, but did not observe any behavioral changes, while Planas et al. (2012) did see changes in behavior with increases in temperature. These studies suggest that these species of seahorse may not be severely affected by rising sea surface temperatures at a behavioral level, but the mechanisms compensating for the effects of increasing temperature to allow the animal to maintain normal functions remain unclear. Furthermore, it has also been proposed that the ability of an ectothermic organism to adapt to a highly variable environment, like the estuarine habitats where seahorses are found, comes at the cost of being able to adapt to long-term changes (Mascaro et al. 2016). This suggests that not just temperature, but duration of exposure to a temperature, will affect how an animal is able to respond to the changing temperatures and currents of today’s oceans.

At the cellular level, organisms respond to the introduction of stresses by upregulation of stress proteins, which can refer to any number of functionally different proteins whose expression changes in response to the stress. These stress proteins can include metallothioneins, cytochrome P450, and heat shock proteins (HSPs) (Iwama et al. 1998, Khalil et al. 2011; Krebs and Holbrook 2011). HSPs are perhaps the most well-conserved and universal cellular stress response mechanisms and are found in all animals with extremely similar amino acid sequences (Iwama et al. 1998; Somero 2002; Werner et al. 2007) (Lindquist 1986; Welch 1993; Morimoto et al. 1996; Al-Whaibi 2011). The expression of HSPs is induced by a wide variety of environmental and biological stressors (Iwama et al. 1998). Many HSPs function as molecular chaperones, which repair or degrade damaged proteins
in the cell (Welch 1993; Iwama et al. 1998; Somero 2002). This role in maintaining proper protein structure makes these HSPs among the most abundant proteins even in unstressed cells (Forsyth et al. 1997; Basu et al. 2002). HSP expression is tightly regulated, likely due to the large energetic cost of HSP synthesis (Krebs and Holbrook 2001). Therefore, the expression level of HSPs can be used to determine the susceptibility of an organism to environmental stressors like temperature (Werner et al. 2007) organism (Feder and Hofmann 1999; Liu et al. 2012). Heat shock protein induction and expression has also been shown to be adaptive over time to changing environmental conditions, referred to as phenotypic plasticity, and is representative of the thermal history of an organism (Feder and Hofmann 1999; Somero 2002; Osovitz and Hofmann 2005).

There are four major families of HSPs: HSP70, HSP90, HSP60, and low molecular weight HSPs (Basu et al. 2002; McLean 2008). The HSP70 family is thought to be the most important family of HSPs involved in protein chaperoning and folding, with HSP70 being present constitutively in the cell cytoplasm under normal conditions. HSP60 is also essential and highly conserved, but is localized to the cell mitochondrion (Kiang and Tsokos 1998; Basu et al. 2002; Mayer and Bukau 2005; Brocchieri et al. 2008; Al-Whaibi 2011; Liu et al. 2012; Wang et al. 2014). Stressors such as heat, hypoxia, pathogens, or toxic metals induce over expression of the inducible forms of HSP60 and HSP70 to either repair or destroy any damaged proteins (Morimoto et al. 1996; Iwama et al. 1998; Brocchieri et al. 2008; Wang et al. 2014). The amino acid sequences defining HSP60 and HSP70
are two of the most highly conserved sequences across an extremely wide range of taxa (42 - 57% from *E. coli* to humans) (Gupta and Golding 1993; Iwama et al. 1998; Molina 2000; Basu et al. 2002), and increased tissue concentrations of these HSPs have been used to detect acute and chronic physiological stress, often in marine invertebrates (Iwama et al. 1998). The highly conserved nature of HSP60 and HSP70, the availability of the sequence of these genes for many species, and the function of these genes in cellular stress response to increased temperature makes them an interesting biomarker tool to identify fish species that are negatively affected by rising sea temperatures.

These cellular indicators have not yet been applied to seahorses in reference to climate change scenarios, and therefore their validity as markers of stressed populations of mobility-limited fish species is unknown. With average sea surface temperatures expected to rise by about 2°C by the year 2100, understanding not only the behavioral and physiological, but also the molecular responses of an organism will be vital in determining the impacts of temperature change on a species (Basu et al. 2002; Lenton et al. 2008; Aurelio et al. 2013). These responses can then be better prioritized to identify “tipping points” for climate change and its effects on ecological and biological systems (Lenton et al. 2008).

The goal of this research is to provide insight into how seahorses, as a representative of mobility-limited marine animals, may react to various expected climate change scenarios by describing how the lined seahorse reacts on a
physiological and molecular level to temperature increases over different durations. Specifically, this study seeks to (1) observe how various physiological processes, including ventilation rates, feeding response, and activity respond to acute and chronic increases in temperature, and (2) quantify the relative expression of \textit{hsp}60 and \textit{hsp}70 in response to short-term (acute: 2, 6, or 10 hours) and long-term (chronic: 28 days) increases in temperature. These future scenarios will not be limited to constant sea surface temperatures warming (simulated by the chronic duration exposures in this experiment), but will also include short-term (simulated by acute durations in this experiment) heat wave events. The lined seahorse, \textit{Hippocampus erectus}, is a species listed as vulnerable on the IUCN Red List by the International Union for Conservation of Nature and Natural Resources, and the aim of this research is to provide a better foundation from which conservation management of these species can be directed.
MATERIALS AND METHODS

Two-hundred thirty-four 4-month-old, captive-bred lined seahorses (Hippocampus erectus) were obtained from the Shenzhen Seahorse Breeding Institute in Guangdong Province, P.R. China, and placed into two large holding tanks (61 cm diameter, 163 liters) at the Chinese Academy of Sciences Key Laboratory of Marine Bio-Resources in Guangzhou, P.R., China. The animals were held at room temperature (22°C) and a salinity of 25, with 12h dark:12h light for 2 weeks, and were fed frozen mysis shrimp at 2% body weight twice per day. The holding tanks were siphoned twice per day, and fresh artificial saltwater was added to the systems after cleaning. Each holding tank was supplied with an airstone and multiple plastic plants to provide habitat for the animals. These animals were approved for use under IACUC #160413 through the Chinese Academy of Sciences.

Twenty-four acrylic tanks (20.3 cm x 30.5 cm x 30.5 cm, 16 liters) were arranged in 3 rows of 8, with Styrofoam sheets placed on all sides in between each tank to assist in maintaining tank temperature. Each tank was supplied with an airstone and a piece of PVC pipe to serve as seahorse habitat, filled with saltwater at a salinity of 25, and stocked with 6 seahorses each from the holding tanks. All tanks were siphoned twice per day, replacing all removed water with new saltwater, and animals were fed frozen mysis shrimp (Hikari® Bio-Pure®) at 2% body weight twice per day. PVC pipes were removed once per week for thorough washing.
Daily logs were kept of tank temperatures (twice per day), salinity (twice per day), ventilation rate, feeding rate, behavior, and mortalities.

Nine of the small acrylic tanks served as housing for the chronic temperature treatments. Three of those tanks were heated to 30°C, 3 were heated to 26°C, and 3 remained at room temperature (22°C). Temperatures were monitored using in-tank thermometers, and heaters were adjusted as needed to keep the tank temperatures within +/- 1°C. Lighting was maintained at 12h dark:12h light. Seahorses were added directly into 22°C tanks, and seahorses intended for the 30°C treatment tanks were placed in bags floating inside of the 26°C tanks for one night to gradually acclimate to the water temperature. The following day, these seahorses were moved from 26°C to the 30°C tanks, and new seahorses were placed directly into the 26°C systems. This was the starting point of the chronic temperature exposure treatment.

The remaining 15 acrylic tanks were maintained with 6 seahorses each at room temperature, allowing the animals to adjust for one week to the tanks in preparation for the acute experiments. Any mortalities were removed, recorded, and placed into a -80°C freezer.

The acute temperature experiment was conducted on a staggered schedule (one replicate of each temperature and duration combination per day) to allow for strict temperature exposure durations, euthanization time, and tissue sampling. Seahorses were moved from their holding tanks at room temperature into pre-
heated tanks at 22°C, 26°C, 30°C, and 32°C for durations of 2, 6, and 10 hours. Opercular movements per minute (opening and closing of the gill covering) were recorded after 1 hour of each treatment. From each tank of 6 seahorses, 4 were sampled, choosing those that did not appear diseased and with similar body size. On day one, 4 seahorses from each temperature and exposure duration (48 total seahorses) were removed from their treatment tanks and euthanized using a sharp cut with a sterile scalpel to the top of the vertebrae. The sampled seahorses were then weighed (g), measured for total length (mm), and then dissected. Sex of the individual was recorded, and female gonad index was recorded using the regime in Table 1. Each organ (brain, tail muscle, liver, heart, gills, gonads, intestines, kidney, and brood pouch if present) was removed using aseptic technique and placed into an individually labelled centrifuge tube and immediately flash frozen with liquid nitrogen. On day two and three, the process was repeated so that there were 3 replicates of each temperature and duration regime, except for the baseline 22°C for 2, 6, and 10 hours, which was only completed on day one. The total number of seahorses sampled throughout the acute experiment totaled 120.

The livers from each sampled seahorse were homogenized. A small portion of each liver was placed into a sterile microcentrifuge tube with 500 µL of Ambion TRIzol® reagent, and the tissue was pulverized using a sterile 23-guage hypodermic needle until no solid tissue pieces could be seen with the naked eye, indicating cells had been sufficiently lysed. The homogenate was then combined
with 200 μL of chloroform, vortexed for 15 seconds, and incubated over ice for 5 minutes. The sample was then microcentrifuged at 12,000 revolutions per minute (RPMs) and 4°C for 15 minutes. The resulting supernatant was carefully transferred to a new centrifuge tube. An equal volume (approximately 500 μL) of isopropyl alcohol was added to each sample, and then the sample was vortexed for 15 seconds and incubated on ice for 10 minutes. This sample was then microcentrifuged at 12,000 RPMs at 4°C for 10 minutes. The resulting supernatant was then discarded, leaving only the pelleted RNA. The pellet was washed 3 times with 1 milliliter of chilled 75% ethanol followed by microcentrifugation at 12,000 RPMs at 4°C for 5 minutes. Any remaining supernatant was removed and the samples were allowed to air dry.

The RNA pellet was dissolved in 20 – 100 μL of diethyl pyrocarbonate (DEPC) water, depending on the pellet size, and stored in a -80°C freezer. The

<table>
<thead>
<tr>
<th>Gonad Index</th>
<th>Ovary Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Small, white, eggs can only be seen with microscope</td>
</tr>
<tr>
<td>II</td>
<td>Larger, slight red color, small eggs visible to naked eye</td>
</tr>
<tr>
<td>III</td>
<td>Eggs visible and red or yellow in color</td>
</tr>
<tr>
<td>IV</td>
<td>Mature eggs that are clear or slightly yellow</td>
</tr>
<tr>
<td>V</td>
<td>Mature, post egg release</td>
</tr>
</tbody>
</table>
RNA was analyzed via gel electrophoresis to determine the quality of the samples by observation of the 28S and 18S rRNA bands. The RNA concentrations were determined using optical density measured by a NanoDrop™ 2000 (ThermoFisher Scientific).

The RNA was incubated at 65°C for 5 minutes to denature any complex structures and an equal amount of RNA was used for cDNA synthesis. Synthesis of cDNA was completed using the ReverTra Ace® qPCR RT Master Mix for reverse transcription followed with a gDNA remover Kit (TOYOBO, Japan) as per the manufacturer’s instructions. The reverse transcription thermocycler (BioRad T100™ Thermal Cycler) parameters used were: 37°C for 15 mins, 50°C for 5 minutes, 98°C for 5 minutes, and 4°C holding. All experimental gene primers (18S, hsp60, and hsp70) were validated by running a polymerase chain reaction followed by gel electrophoresis (Table 2). qPCR analysis was performed using a THUNDERBIRD® SYBR® qPCR Mix Kit (TOYOBO, Japan) to determine threshold cycles of each of the experimental genes in each sample using a LightCycler™ 480 (Roche Diagnostics, USA). For each treatment temperature and duration, at least 12 biological samples were run for qPCR in technical duplicate. By running each biological sample twice, outliers and incorrect reads could be separated out of the data set, using the cutoff of technical replicates with Ct values different by greater than 1.5 cycles. All experimental gene (hsp60 and hsp70) Ct values were normalized to 18S rRNA.
Table 1. *H. erectus* primers used for PCR and qPCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ → 3’)</th>
<th>Primer Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>GTTTCCATCTTTCCACAGAATC</td>
<td>Hsp60 specific primer</td>
</tr>
<tr>
<td>R1</td>
<td>AAACCACAGGCCCACCCCAT</td>
<td>Hsp60 specific primer</td>
</tr>
<tr>
<td>F2</td>
<td>TGGTGCAGGGACGAAAGGGGGTC</td>
<td>Hsp70 specific primer</td>
</tr>
<tr>
<td>R2</td>
<td>TCAGTCCACCTTTCAATGG</td>
<td>Hsp70 specific primer</td>
</tr>
<tr>
<td>F3</td>
<td>CGCCTTTCCAAGCCAGTC</td>
<td>Hsp60 specific primer for RT-PCR</td>
</tr>
<tr>
<td>R3</td>
<td>TGTAACCACGGCTCAAACCTTCAT</td>
<td>Hsp60 specific primer for RT-PCR</td>
</tr>
<tr>
<td>F4</td>
<td>ATGGTGCAGGGACGAAAGGGGGTC</td>
<td>Hsp70 specific primer for RT-PCR</td>
</tr>
<tr>
<td>R4</td>
<td>CCGTGTGGCTCGGGGTTC</td>
<td>Hsp70 specific primer for RT-PCR</td>
</tr>
<tr>
<td>18S - F</td>
<td>CCTGAGAAACGGCTACCACATCC</td>
<td>18S primer</td>
</tr>
<tr>
<td>18S - R</td>
<td>AGCAACTTTTAGTATACGCTATGGAG</td>
<td>18S primer</td>
</tr>
</tbody>
</table>

DATA ANALYSIS

Chronic treatment ventilation rates were recorded as opercular movements per minute for 2 animals per tank, twice daily. The observations from both animals were averaged, and then the 2 daily averages for 1 tank were averaged for a total daily ventilation rate. These ventilation rates were plotted over time, and the slope of the best fit line for each temperature was calculated. These ventilation rates were also divided by week to examine how the seahorses’ ventilation rates were changing over time within each temperature treatment. Student’s T-tests were used to determine significant differences in the ventilation rates from the control temperature of 22°C. Multiple regression analysis was used to determine how
temperature and time spent at that temperature relate to ventilation rates in the lined seahorse, providing a predictive equation of the relationship between the independent variables of time and temperature with the dependent variable of ventilation rate.

Acute ventilation rates were recorded during the first hour of the assigned treatment duration, again using 2 animals from one tank and calculating the average. This was repeated for all three replicates of each temperature and duration combination, except for the 22°C treatments, which were only conducted once each. The Student’s T-test was used to determine significant differences in the ventilation rates within a treatment duration. This data was also used to determine percent change in ventilation rates from 22°C during the treatment durations. Q10 values were calculated for the chronic and acute treatment ventilation rates to define how the physiological process of respiration is affected by temperature increases.

Food intake was recorded for the animals in the chronic treatment by counting the average number of mysis shrimp eaten by one seahorse per tank during the 5 minutes after addition of food to each tank was recorded twice daily (morning and afternoon), and these 2 observations were averaged to estimate food intake as an estimate of appetite. The animals became much less interested in food about five minutes after it was added to the tanks, so this time was chosen as the cut-off for recording number of mysis eaten. The data was distributed by week, and
a Student’s T-test was used to determine if seahorses at 26°C and 30°C ate significantly different amounts of mysis shrimp than those at 22°C. A Pearson’s correlation test was also conducted to determine if there was a relationship between ventilation rate and food eaten.

Relative quantification was conducted using the ΔΔCt method. The Student’s T-test was used to determine significant differences between the control gene expression (chronic: 22°C for 4 weeks; acute: 22°C for 2 hours) and the expression of the genes at the treatment temperatures and durations. The Student’s T-tests were also performed within each temperature at the acute durations (26°C, 30°C and 32°C for 2 hours against 22°C for 2 hours; 26°C, 30°C, and 32°C for 6 hours against 22°C for 6 hours; 26°C, 30°C, and 32°C for 10 hours against 22°C for 10 hours), and within each treatment duration (26°C, 30°C, and 32°C against 22°C for 2 hours, etc.).
RESULTS

To provide insight into how seahorses will react to various expected climate change scenarios, we investigated the physiological and molecular responses of the lined seahorse, *Hippocampus erectus*, to temperature. A range of temperatures was investigated from the acclimation temperature (the temperature at which these specific animals were captively-raised, 22°C) to temperatures near the lined seahorse’s known thermal maximum of 33 °C, a point at which juvenile survivorship at two weeks drops to 26%, (Lin et al. 2008). Three-hundred captive-bred lined seahorses (*Hippocampus erectus*) were exposed to one of two treatment categories, acute (hours) or chronic (days), within which were multiple treatment levels (Figure 1). Those in the acute treatment were exposed to 22°C (control), 26°C (mild stress), 30°C (severe stress), and 32 °C (lethal stress) and measurements were taken at 2, 6, and 10 hours. The temperature of 32°C was chosen based on previous studies identifying low survivorship of this species above this temperature (Lin et al. 2008), and represented a 10°C increase from the control. Those in the chronic treatment were exposed to 22°C (control), 26°C (mild stress), or 30°C (severe stress) for four weeks (Mascaro et al. 2016). When the organisms had completed their assigned treatment, the relative expression of *hsp60* and *hsp70* in
Figure 1. Flow chart diagraming treatment categories. Chronic treatment lasted for 28 days with 18 animals at each temperature. Acute treatments lasted for 2, 6, or 10 hours with 18 animals at each temperature and duration combination, except for the control 22°C treatments, which had 6 animals each.
the liver was quantified using qPCR. The liver was chosen based on its high RNA yield of 2 – 4 µg RNA/mg of tissue (Thermo Fisher 2017) and ease of aseptic removal. For the animals in the chronic treatment, behavioral and physiological data was also collected in the form of eating habits, activity, and ventilation rates counted as opercular movements per minute. This data was used to examine how acute and chronic exposure to increased temperatures alters behaviors, as well as the expression of hsp60 and hsp70.

PHYSIOLOGICAL RESPONSES OF SEAHORSES OVER ACUTE TREATMENT DURATION

Total numbers of seahorses used and mortalities throughout the experiment are recorded in Table 3. No seahorse mortalities occurred at the control or mild stress temperatures at any duration, indicating a 100% survivorship (Figure 2). At severe temperature stress, one entire cohort (6 seahorses) at the 10-hour exposure duration died, indicating a 67% survivorship of these seahorses at 30°C for 10 hours. One seahorse in each of the exposure durations at lethal temperature stress died, resulting in a 94% survivorship of the lined seahorse at any duration at this temperature.
Table 3. Total numbers of *H. erectus* used during the research project.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Temperature</th>
<th>Duration</th>
<th>Total Count at Start</th>
<th>Total Mortalities</th>
<th>Total Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic</td>
<td>22°C</td>
<td>4 weeks</td>
<td>18</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Chronic</td>
<td>26°C</td>
<td>4 weeks</td>
<td>18</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Chronic</td>
<td>30°C</td>
<td>4 weeks</td>
<td>18</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>22°C</td>
<td>2 hours</td>
<td>6</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Acute</td>
<td>22°C</td>
<td>6 hours</td>
<td>6</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Acute</td>
<td>22°C</td>
<td>10 hours</td>
<td>6</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Acute</td>
<td>26°C</td>
<td>2 hours</td>
<td>18</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>26°C</td>
<td>6 hours</td>
<td>18</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>26°C</td>
<td>10 hours</td>
<td>18</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>30°C</td>
<td>2 hours</td>
<td>18</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>30°C</td>
<td>6 hours</td>
<td>18</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>30°C</td>
<td>10 hours</td>
<td>18</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>32°C</td>
<td>2 hours</td>
<td>18</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>32°C</td>
<td>6 hours</td>
<td>18</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Acute</td>
<td>32°C</td>
<td>10 hours</td>
<td>20</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td>236</td>
<td>21</td>
<td>156</td>
</tr>
</tbody>
</table>

Figure 2. Survivorship of seahorses during acute treatment. Numbers in brackets represent total number of seahorses in that temperature group.
Seahorse ventilation rates were recorded as a simple and straightforward indicator of animal physiology during acute temperature stress, which simulates the animal being rapidly introduced to a new temperature regime for a short period of time. This could represent the animal being caught in a changing current or a weather event. Therefore, opercular movements were measured during the first hours of the temperature stress. Measurements were collected for 2 animals per condition and the results were averaged. This was repeated for all three replicates of each temperature and duration combination, except for the 10-hour durations, where only two observations were recorded. It was found that the ventilation rates measured at the halfway point of the defined duration increased with each temperature increase (Figure 3). When compared with the ventilation rates recorded at the control temperature (22°C), animals treated with mild temperature stress (26°C) had average increases in ventilation rates of 46% at 2 hours, 62% at 6 hours, and 33% at 10 hours. At severe temperature stress (30°C), the increases were greater, at 81%, 100%, and 90%, while the increases were even larger at lethal temperature stress (32°C) at 173%, 177%, and 212% (Table 4). This data suggests that ventilation rates increase rapidly upon submersion of the seahorses in increased temperatures, and this increase is proportional to the change in temperature. The ventilation rates were consistent over all acute treatment durations at a given treatment temperature, indicating that if the change in temperature were to elicit an effect on the rate of ventilation, it would occur within the first hour of exposure.
Figure 3. Acute treatment ventilation rates for 3 animals per treatment temperature and duration combination, except the 10 hours treatment where only 2 seahorse ventilation rates were recorded. Error bars represent SEM. Those marked with a * are significantly different (p < 0.05) from 22°C during the same duration.

Table 4. Average percent increase in ventilation rates of acute treatment seahorses when compared with those of animals at 22°C for equal durations.

<table>
<thead>
<tr>
<th></th>
<th>2 Hours</th>
<th>6 Hours</th>
<th>10 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>26°C</td>
<td>46%</td>
<td>62%</td>
<td>33%</td>
</tr>
<tr>
<td>30°C</td>
<td>81%</td>
<td>100%</td>
<td>90%</td>
</tr>
<tr>
<td>32°C</td>
<td>173%</td>
<td>177%</td>
<td>212%</td>
</tr>
</tbody>
</table>
To examine how temperature was able to elicit a change in ventilation rates of lined seahorses, the ventilation rates were used to calculate Q10 values (Figure 4). These values help describe the degree to which the ventilation rate is altered from the control ventilation rate at 22°C. Q10 values are a common metric used to describe how thermally sensitive a species is to changes in temperature; a Q10 value of 2 is expected for any biochemical reaction that is solely affected by temperature, while a Q10 value below 2 indicates the process that the rate is describing is not thermally sensitive, or that there is a compensatory mechanism at work to overcome to thermal challenge. The Q10 values for animals across acute treatment durations were 2.5 for animals held for 2 hours, 2.8 for 6 hours, and 2.5 for those held for 10 hours at their treatment temperatures (Figure 4). The increase of around 2 during mild and severe stress is consistent with a response to temperature that is driven largely by biochemical processes. The larger changes observed for animals exposed to lethal stress conditions for short times was larger, indicative of additional mechanisms, such as tissue-damage, contributing to the response.
RELATIVE EXPRESSION OF \textit{hsp}60 DURING ACUTE TREATMENTS

To investigate the molecular response to acute temperature stress, the amount of HSP60 expression was measured in liver samples from animals in each treatment condition at each time point. 6 animals from each of three tanks were analyzed for each time point, except for the control temperature, which was only completed once with 6 seahorses each. Unexpectedly, there were significant differences (p < 0.05) in \textit{hsp}60 expression in seahorse livers at the control temperature at 2, 6, and 10 hours (Figure 5A), with a 0.49-fold decrease at 6 hours relative to expression at 2 hours, and a 0.75-fold decrease in expression at 10 hours (Figure 5A). These differences, although small, likely reflect an increased expression at the early time points indicative of handling stress. There was no significant difference (p > 0.05) in \textit{hsp}60 expression in seahorse livers at mild
temperature stress for 2, 6, and 10 hours (Figure 5B), although the relative expression of *hsp60* at 26°C for 2 hours was extremely variable, resulting in a large standard error, indicating a large amount of biological variability. During severe stress at 30°C and lethal stress at 32°C, there was a large increase in *hsp60* expression at the 6-hour time points, but again the expression was variable and therefore these differences were not statistically. At later time points, the expression of *hsp60* was similar to baseline, indicating adaptation to the stress. The shape of the response curve in these two conditions featuring induction followed by adaptation is characteristic of the heat shock response in other organisms. As expected, the magnitude of the induction under lethal stress (1550-fold) was much greater than the induction under severe stress (210-fold). The same data is plotted using a box-and-whisker plot in Figure 6. Table 5 shows all possible treatment combination relative expression changes in *hsp60* during the acute treatments.
Figure 5. Relative expression of *hsp60* at A) 22°C, B) 26°C, C) 30°C, and D) 32°C for acute treatment durations. Those marked with a * are significantly different (p < 0.05) from seahorses held at 22°C for 2 hours.
Figure 6. Relative expression of hsp60 at acute treatment durations in box and whisker plots showing quartile 1, median, and quartile 3. Those marked with a * are significantly different ($p < 0.05$) from 22°C for that same duration.

Table 5. Average $\Delta\Delta$Ct values for hsp60 in acute treatment. Those marked with a * are significantly different ($p < 0.05$) from the control (22°C) for that same duration.

<table>
<thead>
<tr>
<th>Hsp60</th>
<th>Control: 22°C</th>
<th>Mild stress: 26°C</th>
<th>Severe stress: 30°C</th>
<th>Lethal stress: 32°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Hours</td>
<td>1.0</td>
<td>5.3</td>
<td>10.7 *</td>
<td>4.0 *</td>
</tr>
<tr>
<td>6 Hours</td>
<td>0.5</td>
<td>1.6 *</td>
<td>210</td>
<td>1550</td>
</tr>
<tr>
<td>10 Hours</td>
<td>0.24</td>
<td>2.0</td>
<td>5.5</td>
<td>2.5 *</td>
</tr>
</tbody>
</table>
RELATIVE EXPRESSION OF \textit{hsp70} DURING ACUTE TREATMENT

To validate the expression patterns of \textit{hsp60}, gene expression was measured for a second heat shock gene, \textit{hsp70}. Similar to \textit{hsp60}, there was a reduction in \textit{hsp70} expression at the control temperature (22°C) at later time points, although this effect was only statistically significant (p < 0.05) for 2 and 6 hours, but not 10 hours (Figure 7A). Also similar to \textit{hsp60}, there was no significant difference (p > 0.05) in \textit{hsp70} expression during mild temperature stress (26°C) for 2, 6, and 10 hours (Figure 7B), although the relative expression of \textit{hsp70} at 26°C for 10 hours was higher and extremely variable, resulting in a large standard error. Severe and lethal stress conditions caused a larger, but also variable increase in \textit{hsp70} expression at all time points, but again these changes were not statistically significant. Aside from a few outliers causing large standard errors, the trend of relative change in \textit{hsp70} was constant throughout treatment temperatures and durations (Figure 8). Table 8 shows all possible treatment combination relative expression changes (\(\Delta\Delta Ct\)) in \textit{hsp70} during the acute treatments.
Figure 7. Relative expression of hsp70 at A) 22°C, B) 26 °C, C) 30°C, and D) 32 °C for acute treatment durations. Those marked with a * are significantly different (p < 0.05) from seahorses held at 22°C for 2 hours.
Figure 8. Relative expression of hsp70 at acute treatment durations in box and whisker plots showing quartile 1, median, and quartile 3. Those marked with a * are significantly different (p < 0.05) from 22°C for that same duration.

Table 6. Average ΔΔCt values for hsp70 in acute treatment. Those marked with a * are significantly different (p < 0.05) from the control (22°C) for that same duration.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Hrs</td>
<td>1.0</td>
<td>3.0 *</td>
<td>6620 *</td>
<td>10820 *</td>
</tr>
<tr>
<td>6 Hrs</td>
<td>0.47</td>
<td>5.6</td>
<td>4760</td>
<td>61410</td>
</tr>
<tr>
<td>10 Hrs</td>
<td>0.6</td>
<td>213</td>
<td>18530</td>
<td>5690</td>
</tr>
</tbody>
</table>
PHYSIOLOGICAL RESPONSES OF SEAHORSES OVER CHRONIC TREATMENT DURATION

Previous studies had identified 33°C as the thermal limit of *Hippocampus erectus* juveniles in a two-week treatment, at which point survivorship dropped to 26% (Lin et al. 2008). Therefore, the maximum chronic temperature chosen for our four-week analysis of chronic stress was 30°C. At the control temperature of 22°C, there was 100% survivorship over the 4-week treatment period, while at 30 °C there was a 39% survivorship (Figure 9). This significantly lower survivorship (p < 0.001) of animals at this temperature indicates that the thermal limit is lower than previous estimates and predicts poor persistence of some *H. erectus* at future climate change scenario predicted temperatures.

This low survivability of seahorses at 30°C for a chronic duration coincided with marked physiological changes. Ventilation rates were recorded twice daily over the course of 4 weeks as a reliable predictor of the metabolic response of an organism to its environment. Ventilation rates were found to increase over the four weeks in all treatment temperatures (Figure 10A). The ventilation rate of control animals (22 °C) was approximately 17 opercular movements (opening and closing of the gill operculum cover) per minute (OM/min) during the first week. This rate increased over time to 21 OM/min by the fourth week, an increase of 24%. The general trend of increasing OM over the course of the experiment at the control temperature is likely due to mass scaling.
of fish (Kiorboe and Hirst 2014), where the resting metabolic rate of a fish is directly related to animal growth over time (Clarke and Johnston 1999). A simple linear regression was performed on these data, returning the linear equations listed on Figure 10A, where the slopes represent the general increase in ventilation rates over time. Animals at 26°C had a ventilation rate of 20 OM/min during the first week, which was 18% faster than the control animals. Ventilations at 26°C increased to 27 OM/min by the fourth week, which is a 35% increase in this rate.
Figure 10. Chronic treatment ventilation rates for 6 animals per treatment temperature, measured twice per day. A) Average ventilation rates at AM and PM per treatment temperature with linear trendlines. B) Average weekly ventilation rates for each treatment temperature with SEM. Those marked with a * are significantly different (p < 0.05) from 22°C during the same week.
at this temperature over the duration of the experiment. The increases in ventilation rates of animals at 26°C were significantly different from the ventilation rates of animals at 22°C during week 3 (p < 0.05) and week 4 (p < 0.001). Animals at 30°C had a rate of 23 OM/min in the first week (50% faster than control animals), and 39 OM/min by the fourth week, an increase of 70%. Therefore, the increase over time at 30°C was almost twice as large as the increase over time at the control temperature (24% at 22°C), and was significantly different than animals at 22°C (p < 0.05 for week 1, p < 0.001 for weeks 2, 3, and 4). These ventilation rates were compiled into weekly averages (Figure 10B) to provide a more succinct representation of how ventilation rates were changing through the 4-week experiment, and a significant difference (p < 0.05) is found between all three treatment temperatures, except for the animals at 26°C during the first and second weeks of treatment. A multiple regression analysis was performed to determine if a descriptive equation could be derived to explain how ventilation rates of the seahorses change with time and temperature. The statistically significant terms returned (p < 0.001) were day and squared temperature, and derived the equation \( \ln(\text{ventilation rate}) = 2.1 + 0.0011(\text{temp}^2) + 0.0147(\text{day}) \), with an adjusted \( r^2 \) value of 0.59.

The ventilation rates from the chronic experiment were used to calculate Q10 values to examine how temperature was able to elicit a change in ventilation rates of lined seahorses. The average Q10 calculated for the chronic experiment animals was 1.67 for animals at 26°C, and 2.00 for animals at 30°C (Figure 11A,
indicating that the ventilation rates of animals at 26°C were beneath the expected change due to temperature likely due to some compensatory mechanism that counteracts the effects of temperature on biochemical reactions. For animals at 30°C, the Q10 value of 2.00 indicates that the ventilations rates were affected directly by the change in temperature, and by week 4 of the treatment, average values were above 2.00, indicating a breakdown of the biochemical reactions required to sustain ventilation rates (Figure 11). The Q10 values were significantly different from each other (22°C - 26°C versus 22°C – 30°C) during week 3 (p < 0.05), but the overall averages were not significantly different from one another. When the data is separated into weekly averages (Figure 11B), it becomes apparent that seahorses at 26°C are able to compensate for a longer period of time, indicated by a Q10 value below 2.00, while those at 30°C reach a Q10 above 2.00 much earlier, and maintain this elevated Q10 value throughout the duration.

This increase in ventilation rates suggests that the biochemical processes underlying the rates would require more energy to maintain normal biological function of the organism. This was assessed by monitoring how many mysis shrimp were eaten by a seahorse over a course of 5 minutes twice per day in each chronic treatment tank. As expected, the general trends for control and mild stress were that food intake slightly increased over time. Surprisingly, animals exposed to severe stress at 30°C decreased their food intake over time (as seen by the negative slop of the trend line, Figure 12A). When this data is average into weeks to provide a more generalized way to view the data, this pattern becomes more apparent (Figure 12B).
Animals at 22°C and 26°C started and ended the experiment eating approximately 5 and 6 mysis shrimp per feeding, respectively, but the number of mysis eaten at 26°C during weeks 1 and 2 were significantly different than those of animals at 22°C (p < 0.001). Seahorses at 30°C however, started the experiment eating approximately 6 mysis shrimp per feeding, and ended eating 5 mysis shrimp per feeding, with significant difference from animals at 22°C during weeks 1 and 2 (p < 0.05). This decrease in food intake does not fit with the expected trend that increasing ventilation rates would require more energy to sustain normal physiological functions. This was also an observation made by Mascaro et al. (2016), where seahorses were expected to consume more food at higher temperatures, but did not.

This raised the question of whether the seahorses were gaining size over the course of the chronic treatment, as anticipated of juvenile animals. To answer this question, initial and final weights (total body, g) and heights (top of crown to tip of tail, mm) were recorded for all sampled seahorses (Figure 13). The data indicate that there was not a change in either height or weight over the course of the experiment, although there was a very small trend towards smaller organisms in the severe stress conditions.
Figure 11. Q10 values for chronic treatment seahorses. A) Daily Q10 values over duration of 4 week treatment. Linear regression equation for 22°C-26°C is \( y = 0.03x + 1.27 \) (\( r^2 = 0.87 \)) and 22°C-30°C is \( y = 0.04x + 1.36 \) (\( r^2 = 0.31 \)). B) Average Q10 values for each week of treatment at each temperature increase.
Figure 12. Chronic treatment mysis eaten for 6 animals per treatment temperature, measured twice per day. A) Average number of mysis eaten at AM and PM per treatment temperature with linear trendlines. B) Average mysis eaten weekly for each treatment temperature with SEM. Those marked with a * are significantly different (p < 0.05) from 22°C during the same week.
Figure 13. Height measurements of each seahorse were recorded from tip of crown on head to tip of straightened tail (ProjectSeahorse.org).
Figure 14. Change in body morphometrics, calculated as difference in A) initial and final height (mm) and B) initial and final weight (grams). Open triangles (△) represent mean values for that temperature.
This trend was further confirmed by the results of a Pearson correlation performed between the number of mysis eaten per seahorse and the observed ventilation rates at each temperature. No significant correlation was determined between these two dependent variables at any chronic treatment temperature (22°C r = 0.008, 26°C r = 0.17, 30°C r = -0.12, all p-values > 0.05), as none of the Pearson correlation r values were significantly different from 0.

As another measure of seahorse behavior, the activity of each seahorse was recorded once per day in each of the chronic treatment tanks, following the descriptions listed in Table 3. Animals at 22°C were hitching 71% of the time, while those at 26°C and 30°C were hitching 61% and 47% of the time, respectively. Animals at 22°C were swimming 22% of the time, while those at 26°C and 30°C were observed swimming 21% and 36% of the time, respectively. For resting behavior, the observations recorded were 5%, 18%, and 17% for 22°C, 26°C, and 30°C (Figure 15). The overall observed condition of seahorses at each temperature showed that those being held at 30°C were visibly agitated and stressed, and this is consistent with the number of mortalities (12 seahorses) seen at this temperature (Table 7). A logistic regression analysis was performed on the activity data to determine the odds of a seahorse hitching at each treatment temperature. The odds of hitching at a certain temperature can be described as

\[ \ln(p/1-p) = 9.39 - 0.37(\text{temp}), \text{ or } e^{(9.39 - 0.37(\text{temp}))}. \]

This provides the odds for hitching at 22°C as 3.65, 26°C as 0.84, and at 30°C as 0.19. Based on the odds ratio between 22°C and 30°C, the odds of these seahorses hitching at 22°C is 19 times
the odds of seahorses hitching at 30°C. Hitching is described as a normal seahorse behavior in times of non-stress (Faleiro et al. 2008), and it can therefore be deduced that the seahorses being held at 22°C were in a more comfortable state of activity than those at 30°C.

Figure 15. Behavioral observations of all seahorses at each chronic treatment temperature, recorded as one of three defined behaviors. Results were pooled for entire 4 week treatment period.

Table 7. Description of seahorse activities used during chronic treatment.

<table>
<thead>
<tr>
<th>Behavior Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hitching</td>
<td>Seahorses tail wrapped around substrate with slight movement of the body</td>
</tr>
<tr>
<td>Swimming</td>
<td>Seahorse actively moving around tank, without making contact with any substrate</td>
</tr>
<tr>
<td>Resting</td>
<td>Seahorse making contact with bottom of tank, while not having tail wrapped around any substrate</td>
</tr>
</tbody>
</table>
RELATIVE EXPRESSION OF *hsp60* AND *hsp70* DURING CHRONIC TREATMENT

The physiological responses of seahorses to chronic temperature stress led to the question of what was occurring on the cellular level. Heat shock protein 60 and 70 mRNA expression was used as a quantitative method to assess the cellular stress response of animals held at different temperatures. The expression of *hsp60* and *hsp70* in seahorses exposed to the control treatment temperature of 22°C for a 4-week period showed high levels biological variability, with the basal levels of *hsp60* and *hsp70* found in individual seahorses varied within a wide range (0.01 – 100) (Table 8). The median normalized ΔΔCt for these animals was 16, while the mean value was 91. *Hsp70* expression showed a similar but less drastic trend in 22°C seahorses, with a median value of 90, and a mean value of 105. Seahorses held at 26°C for a 4-week period showed an increased expression of *hsp60* (median = 270, mean = 380) and *hsp70* (median = 615, mean = 620) from control animals, indicating activation of the heat shock response. Again, a large amount of variability was found in the samples, preventing the induction from reach statistical significance. Unexpectedly, seahorses exposed to severe stress at 30°C showed down-regulation of heat shock gene expression compared to 26°C animals. The median and mean values for *hsp60* were 0.01 and 290, respectively, while the values for *hsp70* were 0.12 and 600. This points to two important and interesting ideas: first, the values are all lower than those seen in 26°C animals, indicating that the heat shock response is being induced as would be expected. Second, due to the
high degree of biological variability, some animals were appeared competent to activate a cellular stress response, while others were unable to do so. The overall failure of the cellular stress mechanisms to respond at this 30°C treatment temperature correlates with the low survivorship of animals in this treatment group.

Table 8. Average ΔΔCt values for hsp60 and hsp70 from chronic treatment as mean and median values.

<table>
<thead>
<tr>
<th></th>
<th>22°C</th>
<th>26°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp60 (mean)</td>
<td>91</td>
<td>380</td>
<td>290</td>
</tr>
<tr>
<td>hsp60 (median)</td>
<td>16</td>
<td>270</td>
<td>0.01</td>
</tr>
<tr>
<td>hsp70 (mean)</td>
<td>105</td>
<td>620</td>
<td>600</td>
</tr>
<tr>
<td>hsp70 (median)</td>
<td>90</td>
<td>615</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Figure 16. Relative expression of A) hsp60 and B) hsp70 at chronic treatment duration of 4 weeks shown as box and whisker plots with quartile 1, median, and quartile 3 on a log scale.
DISCUSSION

PHYSIOLOGICAL RESPONSES OF SEAHORSES TO CHRONIC AND ACUTE TEMPERATURE INCREASES

The ventilation rates of the seahorses over the course of the chronic treatment gradually increased for all temperature treatments over the four-week treatment period (Figure 10, page 35), which is in agreement with the accepted relationship between growth and ventilation requirements (Kiorboe and Hirst 2014). The seahorses used during this experiment were 4-months old at the start of the treatments. These animals reach adult size between 8 and 10 months and have a lifespan of 2 – 4 years (Vincent et al. 2011). This likely indicates that the seahorses used in this research were still in the juvenile stages, where energy demands are greatly elevated due to the requirements of rapid somatic cell growth. This is in agreement with the general increasing ventilation rates over time for all chronic treatment seahorses, but it does not explain why ventilation rates were different between each temperature treatment. The solubility of oxygen in water increases with decreasing temperature, meaning that more oxygen can dissolve in cooler water than warmer water, and therefore be more readily available for uptake by biological organisms (Perlman 2017). This does provide some guidance on the differences in ventilation rates between the chronic temperature treatments, where, at 30°C, ventilation rates were greatly increased compared to the 22°C and 26°C.
treatments. The main conclusion from this piece of data is that ventilation rates of lined seahorses are elevated with temperature.

Q10 is a metric used to describe how the rate of a biological process will change with every 10°C increase in temperature, and requires two rates measured at two different temperatures to calculate. Following the strict rules of chemistry, the expected Q10 for any reaction would be 2, referring to a doubling of the measured rate. This is often not the observed Q10 in practice though, as biological organisms are dynamic, with multiple response mechanisms to protect and overcome these environmental challenges. Turingan and Sloan (2016) found that invasive fishes like the Mayan cichlid (*Cichlosoma urophthalmus*), lionfish (*Pterois volitans*), and pike killifish (*Belonesox belizanus*) could maintain their optimal feeding kinematics despite being subject to increased temperatures, indicating the presence of a compensatory mechanism within their biology. These compensatory mechanisms are thought to include *hsp*5 and other chaperone proteins, but it is also apparent that there is a point in the thermal exposure of fishes that these compensatory mechanisms cannot overcome the level of stressor, as mortality still occurs at certain temperatures.

The Q10 values calculated by week during the chronic treatment can be viewed in Figure 11 (page 39). Throughout the 4-week exposure time of seahorses to 22°C, their average breaths per minute increased by 5, compared to an increase of 10 breaths per minute at 26°C and 15 breaths per minute at 30°C. This suggests
that there is a compensatory mechanism at work to overcome to thermal challenge (Turingan and Sloan 2016). The average Q10 calculated for the chronic experiment animals was 1.68 for animals at 26°C, and 2.07 for animals at 30°C, indicating that the ventilation rates of animals at 26°C were beneath the expected change due to temperature likely due to some compensatory mechanism.

It was expected that, as the seahorses aged and ventilation rates increased, they would require larger amounts of food to maintain themselves. This is especially true of juvenile fishes, as different life stages have different energy demands, and the juvenile stage exhibits the fastest somatic cell growth and therefore the largest energy requirements (Peck et al. 2003). This was not observed in this experiment to any level of significance (Figure 12, page 40), which is in conflict with the findings of many other studies, where increases in environmental temperature cause fish to increase their food consumption (Baltz et al. 1987; Jobling 1997; Freitas et al. 2015), but in agreement with others (Faleiro et al. 2015). In this case, the seahorses may be adjusting their feeding behavior in ways not identified in this research, or feeding activity was not an energetic priority able to be fulfilled by the animals.

RELATIVE EXPRESSION OF \textit{hsp60} AND \textit{hsp70} IN SEAHORSES EXPOSED TO ACUTE AND CHRONIC TEMPERATURE INCREASES

\textit{Hsp60} and \textit{hsp70} are molecular chaperone genes that are translated into the proteins HSP60 and HSP70, which assist in the sequestering and re-folding of
damaged proteins. Proteins that are misfolded can form large aggregates, have unnaturally exposed active sites, and generally cannot function in their intended physiological processes (Kregel 2002; Khalil et al. 2011). Specifically, HSP60 (translated from the gene hsp60) is a protein that is active in the mitochondrion, and functions to help re-fold, prevent aggregation, and/or guide the denatured proteins to an apoptotic pathway. HSP70 is a protein found in the cell cytosol, functioning as a molecular chaperone to aid in the re-folding of denatured proteins (Kregel 2002). As an ectothermic organism, like a fish, is exposed to changes in temperature, their cells are also subject to this change in conditions, leading to cell stress.

A natural and necessary heat shock response has been documented in many marine invertebrates that are exposed to either diel migrations or intertidal temperature changes. One amphipod species (Phronima sedentaria) typically undergoes a diel migration involving an abrupt 15°C temperature change. The diel exposure lasts for 9 hours on average, and a small heat shock response is mounted to allow these migrations. When these amphipods are exposed to this 15°C temperature increase for longer than this natural exposure duration, their heat shock response is greatly upregulated. Any duration exposure of these amphipods to temperatures above their thermal limits lead to immediate upregulation of hsp6s, as well as a 50% mortality (Elder and Seibel 2015). The temperature stress causes proteins inside of the cell to misfold, preventing them from functioning properly, or even causing them to interact improperly with other proteins. This stress induces
the up-regulation of these HSP molecular chaperones, which can be confirmed by an increase in the presence of the genes coding for these proteins in the organism’s mRNA. The heat shock response mounts quickly to ensure minimal damage can occur in the cell, and once the damaged proteins have been appropriately managed, the expression of these \textit{hsp} genes can be downregulated (Iwama et al. 1998; Basu et al. 2002; Wang et al. 2014). In the absence of a mounted heat shock response, cells exposed to, and therefore damaged by, temperature stress will undergo programmed cell death. This has been documented \textit{in vitro} in a tumorigenic mouse cell line (BC-8) that fails to mount a heat shock response when exposed to increased temperature. Upon introduction of the heat stress, the cells may quickly undergo apoptosis (Sreedhar et al. 1999).

The expression of \textit{hsp60} and \textit{hsp70} in the livers of seahorses held at 22°C for 2, 6, and 10 hours was expected to remain unchanged, as this temperature represents the natural setting in which these animals were reared, and has been shown to be within the range of favorable temperatures for growth and survival of this species (Lin et al. 2008). This was not the case however, as the expression of \textit{hsp60} (Figure 5, page 28) and \textit{hsp70} (Figure 7, page 31) were downregulated at 6 and 10 hours. This indicates some sort of stressor inducing a heat shock response initially (at the 2-hour treatment), but resolving itself by 6 and 10 hours. Handling stress could cause this mounting of the heat shock response, as this has been recorded in mammals (Blake et al. 1999). This was refuted in fish models by Ackerman et al. (2000), and it was generally accepted from therein that short term
handling of fish does not elicit a heat shock stress response. The data presented here suggests that some fish do in fact mount a heat shock response when handled, while also suggesting that there is great biological variability between individual organisms.

The expression of *hsp60* and *hsp70* at 26°C for 2, 6, and 10 hours were different from one another, although neither gene showed any statistically significant difference to 22°C at this temperature. *Hsp60* (Figure 5, page 28) again showed an initial increase in expression, but the response was decreased at longer durations. This suggests that the abrupt temperature change from 22°C to 26°C was enough of a stressor to immediately induce the upregulation of *hsp60* to repair damaged proteins in the mitochondrion, but the animal’s cells were able to acclimate over time to the new temperature. As the seahorses became more acclimated to the new temperature of 26°C, the cells become less negatively affected by the temperature, and HSP60 is no longer needed in such large quantities. *Hsp70* (Figure 7, page 31) expression gradually increased with longer durations at 26°C, indicating that the heat shock response in the cytosol was delayed compared to that of the mitochondrion. This could mean that 1) proteins in the cytosol are less negatively affected by the change from 22°C to 26°C, or 2) are affected at a slower rate, so that the accumulation of denatured and misfolded proteins ensues more slowly. This would explain why the upregulation of *hsp70* is occurring more gradually. There was also a large standard error in the 10-hour
duration, showing that there is large biological variation in the upregulation of hsp70.

The expression of hsp60 at 30°C and 32°C for 2, 6, and 10 hours creates a bell shaped curve, with a large induction of the gene at 6 hours, followed by downregulation at 10 hours (Figure 5, page 28). This shape is typical of the heat shock response, indicating a mounting induction of hsp60 that reaches a peak, and then a recovery period as the cells adapt to the surrounding environment. The expression of hsp70 at 30°C showed a large initial induction at 2 hours, and a quick recovery defined by downregulation of the gene at 6 and 10 hours (Figure 7, page 31), indicating that the animal is able to adapt over time to that temperature regime. At 32°C, hsp70 expression remained elevated at all durations (Figure 7, page 31), suggesting that this temperature is above the maximum threshold of tolerance for this species. The prevailing trend in hsp60 expression across all acute treatment temperatures and durations is a slight increase at 2 hours, a larger and plateauing increase by 6 hours, and a decrease in expression by 10 hours (Figure 6, page 29), with greater levels of upregulation observed with higher temperatures. At 2 hours, 30°C and 32°C -treated seahorses showed a significant increase in the expression of hsp60 when compared to the control treatment of 22°C for 2 hours (p < 0.001). At 6 hours, 26°C-treated seahorses showed a significant increase in the expression of hsp60 when compared to the control treatment of 22°C for 6 hours (p < 0.05). At 10 hours, 32°C-treated seahorses showed a significant increase in the expression of hsp60 when compared to the control treatment of 22°C for 10 hours (p < 0.05).
These significance values are difficult to interpret with relative gene quantification data, and the overall represented trend (Figure 6, page 29) provides the most accurate depiction of how hsp60 gene expression is responding to acute temperature changes in the lined seahorse. The trend of hsp70 expression throughout the acute experiment is different than that of hsp60 (Figure 8, page 32), showing a general increase in expression at 2 hours, followed by downregulation, although downregulation only occurred in the 26°C and 30°C animals. At 2 hours, 26°C and 32°C -treated seahorses showed a significant increase in the expression of hsp70 when compared to the control treatment of 22°C for 2 hours (p < 0.05). At 6 and 10 hours, there was no significant difference in expression of the gene at the treatment temperatures when compared to the 22°C at the same durations. As with the hsp60 trends above, these significance results can gloss over the real trends occurring in expression, which is that seahorses exposed to increased temperatures on an acute duration show the appropriate expected molecular stress responses indicative of a biological system reacting as necessary.

Three main sources of variability have been described in the use of qPCR (Bustin 2010), raising concern as to the need for standardized protocols and reporting methods. One portion of variability arises from poor experimental design, as with any research related project. Another source of variability is that inherent to the instruments used in qPCR and biochemical reaction inconsistencies. The third source of variation arises from the intrinsic dynamic nature of biological systems, which can help define why the standard errors of gene expression in these
experiments were so large. All animals have genetic variability, followed by phenotypic variability and discrepancies in environmental exposure histories, allowing different biological systems to react to the same conditions in different ways. This, in essence, is how evolution occurs. Even the location of mRNA within an organism’s cells can alter how one treatment affects the expression of that mRNA (Bustin 2010), which is why two experimental genes were used during this experiment. Hsp60 is found within the mitochondria of the cell, while hsp70 is found free in the cytosol (Kregel 2002), so each gene is differently exposed to environmental stressors.

The expression of hsp60 (Figure 16A, page 46) and hsp70 (Figure 16B, page 46) following the chronic treatment also showed large variability from different biological organisms. The relative expression of hsp60 in seahorses held at 26°C for 4 weeks was upregulated 300-fold from that measured in seahorses held at 22°C for the same duration, while those held at 30°C for 4 weeks expressed a 200-fold increase in expression from animals at 22°C. The observed expression values of hsp60 in 26°C animals during the acute treatments were upregulated 2 – 5-fold (Figure 5, page 28), compared to the chronic 300-fold increase. The observed expression values of hsp60 in 30°C animals during the acute treatments, however, were upregulated 10 – 200-fold (Figure 5, page 28), suggesting that some seahorses in the acute treatment reached the average chronic relative expression levels of hsp60, while others were able to maintain a less heat shock responsive system at this temperature. In other words, many individual seahorses failed to
mount an appropriate heat shock response at 30°C. The relative expression of
*hsp70* in seahorses held at 26°C for 4 weeks was upregulated 200-fold from that
measured in seahorses held at 22°C for the same duration, while those held at 30°C
for 4 weeks expressed a 100-fold increase in expression. The observed expression
values of *hsp70* in 26°C animals during the acute treatments showed a great deal of
variability, as levels were upregulated 3 – 200-fold (Figure 7, page 31), where
some animals reached the maximum 4-week 200-fold increase in *hsp70* expression.
The observed expression values of *hsp70* in 30°C animals during the acute
treatments, however, were upregulated 5 – 19000-fold (Figure 7, page 31),
suggesting that some seahorses in the acute treatment were extremely stressed by
the instantaneous exposure to such an elevated temperature, while those animals
exposed to such a temperature for a longer period of time were able to adjust
adequately to a less stressed state.

The heat shock response is typically considered a beneficial molecular
reaction to adverse conditions, as the re-folding and/or sequestering of damaged
proteins prevents inappropriate interactions within the cell. Over-expression of
these genes indicates two important factors – the cell is experiencing adverse
conditions, and the cell is able to respond to these conditions. Continued production
of *hsp70* has been shown to improve tolerance to unfavorable conditions (Khalil et
al. 2011), and this assists in explaining why seahorses exposed to increased
temperatures acutely show such a wide variability in expression, but those given a
chronic period of time at the same temperature are able to level out their expression
to the adequate level to maintain proper cell function. This view may be slightly too simplistic though, as overexpression of hsp is not always beneficial. In treatment of human cancers, hsp have come into question as hsp can inhibit the action of chemotherapeutics used to treat such diseases (Khalil et al. 2011). In other words, a drug is administered to a cancer patient to degrade certain proteins, but this action triggers hsp to upregulate and repair these same proteins that were the target of the drug. Another fact of hsp to consider is that they are energetically costly to make in the cell, taking energy away from other biological processes like ventilation, growth, and reproduction, in order to fuel the production of the molecular chaperones (Silbermann and Tatar 2000; Al-Whaibi 2011). Transgenic Drosophila melanogaster with greatly upregulated hsp showed a lower incidence of death related to aging, at the cost of reproductive success, namely a decreased number of hatched eggs per transgenic individual (Silbermann and Tatar 2000). So although the presence of hsp is indicative of a proper cellular response to stress, the continued production of these proteins at high levels can be detrimental to the organism’s cellular system.
CONCLUSION

In conclusion, these data suggest that the lined seahorse, *Hippocampus erectus*, has developed robust phenotypic plasticity for acute temperature challenge situations, but it may be less able to cope with temperature challenges on a chronic timescale. These results are also supported by the recent findings of Mascaro et al. (2016). Seahorses treated with acute temperature increases for 2, 6, or 10 hours, mounted an appropriate heat shock response in both the mitochondrion (*hsp*60) and cell cytoplasm (*hsp*70) at all treatment temperatures, with the expected recovery defined by downregulation by the 10-hour treatment. Seahorses treated with increased temperatures for a chronic period of time showed a different trend, where animals held at an increased 26°C mounted an appropriate heat shock response of both *hsp*60 and *hsp*70. This response was reflected in the Q10 value of 1.67 for animals at this temperature, as it indicates there is a compensatory mechanism, like the heat shock response, working to assist the biological system to maintain normal stasis. Animals held at 30°C for the chronic duration however showed a complete failure of molecular response mechanisms in the form of the heat shock response. Both *hsp*60 and *hsp*70 were downregulated from the control 22°C seahorses and 26°C seahorses, and this was also reflected in the Q10 value of 2.07, indicating there was no compensatory mechanism at work to assist in recovery of the biological system. This was further supported by the mortalities seen in the 30°C chronic treatment seahorses. There were, however, some seahorses in the 30°C treatment that did attempt to mount a heat shock response when thermally
challenged, leading to a large degree of biological variability in the fold change expression values. These findings support that seahorses will be negatively affected by global climate change, as sea surface temperatures rise, creating a chronic period of increased temperature exposure. Seahorses can further be used a model of mobility-limited fish species that do not have the capabilities to shift their ranges as effectively as more mobile species.


Fisheries Centre Research Reports. 2016. Seahorses (Hippocampus spp.) and the CITES Review of Significant Trade. Institute for the Oceans and Fisheries, The University of British Columbia, Canada.


