Upper thermal limits in *Mytilus trossulus and Mytilus californianus:* Glucose as Major Fuel for Heat-stress Response

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Abstract. A range of upper thermal limits and glucose levels of heat-stressed mantle tissue were examined in mussels (*Mytilus trossulus and Mytilus californianus)*. When placed in temperatures increasing at 1°C/10 min^{-1} the upper thermal limit was found to range between 35°C and 40°C as recovery time ranged from 7-10 days. After seven days post-heat stress, experimental *M. californianus* glucose levels were significantly decreased compared to control values. Due to a high standard error, *M. trossulus* data was not considered.

Introduction

Intertidal and subtidal organisms have a unique ability of coping with daily fluctuations in salinity, pH, temperature, and irradiation. In the event of environmental perturbations, such as global warming, these animals may not have the ability to survive constant temperature increases. Lethal upper limits have been determined for many bivalve animals, but the various methods used have made interpretations problematic (Hicks, D.W. & McMahon, R.F.). Upper thermal limits for *Mytilus trossulus* and *Mytilus californianus* have not yet been determined, and have been examined here.

Only one species has been found to not synthesize heat-shock proteins (Hsps) in response to a small increase of temperature above the normal range. During a heat-stress event, Hsps are the major proteins synthesized (Roberts, et al., 1997). These molecular chaperones work to prevent aggregation and unfolding of damaged proteins. Therefore, Hsps work to repair the effects of a heat-shock event to return cellular processes to normal. To express a sudden abundance of non-endogenous Hsps an immediate supply

of energy, glycogen, is required. Glycogen is the major storage site of energy for bivalves (Patterson, et al., 1999). The digestion of glycogen, to glucose, allows an immediate entrance to aerobic metabolism to drive the survival attempt.

Mytilus trossulus and *Mytillus californianus* were chosen as experimental species, due to the lack of upper thermal limits, thus far, determined in these species. This paper examines (1) upper thermal ranges that may, or may not, prove lethal for these species; and (2) significant differences of glucose concentrations, post-heat stress, taken from mantle tissue.

Methods & Materials

Upper thermal limits

M. trossulus and *M. californianus* were collected from the boat docks in Charleston, OR. Mussels were transported to the lab with flow-through seawater. Shells were cleaned of encrusted organisms, separated by species into separate baskets, and placed within an aerated 11° C seawater table. *M. trossulus* were acclimated for 11 days and had an average length of 5.02 cm. *M. californianus* were acclimated for 14 days and had an average shell length of 7.36 cm.

Ten of each mussel species were used per experiment, five in each experimental treatment and five for the control. The control animals, in all cases, were kept at 11° C in an aerated sea table and were left undisturbed during the duration of each experiment. For each heat stress assay, mussels were placed within a beaker containing 11° C seawater, with an aerator. The beaker was then placed into a 15° C, pre-heated water bath. Every 10 minutes the water temperature was increased by 1° C, the mussels were removed and observed for mortality (gaping valves), and then placed back into the water bath. This procedure continued until reaching the maximum temperature. Once complete, the mussels were left in the beaker and left at room temperature for 20 minutes. To further allow gradual cooling, the beaker was placed within the sea table for 30 minutes. Finally, the animals were placed back into the sea table and were observed for mortality over the next week. Mussels that were gaping and unresponsive to touch were

determined to be dead. Two experiments were run: the first experiment increased to a maximum of 40° C, while the second experiment was increased to a maximum of 35° C.

Glycogen Assay

Animals that survived seven days post-heat stress were analyzed for glycogen content of mantle tissue. The tissue was carefully taken from the upper right hand corner of each *Mytilus* shell (of right shell when shell placed open and right side up).. The samples were then frozen using liquid nitrogen and stored at -80° C.

Mantle samples of ~50 mg were then homogenized with 20x volumes of sodium citrate buffer. Blue plastic pestles were used to mascarate the tissue, as well as possible. After the initial homogenation, samples were further degraded by use of a heat block. Here, samples were warmed at 90° C for three minutes and re-homogenized. Due to the difficulty in breaking down the mantle tissue, this process was repeated twice. The homogenate was then centrifuged at 14,000 g for three minutes. The supernatatant was then aliquotted into three separate sets of microcentrifuge tubes (samples stored on ice throughout assays): 1) 20 uL for the protein quantification assay, 2) 400 uL with the addition of 20 uL of the digestion enzyme (0.5% amyloglucosidase), 3) 400 uL with the addition of 20 uL sodium citrate buffer (only four randomly chosen samples) for the undigested data. The digested and undigested samples were then incubated at 55° C for two hours, centrifuged at 130,000 g for 30 minutes, 333.3 uL of supernatant was then transferred to new microcentrifuge tubes, and stored overnight in the refrigerator.

The protein quantification assay was performed in (1) two sets of BSA standards and; (2) one tube each of the homogenate supernatant from each sample. The glycogen concentrations were later used to normalize the glucose concentrations. The same protocol was utilized from Laboratory #3 – Marine Animal Physiology (Caren Braby, 2006) with the exception of sodium citrate buffer (100mM, pH 5) included with the BSA stock volume. Two sets of each standard were processed to determine the technique efficiency.

To determine the final glucose concentrations, we used the Glucose (GO) Assay Kit (GAGO-20) from SIGMA. The kit had recommended using samples of glucose equaling 0.02-0.08 mg of glucose/mL. The adductor muscle in bivalves has been shown as 7-12 mg glycogen/gram of tissue and is ~10% of the total found in flounders. With flounder exhibiting 1.44 mg glucose/mL, (Carr & Nerr, 1984) we were able to determine a 10% estimate for *Mytilus* at 0.144 mg glucose/mL. Therefore, 1:20 volumes were used for the homogenization samples rather than 1:10 values that were used by Carr and Neff. Our final digested/undigested samples were expected to be in the range of 0.01-0.075 ug of glucose/mL. The SIGMA protocol was then followed with the exception of 1mL assays performed rather than 3 mL.

Results

Range of upper thermal limits

The upper thermal temperatures and mussel mortality/survival percentages are listed in Table 1. When exposed to 40°C, 100% mortality was observed. *M. trossulus* survived at 40°C for 10 days and *M. californianus* survived 7 days post-heat stress. At 35°C exposure, 100% of both species survived seven days and were dissected for mantle tissue. A range of upper thermal limits has been determined as 35°C-40°C when duration of recovery is one week.

Glucose assay

The protein quantification assay results are graphed in Figures 1 and 2. Fig. 1 shows a linear relationship of BSA concentrations compared to the absorbency of the standards, suggesting sufficient experimental techniques. The equations were used to calculate the concentrations of protein and glycogen concentrations, respectively. The final concentrations were then utilized to find the normalized glucose concentrations.

From previous work (Carr & Neff, 1984), we expected glucose concentrations to fall within the range of 0.02-0.144 ug glucose/mL. We were near these values at 0.11-0.23 ug glucose/mL. However, these reported values are only including the experimental *M. californianus* and the control. There was a large standard error associated with the

M. trossulus controls. Therefore, all values for *M. trossulus* will not be reported. A significant decrease in glucose concentration was found in the heat-stressed *M. californianus* when compared to control values (Fig. 3). This suggests that these mussels had depleted glucose levels in response to the heat stress.

A problem also occurred with the absorbency reading of the undigested samples. Two of the, four randomly chosen, undigested samples were at zero while the other two had values resembling the digested glucose samples. Khoury Hickman (another student also running this assay) did have zero values for all undigested samples, suggesting that an error was made by this experimenter. This oddity was most likely due to the addition of amyloglucosidase to the two undigested samples, in error.

Discussion

Total mussel mortality was seen when thermal stressed to 40°C, within 10 days of treatment. However, when exposed to upper thermal limits of 35°C both *Mytilus* species did survive during a week period suggesting that this isn't a lethal temperature (Fig. 1). When dissecting the lower temperature mussels, it was noted that two *M. trossulus* did not appear healthy. If these animals were left in recovery for two, three, or maybe four weeks, higher levels of mortality may have been seen.

The treatment of 35°C was not lethal, but it was high enough to stimulate a heat-shock response in *M. californianus* (Fig. 3). Previous studies agree with this conclusion, one reported a 0° to 34° value for *M. californianus* collected along the Oregon coast (Elvin, D.W., and Gonor, J.J., 1979). Data won't be reported for *M. trossulus* in this paper. However, during midday low tides on the Washington coast, *M. trossulus* tissue temperature was recorded as high as 32°C during emersion (Somero, 1995). Studies looking at both temperature stress and heat-shock induction are key in understanding how marine euryhaline animals cope with their ever changing environments. Furthermore, one can determine how and if these species will continue to survive if upper thermal limits are reached and continue to climb on a long-term basis, such as in global warming.

The mussel energy budgets are also important. Synthesizing Hsps is an expensive chore. It was previously estimated that 20-25% of *Mytilus edulis* total energy was spent

in the synthesis of proteins (Hawkins, 1991). This agrees with the depletion of glucose concentrations when comparing experimental *M. californianus* with the controls (Fig. 5). Numerous scientific viewpoints do agree that the heat-stress response comes with a high price tag to organisms (Krebs, R.A. and Loeschcke, V., 1994). This leads us, to all the more reason, to further study and understand these complex processes.

Graphs & Tables

Table 1. Upper Thermal Limit assay. Temperatures at which *Mytilus* survived or experienced mortality. At 35°C surviving mussels were dissected 7 days post-heat stress. At 40°C *M. trossulus* survived 10 days while *M. californianus* survived 7 days.

Mussel Mortality/Survival		
Species	35°C Maximum Temperature	40°C Maximum Temperature
M. trossulus	100% Survival	100% Mortality
M. californianus	100% Survival	100% Mortality

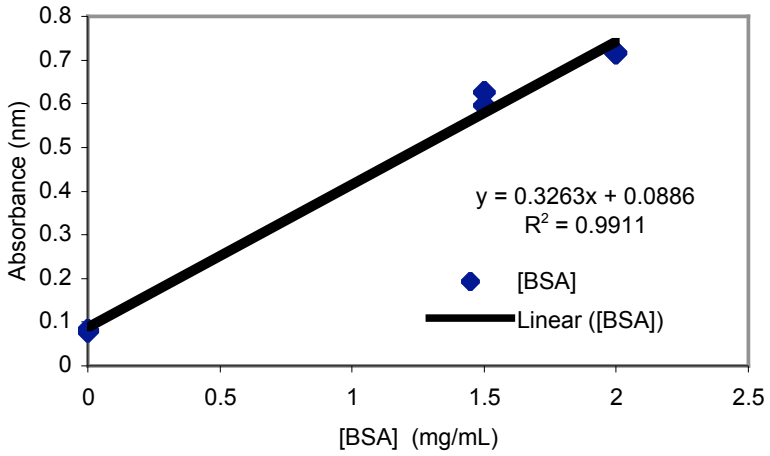

Fig. 1. Protein Quantification Assay. Linear relationship of BSA concentrations vs. absorbance of standards.

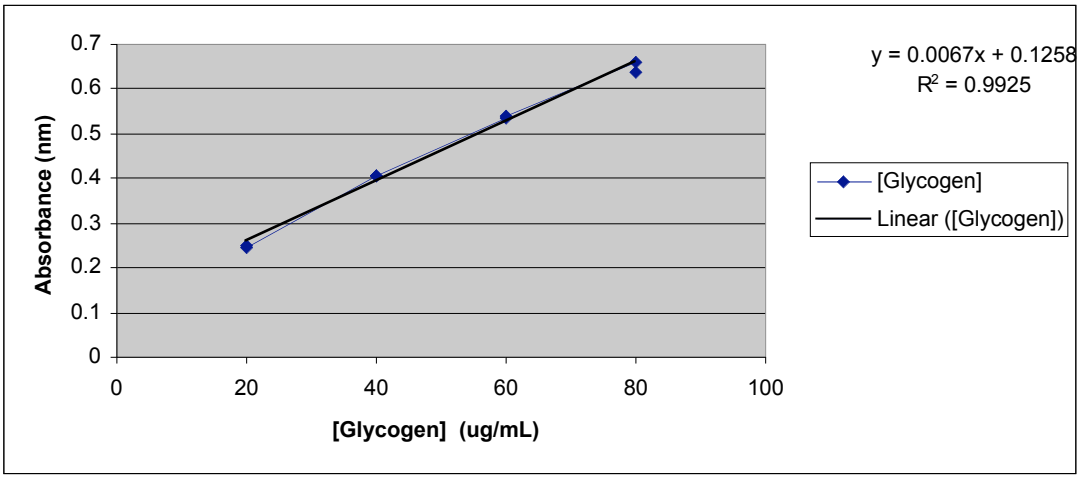

Fig. 2 Glycogen Assay. Glycogen vs. absorbance at varying concentrations used to normalize final Glucose concentrations.

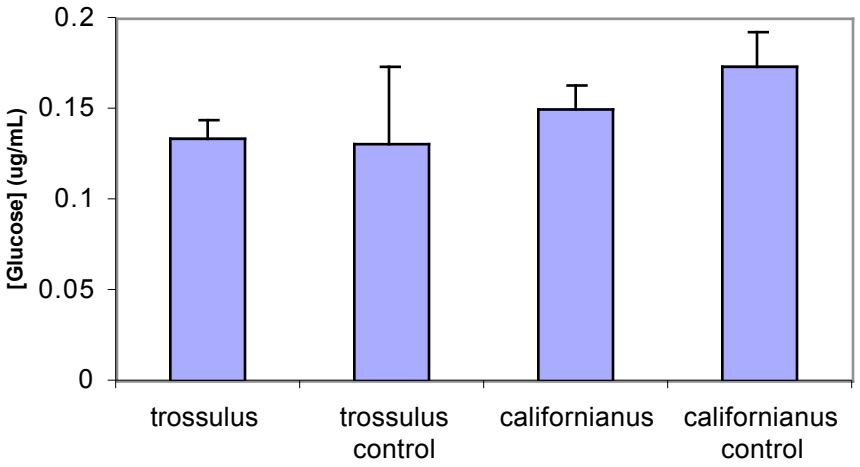

Fig. 3 Glucose Assay. *M. californianus* had a decrease in glucose concentration post-heat stress, as compared to the control. *M. trossulus* control values exhibit a large standard error and shouldn't be trusted.

Table 2 Glucose Assay. Two of the four randomly chosen undigested samples showed high levels of glycogen. This could be due to the addition of the digestion enzyme to these samples by error.

Mytilus Sample	Absorbance (nm) Glycogen	Concentration (ug/mL) Glycogen
t1	0.5751	67.05970149
t2	0.603	71.2238806
t3	0.5806	67.88059701
t4	0.5871	68.85074627
t2c	0.5854	68.59701493
t3c	0.4267	44.91044776
t4c	0.3472	33.04477612
t5c	0.7585	94.43283582
cal1	0.5718	66.56716418
cal2	0.5851	68.55223881
cal3	0.5866	68.7761194
cal4	0.583	68.23880597
cal5	0.6064	71.73134328
cal1c	0.578	67.49253731
cal2c	0.6699	81.20895522
cal3c	0.6257	74.6119403
cal4c	0.5911	69.44776119
cal5c	0.6144	72.92537313
t1 special	0.5256	59.67164179
t2c special	0.591	69.43283582
cal3 special	-0.0467	-25.74626866
cal5c special	0.0283	-14.55223881

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Upper Thermal Limit & Glucose Assay - Final Protocol

Supplies: 1-Thermometer (long),1- 2 L glass beaker, water bath, 6-baskets, tape, and clothespins (for labeling baskets)

Collect: 15-*Mytilus californianus* (all around same size)
15-*Mytilus trossulus* (all around same size)

Collect mussels from boat dock in Charleston, OR.
-cut byssal threads when collecting, do not pull mussels from docks
-transport mussels in bucket to lab
-scrape shells clean of sea life
-place species in separate, labeled baskets
-place baskets in sea water table at 11°C, with aerator

Experiments 1 & 2:

M. californianus & *M. trossulus* (of each species)
-5 used for upper thermal limit experiment
-5 used for control in 11°C sea water

Upper Thermal Limit Experiment:

Water bath pre-heated to 15°C with tap water.
Mussels prepared: 10 *M. trossulus*
5 – Sample 1, measure length & record
5 – Control, measure length & record
Measure temperature of holding tank, record.
Place 5 control *M. trossulus* in basket with aerator, in sea table.
Use seawater from holding tank to partially fill 2 L beaker
-add sample #1-(5) *M. trossulus* to beaker
-place beaker (w/mussels and sea water) into water bath
-add additional sea water to beaker until water line matches that of the water bath level
-add aerator to beaker
Once sample is held at 15° C for 10 minutes
-increase water bath temperature by 1°C
-remove beaker from bath and check sample for signs of mortality (gaping valves)
-mentally make observations (byssal attachment, gaping, etc)
-return beaker to water bath
-set timer for another 10 min
-record observations
-repeat this procedure every 10 minutes until have maintained 10 minutes at 40° C
To slowly decrease temperature of mussels, do in order at completion:

-place beaker with contents and aerator at room temperature for 20 minutes.
-place beaker with contents and aerator into 11° sea table for 30 minutes.

Replace living mussels back into labeled baskets in water table with aerator.
Record any observations of mussel activity during duration of entire experiment.
Check daily for mortality and record.
Test mortality by touching gaping valves and waiting for response.

Repeat process with *M. californianus*.

Upper Thermal Experiment #3 (longer time/lower temp)
(Preparation for glycogen assay)

All collections, transportation, handling and procedures were identical to experiments 1 & 2 with the exceptions of:

-5 of each species were placed together, rather than separately, into the beaker, sea water, and water bath.
-temperature was taken to 35° C and maintained for 10 minutes, rather than 40° C.
-after seven days post-experiment, both species and controls were used to dissect a small portion of the mantle tissue of each mussel.

Dissection of mantle tissue (*M.* trossulus, *M.* californianus, & controls):

-microcentrifuge tubes labeled for tissue samples (also labeled w/date & my initials)
    M. trossulus samples (t1-t5)
    M. trossulus controls (t1c-t5c)
    M. californianus samples (cal1-cal5)
    M. californianus controls (cal1c-cal5c)

Measured samples and controls to identify mussels 1-5, directly before each dissection.
Carefully dissected mantle tissue from upper right hand portion of shell from each mussel (if shell open and right side up).
Placed mantle tissue in correct tube.
Once finished dissecting all *M. trossulus* samples and controls
-froze samples (in tubes) with liquid nitrogen
-placed tubes on ice
Repeated with *M. californianus* and controls
Placed all frozen tubes into labeled box and put into -80° C freezer.

Glycogen Sample Preparation

Prepared samples

-retrieved *Mytilus* samples from -80° C freezer, (on ice)
-transferred ~50mg of each tissue to new microcentrifuge tube, (on ice)
-recorded each weight

Homogenized samples:

-placed blue plastic pestles in order of use on paper towel (used each three times in same sample)
-added 1000uL of sodium citrate buffer to each sample (1:20 volume)
-plastic pestles were used to break up tissue in each tube
-samples placed on 90° C heat block for 3 minutes
-pestles used again to break up tissue, to heat block again (3 min), and final try to break up tissue
-centrifuged samples for 3 minutes at 14,000 g

Protein quantification, digestion, & undigested samples:

-labelled tubes for:

1) Protein quantification (for each sample)
2) Digested samples (for each sample)
3) Undigested samples (two random samples only)

-aliquotted: (immediately to ice)

1) Protein quantification tubes:
   20 uL supernatant
2) Digested sample tubes:
   400 uL supernatant
   20 uL digestion buffer (0.5% amyloglucosidase)
3) Undigested samples (two random samples)
   400 uL homogenate
   20 uL sodium citrate buffer
   (Processed these samples identically as the digested samples.)

-Placed remaining pellet and supernatant in fridge until assay was complete.

Digested/undigested samples:

-incubated at 55° C in pre-heated water bath
added 55° C pre-heated water to a Ziploc baggy
added samples
allowed baggy and contents to float in water bath for 2 hours
-centrifuged samples at 130,000 g for 30 minutes
-labeled new tubes
-aliquotted 333.3 uL of supernatant to new tubes (to fridge until needed)

Protein quantification assay

Followed the Protein quantification protocol by Caren Braby, (Marine Animal Physiology-lab #3) with the exception of:

-buffer used for standards was sodium citrate (100mM, pH 5)

Glucose spectrophotometric assay

Followed the Sigma (GO) Assay kit (GAGO-20) – Methods 1 & 2 - with the exceptions of:

-volumes were reduced to1 mL assays rather than 3 mL

-duplicate sets of glucose standards were made with 1.5 mL sodium citrate buffer

-digested/undigested samples were (333.3 mL samples) used for the assay (previously made & refrigerated)