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Differential Response Of AMP Activated Protein Kinase (AMPK) And Hsp70 To Temperature Stress In The Gastropod, Nucella Lapillus

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Differential response of AMP activated protein kinase (AMPK) and Hsp70 to temperature stress in the gastropod, *Nucella lapillus*.

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Abstract

Populations of the gastropod *Nucella lapillus* are polymorphic for shell color, with light-colored shells predominating on warmer, wave-protected shores and dark-colored shells limited primarily to cooler, wave-exposed shores. During thermal stress, darker shells attain higher body temperatures than lighter shells. These results suggest that heat stress may determine field distribution patterns. However, there is currently little evidence of physiological consequences of thermal stress in these organisms. Following the guiding hypothesis that heat stress leads to cellular energy depletion, we explored whether the central energy regulator AMP-activated Protein Kinase (AMPK) is activated by heat stress. We compared this response in both color morphs to the expression of a heat shock protein (Hsp70) in field and lab experiments. For the field experiments, two color morphs of snails were tethered to a rock and exposed to heat stress on two hot summer days. For the laboratory experiments, snails were exposed to 24°C, 28°C, and 34°C using a heat lamp, and sampled after 0, 2 and 4 hours of heat stress. Samples were analyzed using western blot and quantitative real-time PCR. AMPK activity increased after 2 hours exposure to 28-30°C in dark morphs and 34°C in light morphs. In contrast, Hsp70 began to show increased mRNA expression after 4 hours at 34°C in light morphs, but no increased in Hsp70 protein was observed. Interestingly, AMPK activity had decreased again by the onset of Hsp70 expression. Our results suggest that AMPK may be an early indicator of thermal stress in gastropods and a useful tool for studying thermal stress. The delayed stress response in light morphs relative to dark morphs suggests that light morphs are more thermotolerant to higher temperatures. The predicted temperature rise due to climate change will increase stress events, possibly causing mortality in dark morphs, favoring the spread of white morphs. This change in color morph distribution may be a useful integrative indicator of climate change.

Introduction

Air temperatures in coastal Maine are predicted to rise 3-4°C by the year 2100 (IPCC 2001). Increased temperatures may have a large impact on intertidal flora and fauna, which regularly cope with alternating aquatic (when immersed) and terrestrial (when emersed) environmental conditions. Steep gradients in predation, energy availability, desiccation, wave energy exposure, and thermal stress create a highly variable and stressful environment (Helmuth, 1999; Hofmann et al. 2002). This variation makes the intertidal zone an ideal model for examining the effects of the physical environment (e.g. climate) on the ecology, physiological performance, and distribution of ectotherms, such as gastropods (Helmuth et al. 2006).

In the rocky intertidal zone, physiological performance and possibly organismal distribution are determined largely by temperature (Helmuth and Hofmann, 2001). Thermal stress can have detrimental effects on behavior (foraging and feeding rates) and protein synthesis and function (Dahlhoff, 2004). Most intertidal organisms live at the upper end of their thermal limit of protein synthesis (Stillman and Somero, 2000; Tomanek, 2005). Small increases in extreme temperatures, such as predicted by the IPCC, may be enough to stress intertidal ectotherms beyond their thermal limits (Tomanek, 2005). This consequence is especially probable when low tides occur at midday, causing invertebrate body temperatures to increase 15°C or more on even moderately warm days, increasing at a rate of 3°C per hour or greater (Dahlhoff, 2004; Hofmann, 1999). A better understanding of the physiological responses of these organisms to heat stress in a natural context may allow better predictions of the effects of climate change.

The dogwhelk *Nucella lapillus* is a common, carnivorous marine snail of Northern Atlantic rocky shores. Unlike most marine invertebrates, the dispersal range of *N. lapillus* is

limited by its crawl-away larva (Bell and Okamura, 2005; Palmer, 1984). The low lifetime dispersal distance (30m in 10 years) allows variability in microhabitats to lead to significant population differentiation in local morphology (Bell and Okamura, 2005). Considerable morphological variation in *N. lapillus* has been attributed to differential wave exposure (Etter, 1988; Rolan et al. 2004). Larger, thicker-shelled whelks occur on wave-protected shores and smaller, thinner-shelled whelks occur on wave exposed shores (Avery and Etter, 2006). *Nucella lapillus* also exhibits a genetically controlled color polymorphism that correlates with wave exposure and may be explained by the varying thermal features of light and dark shells.

Shell color has been found to correlate with environmental gradients of wave exposure and temperature: dark whelks occur on cooler, wave exposed shores and white whelks occur on warmer, wave protected shores (Etter 1988; Harris and Jones 1995; Palmer, 1984). Etter (1988) found that darker morphs heated up more quickly, attained higher temperatures, and suffered higher mortality than white morphs. Whelks on wave-exposed shores experience less thermal and desiccation stress than on wave-protected shores due to wave-splash and the cool, shaded crevices in which whelks take shelter from the wave action (Harris and Jones, 1995). Conversely, whelks with light shells reflect solar radiation, reducing maximum body temperature on protected shores; however, changes in shell coloration (due to algae growth on shells) can modify the reflectivity and thus body temperature of whelks (Etter, 1988; Helmuth, 1999). This association suggests that thermal stress dictates morph-specific distribution patterns.

However, we do not yet know whether physiological symptoms of heat stress are consistent with distributional data. Biochemical indicators, such as heat shock proteins, are much more sensitive to changes in the environment than growth rate or survival rates alone (Dahlhoff, 2004). The heat-shock proteins (Hsps), especially the 70 kilodalton (kDa) class, are the most

commonly used biochemical indicator of heat stress in eco-physiology studies (Dahlhoff, 2004). Hsps are evolutionarily highly conserved, occurring in almost every species investigated (Hofmann et al. 2000, found no Hsp expression in Antarctic eelpout species). These molecular chaperones help maintain a cellular protein homeostasis between protein synthesis, protein degradation (due to various stressors, including thermal stress, cellular energy depletion, and extreme concentrations of ions, other osmolytes, gases, and various toxic substances), and protein refolding (Feder and Hofmann, 1999; Hofmann et al. 2002). Temperature is thought by some authors to be the primary cause of the induction of Hsps in intertidal organisms (Halpin et al. 2002).

The well-described heat shock response is plastic. The threshold induction temperature occurs very close to the upper thermal limits of organisms, although it varies as a function of recent thermal history of organisms (e.g., geographic and seasonal) and is dependent on acclimation and acclimatization (Berger and Emlet, 2007; Buckley et al. 2001; Dahlhoff, 2004; Feder and Hofmann, 1999; Halpin et al. 2002; Hofmann et al. 2002; Tomanek, 2002). Studies of many intertidal organisms have shown that acclimation to higher temperatures (i.e., higher thermotolerance) correlates with higher levels of Hsp70, providing a potential mechanism linking environmental stress and organisms' distributions (Feder and Hofmann, 1999; Sorte and Hofmann, 2005). Highly thermotolerant species living at the upper physiological limit of thermal stress have a relatively low capacity to further adjust the stress response to warmer temperatures because they produce Hsps slower and at higher temperatures relative to less thermotolerant species (Hofmann et al. 2002; Stillman and Somero, 2000; Tomanek, 2005). In mussels and marine snails, less heat stress (a lower total Hsp70 level) was experienced at wave-exposed than wave-protected locations (Dahlhoff, 2004; Sorte and Hofmann, 2004; Tomanek, 2005). This

difference was most pronounced in summer, when mid-day low tides coincided with high air temperatures and sunny conditions (Dahlhoff, 2004; Halpin et al. 2002; Roberts et al. 1997).

The stress response and synthesis of Hsps may contribute to the physiological tolerance that sets species distribution limits (Hofmann et al. 2002). Hsp70 is widely studied in marine organisms, and (in addition to temperature) is induced by other types of stress, such as anoxia in fruit flies (Ma and Haddad, 1997), salinity and water stress in rice plants (Pareek et al. 1997), and toxins in fish and mollusks (Feder and Hofmann, 1999). Hsps play diverse roles in unstressed cells, facilitating folding, assembly, and regulation of proteins. In addition, when mild stressors act in combination, even mild stress levels can induce Hsps (Feder and Hofmann, 1999). Following a stress event, there is a considerable time lag before the heat-shock response is expressed, ranging from one to three hours in the marine snail *Tegula* sp. (Tomanek and Somero, 2000), four hours in mussels (Halpin et al. 2004) and copepods (Voznesensky et al. 2004), to more than 24 hours in oysters (Hamdoun et al. 2003). This time lag suggests that an additional faster mechanism may help organisms withstand thermal stress. More recently, Frederich et al. (2009) suggested that AMP activated protein kinase (AMPK) might be an earlier indicator of temperature stress than Hsp70.

AMPK is sensitive to cellular stress and regulates the energy metabolism of cells. In mammalian cells, AMPK is activated by glucose deprivation and other stresses that reduce ATP levels either by inhibiting its synthesis, such as hypoxia, or by accelerating its consumption, such as muscle contraction (Hardie, 2005). Rising AMPK activity indicates decreased available energy (ATP) caused by starvation for a carbon source or other stress (Hardie, 2005). AMPK then limits further ATP use by switching on ATP-generating catabolic pathways while switching off ATP-requiring processes (by inhibiting enzymes) that are not essential to the short-term

survival of the cell, such as the synthesis of glycogen, fatty acid, and cholesterol (Fig. 1; Hardie, 2005; Kemp et al. 1999). AMPK initiates a series of compensatory changes to maintain a constant cellular ATP concentration via phosphorylation and dephosphorylation (Kemp et al. 1999). For example, AMPK activation promotes increased uptake of glucose and fatty acids into the cell and mitochondria, respectively, to accelerate ATP synthesis (Hardie, 2005). AMPK is activated through phosphorylation by an upstream kinase kinase and through binding of AMP (Hardie, 2005). AMPK is highly conserved during evolution and is a known regulator of energy metabolism in vertebrate systems, but has been rarely studied in invertebrates.

Increased temperature leads to anaerobiosis in intertidal organisms (review by Portner, 2002). Anaerobic metabolism depletes cellular ATP levels while increasing AMP levels, which activates AMPK. This study combined the traditional approach of investigating Hsp70 levels with the newer hypothesis that AMPK is involved in thermal tolerance in invertebrates. Few studies have investigated heat stress (Hsp levels) under natural conditions in the field, and lab-based predictions about the ecological role of the heat-shock response have rarely been tested in the field (for review see Feder and Hofmann, 1999). I hypothesized that AMPK is activated by heat stress and compared the response to the response of Hsp70. I also investigated whether there is differential expression in the two color morphs of *Nucella lapillus,* both in a controlled laboratory setting and in the field. The distribution of the color morphs may be determined environmentally by microhabitat differences (wave-exposure) that affect thermotolerance. The warmer air temperatures predicted for coastal Maine by the IPCC may change this distribution pattern, possibly making color morphs a useful indicator of climate change.

Materials and Methods

Overview

To investigate the differential thermal stress in the color morphs, reciprocal field transplant experiments were conducted. Field sites were surveyed prior to transplants to determine distribution of color morphs and whelk tissue temperature. The purpose of the transplants was to measure variable thermal stress in the natural habitat of dogwhelks and compare stress in dark- and white-shelled whelks on both a wave-protected and a wave-exposed site. Results obtained from these experiments were inconclusive, so experiments were moved into a controlled laboratory setting. The lab trials investigated differential heat stress between dark- and white-shelled whelks exposed to the same thermal regime. For all experiments, biochemical indices (Hsp and AMPK) were quantified using western blots, with additional quantification of Hsp using qPCR.

Field Site

Wood Island, off Biddeford Pool, Maine, was selected as a study site due to the proximity of a wave-exposed and wave-protected shore (Fig. 2). Around 70 whelks were surveyed on both shores. Body temperature measurements from these whelks were taken using a hypodermic temperature probe during midday low tide series, when the warmest temperatures occur due to solar radiation (Fig. 3).

Field Transplant

A field reciprocal transplant experiment was conducted in the summer of 2007 to investigate differential heat stress in dark- and white-shelled whelks in their natural habitats, but

numerous confounding factors (the whelks were not tethered, resulting in microhabitat effects) in addition to a low recapture rate (50%) produced inconclusive results. In the summer of 2008, two more field reciprocal transplant experiments were conducted. Two flat areas of bedrock at similar tidal heights on the exposed and protected shores were scraped clean of algae and barnacles. Whelks (80 white, 80 dark) were tethered (using fishing line and superglue) to grids of fishing line secured to the bedrock at the wave-exposed and wave-protected sites for each transplant. The largest dark whelks (17 to 20 mm) and smallest white whelks (19 to 24 mm) were used to reduce size-related effects (Fig. 4). No algae-covered whelks were used for the experiments. During the first transplant of 2008 (Transplant A), the wave-exposed site was too low and remained immersed much longer than the wave-protected site, so only the wave-protected site was used, with dark- and white-shelled whelks tethered to the bedrock to compare heat stress between the two color morphs in the natural environment. A new wave-exposed site was cleared for the second transplant of 2008 (Transplant B).

	Dark Whelks	White Whelks
Exposed Site	40	40
Protected Site	40	40

The whelks were tethered at the beginning of a midday low tide series with predicted sunny days and warm temperatures. Samples were collected the following day after four hours of aerial exposure, at the time of expected Hsp70 and AMPK maximum expression after emersion. Only the foot muscle was dissected from the whelks because it is easily isolated from surrounding tissues, and to exclude other organs that are not quickly isolated and that may respond differently to heat stress. Half of the whelks from each treatment (n = 20) were flash frozen with tongs pre-cooled in liquid nitrogen and stored at -80°C for western blots to assay

Hsp protein and AMPK activity levels, and half (n = 20) stored in RNAlater® solution for quantitative real-time Polymerase Chain Reaction (qPCR) to assay Hsp mRNA levels.

Lab Experiments

Due to the numerous confounding variables in the field, the temporal pattern of Hsp70 and AMPK mRNA expression post stress was also studied in a controlled lab setting. Whelks were collected from Wood Island and acclimated to lab conditions for several days before experimentation. Whelks were tethered to a rock in a flowing seawater tank 12 hours before the experiment. The rock was emersed and heated with a 250 watt infrared heat lamp to either a control temperature of 24°C (below any reported threshold of Hsp70 in marine snails), a low-stress temperature of 28°C (similar to the induction threshold of Hsp70 in *Tegula sp.*; Tomanek, 2005), or a high-stress temperature of 34°C (near the maximum temperature experienced in the intertidal in Maine, but lower than the lethal temperature of 36°C for *Nucella* sp. reported by Sandison, 1967 and Sorte and Hofmann, 2005). Temperature was recorded using thermocouples inserted in four seawater-filled whelk shells sealed with silicone (Fig. 5). Foot tissue of the heated whelks was collected after zero, two and four hrs of exposure to 24°C and 28°C, and after zero, three, and five hrs of exposure to 34°C. Timing of the exposure duration began immediately after the rock was emersed from the water and the heat lamp turned on. The sampling times replicated the emersion time experienced by whelks in the field. Half the samples were flash frozen for western blots to assay Hsp protein levels and AMPK activity (n = 4 to 7) and half stored in RNAlater® solution for qPCR to assay Hsp mRNA levels (n = 6).

Characterization of Biochemical Indices

Total Hsp70 protein and AMPK activity (phosphorylated AMPK) were quantified by western blots. Samples of tissue were ground in liquid nitrogen and homogenized in a buffer containing phosphatase inhibitors to prevent dephosphorylation of AMPK as described by Frederich et al. 2009. An 8% polyacrylamide/SDS gel at 200 V for 30 min was used to separate 50µg of the homogenate. The proteins were then transferred to a nitrocellulose membrane at 70 V for two hrs. After blocking the membranes with 3% non-fat dry milk, primary mouse anti-Hsp70 (Sigma) and rabbit anti-phospho-AMPK (Upstate) antibodies and secondary goat anti-mouse or goat anti-rabbit antibodies (BIORAD) were used to detect a signal. Anti-actin antibodies (Sigma) were used as a positive loading control. Each gel was loaded with samples from all treatment groups (controls and respective exposure duration). The membranes were scanned and the bands quantified using ImageJ software. The intensities of the sample bands were divided by the intensities of the actin loading control to allow comparison among gels.

Degenerate primers were designed with the idtDNA primer design tool (www.idtDNA.com) based on Hsp70 gene and protein sequences from various invertebrate species found by searching GenBank, and aligning these sequences using MultiAlin (bioinfo.genotoul.fr/multalin/multalin.html). Degenerate primers for PCR were designed based on highly conserved parts in the aligned sequences, using *Littorina* sp. sequences as the target sequence (due to the relationship with *Nucella* sp.; Fig. 6, Table 1). Total RNA was purified from whelk tissue stored in RNAlater® Solution using the Promega Total RNA Isolation System. RNA was then tested for purity, quality, and quantity by a UV/VIS spectrophotometer at 260 and 280nm wavelengths. The Invitrogen Super-Script First Strand Synthesis System was used for reverse transcription from RNA to cDNA, which was then amplified by PCR with an

annealing temperature of 45°C. The cDNA sequences obtained were separated from the sequences of interest by an agarose (8%) gel electrophoresis and were extracted from the gel using a Qiagen MinElute Gel Extraction kit. DNA sequencing was then performed by the Mount Desert Island Biological Laboratory (MDIBL) sequencing core facility on an ABI 3100 sequencer. The DNA sequences obtained were converted to a predicted amino acid sequence using the NCBI open reading frame finder (www.ncbi.nlm.nih.gov), and a BLAST search confirmed the cDNA as Hsp70 or AMPK, respectively (Fig. 7).

Quantitative real-time polymerase chain reaction (qPCR) was used as a more sensitive way to measure Hsp mRNA levels. Elevated mRNA levels are a precursor for increased protein levels (which are translated from mRNA). Specific primers for qPCR were designed with the idtDNA primer design tool (www.idtDNA.com) based on the sequences obtained with the degenerate primers. The Stratagene Brilliant SYBR Green qPCR Kit (Stratagene, La Jolla, CA) was used on a Stratagene MX3005 Real-Time PCR instrument. Samples were analyzed in triplicates (undiluted, and diluted 1:10, 1:100, 1:1000) after 40 cycles. The gene 18s was used as a reference: a strong 18s qPCR signal despite a weak Hsp70 signal shows that the weak signal is not caused by poor mRNA quality or other methodological problems. Specific 18s primers were designed for *Cancer irroratus* by Frederich et al. 2009, and were used for *N. lapillus* successfully.

Data Analysis

Temperatures of dark and white whelks were compared using a t-test for each field transplant site. AMPK and Hsp data from transplants were normalized to the mean of the exposed shore whelks within each color morph, resulting in relative unit levels. A two-way ANOVA was used to compare dark and white whelk data from the exposed and protected field

sites, and to test the interaction between site and shell color. T-tests were used to compare Hsp expression and AMPK activity in non-stressed, non-heated dark and white whelks (used as controls in the lab experiments) to one another. To compare Hsp expression and AMPK activity over time in cold-acclimated white whelks, a one-way ANOVA was used. Lab data were normalized to the control mean for each color morph (i.e., dark treatments were normalized to the mean of the dark control, etc.), resulting in relative unit levels. Two-way ANOVAs with Bonferroni *post-hoc* analyses were used to analyze variation in morph-specific AMPK and Hsp levels over time, and to test the interaction between the two groups. The mean temperature over time for dark and white whelks during lab experiments was compared using a t-test comparing the heating slope from two hours exposure to four hours exposure for each whelk. An alpha level of 0.05 was used in all tests. Data are shown as means ± standard error. All statistical analyses were performed using Systat11.

Results

Field Transplants

The cooler, wave-exposed shore provided habitat for predominantly small, dark whelks, whereas the warmer, wave-protected shore provided habitat for predominantly larger, white whelks (Fig. 8). In the field transplants of 2008, dark whelks tethered on both the exposed and protected shores tended to be around 1°C warmer than white whelks (Fig. 9), although this trend was not significant (exposed shore, $T_{20} = 1.122$, $p = 0.275$; protected shore, Transplant A, $T_{18} = 1.877$, $p = 0.084$; protected shore, transplant B, $T_{18} = 0.624$, $p = 0.541$). Despite the warmer trend in dark whelk body temperatures, there were no apparent trends (two-way ANOVA) in AMPK activity or Hsp70 expression in these whelks (Fig. 10, Table 2).

Lab Experiments

Whelks left in the seawater tank (i.e., the control group with no exposure to heat) were sampled pre-exposure to test for inherent differences between colors (Fig. 11). Seawater temperature was between 15 and 18°C for the duration of the experiments. As expected, no differences were found between the color morphs (t-test Hsp $T_{12} = 0.847$, $p = 0.414$; AMPK $T_{12} = 1.054$, $p = 0.313$), indicating any subsequent experimental effects are probably due to differential responses to heat exposure. The next group of whelks was collected in February and was heated to 24°C as a control. Only white whelks were used in this experiment. Again, there was no significant change in Hsp70 expression (one-way ANOVA $F_{1,11} = 9.330$, $p = 0.289$) or AMPK activity (one-way ANOVA $F_{1,11} = 1.860$, $p = 0.201$) in these cold-acclimated whelks (Fig. 12).

In the next experimental run, whelks were heated to around 28-30°C, similar to previously reported Hsp70 induction temperatures in marine snails. Both dark and white whelks (collected in May) were heated to test for differential activity. Dark whelks had significantly higher levels of Hsp expression and AMPK activity (two-way ANOVA, $F_{5,30} = 6.496$, $p < 0.005$) than white color morphs (Fig. 13, table 3). There was also a significant interaction effect between morph color and time ($F_{5,30} = 6.139$, $p = 0.005$). After 2 hours of exposure, dark whelks had higher AMPK activity than white whelks. Interestingly, AMPK activity in dark whelks decreased after four hours. There was no significant difference in Hsp70 expression between either the color morphs or the exposure durations. Due to the range of exposure temperatures between 28 and 30°C in the preceding experiment, a new set of whelks were exposed to 28°C (Fig. 14). No significant differences were found (two-way ANOVA, Table 4).

Whelks (collected in late August) were then heated to 34°C to create a high level of sub-lethal stress. Differential heating and physiological responses were observed in whelk color morphs heated to 34°C, with dark whelks significantly warmer than white whelks by around 1°C after two hours and 2°C after four hours of exposure (t-test, $T_{10} = 4.03$, $p < 0.005$; Fig. 15). Despite dark whelks attaining higher temperatures, only white whelks showed increased Hsp70 expression and AMPK activity (Fig. 16). White whelks heated to 34°C showed increased AMPK expression after two hours of exposure (Table 5; 2-way ANOVA with Bonferroni *post-hoc*, $F_{5,27} = 5.29$, $p < 0.05$). Under the same conditions, there was no significant elevation in either Hsp70 mRNA or protein levels (2-way ANOVA, Table 6). However the mRNA did begin to increase after 4 hours of exposure (Fig. 17). In dark whelks heated to 34°C, AMPK and Hsp70 levels did not vary from control whelks (no exposure to heat).

Discussion

Lab experiments

No difference in Hsp70 or AMPK levels were observed in organisms heated to 24°C, indicating that this temperature is below the threshold necessary to induce a heat response in *Nucella lapillus*. This result is not surprising, as 24°C is much cooler than the induction temperature found by Tomanek (2005) for Hsp70 in the marine snail *Tegula* sp. (around 28°C). An alternative explanation is that the whelks used in this experiment were collected in late February when ambient temperatures (air and water) are colder than during the summer, possibly indicating that winter acclimated animals respond differently than summer acclimated animals (such as the ones heated to 28°C and 34°C). Further experiments are needed to investigate differential response in winter- vs. summer-acclimated animals.

Whelks heated to around 28-30°C (similar to the induction temperature for *Tegula* sp.) exhibited higher Hsp70 expression and AMPK activity in dark morphs than in white morphs. The significant interaction effect between color and time in AMPK activity indicates that dark whelks had higher AMPK activity after 2 hours of exposure (Fig. 13). This may indicate that dark whelks experienced more thermal stress than white whelks. The lack of significant results in whelks heated to 28°C (Fig. 14) may indicate that this temperature is not stressful enough to induce a stress response. However, the results from whelks heated to around 28-30°C suggest that exposure to 30°C for two hours may cause enough thermal stress to elevate AMPK activity in dark morphs. Dark whelks, common to wave-exposed shores, regularly experience cooler temperatures than white whelks, common to wave-protected shores (Etter, 1988). In addition, dark whelks are rarely observed on sunny, open rocks at low tide, possibly to avoid the thermal stress response. However, 30°C is commonly experienced by white whelks on the wave-protected shore during summer low tides. White whelks showed no stress response at this temperature, suggesting that they may be acclimated to this temperature. In addition, the white shells may reflect more of the incoming solar radiation, keeping the whelk body temperature lower than that of dark whelks (Etter, 1988). These factors are consistent with the observation that white whelks are abundant on rocks in direct sun at low tide.

To further characterize thermal stress in dogwhelks, especially in the white morphs, whelks were heated to 34°C. As expected, dark morphs attained significantly higher temperatures (between 1 to 2°C) than white morphs (Fig. 15). Surprisingly, no significant Hsp70 expression or AMPK activity was observed in the dark whelks. The heat stress response is energetically costly, and can compromise the ability of an organism to function, potentially resulting in mortality (Feder and Hofmann, 1999; Hofmann et al. 2002). Dark whelks may have

experienced maximal thermal stress at 34°C, preventing them from maintaining the stress response. This temperature may be too close to the upper lethal limit for dark-shelled whelks.

In contrast, white morphs exhibited significantly higher AMPK activity after two hours of exposure to 34°C. This temperature is slightly lower than the previously reported lethal temperature of around 36°C for *Nucella* sp. (Sandison, 1967; Sorte and Hofmann, 2005). White morphs were expected to be more thermotolerant and thus less stressed when exposed to high temperatures due to regular exposure to higher temperatures in the field and the reflective capacity of the lighter shells (Etter, 1988; Harris and Jones, 1995). The delayed stress response may be due to increased thermotolerance in white morphs. No differential Hsp70 protein expression was observed in either color morph. However, Hsp70 mRNA expression began to increase after four hours of exposure to 34°C. Elevated mRNA expression must precede elevated protein levels because proteins are translated from mRNA. Based on this basic biosynthesis process, the elevated mRNA expression that began to appear suggests that increased Hsp70 protein expression could have been observed at a longer exposure time.

These results support the hypothesis that AMPK is activated earlier in the heat stress response than Hsp70. AMPK maintains cellular concentrations of ATP, which allows organisms to endure short exposures to temperatures above the average maximum temperature of their habitat (such as during midday summer low tides). It is highly conserved across many diverse phyla, indicating the importance of this energy regulator (Frederich et al. 2009). AMPK activity peaked two hours earlier than mRNA expression of Hsp70 began to increase. Hsp70 has been well studied in numerous organisms and habitats, including intertidal invertebrates (Dahlhoff, 2004; Feder and Hofmann, 1999). However, this study indicates that AMPK is an earlier signal for temperature stress than Hsp70, and may be a new useful tool for studying thermal stress.

Field transplants

Although dark whelks tended to attain warmer temperatures than white whelks, there were no significant trends in Hsp70 expression or AMPK activity. The heat stress response varies in natural populations across environmental gradients and with thermal microhabitat (Hofmann et al. 2002). Along rocky intertidal shores, higher temperatures and Hsp70 levels have been found in organisms on open, horizontal, flat microhabitats than those found in crevices, on vertical or north facing rock slopes, in algae beds, or in tide pools (Dahlhoff, 2004; Halpin et al. 2002; Helmuth and Hofmann, 2001). Whelks were tethered to the cleared sites to increase recapture rate and to decrease microhabitat effects.

Based on the lab experiments, it is not surprising that no differential effect on whelk stress levels was observed. During Transplant A, dark whelks tethered on the protected shore attained around 34°C. Dark whelks heated to this temperature in the lab exhibited no response, possibly because it is too close to the upper lethal limit for this color morph. White whelks tethered on the protected shore attained around 32°C, below the induction temperature observed in the lab (34°C). The average temperature during Transplant B for all treatments was around 28°C, below the induction temperature observed in lab for dark whelks (30°C). In addition, the lack of an observed differential effect on whelk stress levels may be explained by the factors affecting the body temperatures of intertidal ectotherms, including solar radiation, wind speed, relative humidity, cloud cover, and air and ground temperatures, as well as the shape, color, and mass of the organisms (Helmuth et al. 2006). Although temperatures during field transplants did not reach stress induction levels, these other factors may have further confounded any trends in relation to the stress response of the whelks.

Conclusion

Dogwhelks on the coast of Maine currently experience 30°C temperatures regularly, and experience 34°C temperatures during one or two weeks every year (based on the temperature surveys of the study sites), when midday low tides coincide with the summer's hottest temperatures. With temperatures predicted to rise 3-4°C by the year 2100, both color morphs of dogwhelks may soon be experiencing stressful temperatures on a more regular basis (IPCC 2001). Stress responses, such as elevated AMPK activity and the upregulation of Hsp70, require high energy costs and can result in mortality if the stress event is too extreme or of long duration (Feder and Hofmann, 1999). By understanding more thoroughly the physiological processes involved in withstanding temperature stress, more accurate predictions of the potential impacts of temperature change on organisms may be made (Osovitz and Hofmann, 2007). The thermotolerance of dogwhelk color morphs may result in differential survival when exposed to warmer temperatures. The possible resulting change in the distribution of color morphs may be a useful indicator of climate change.

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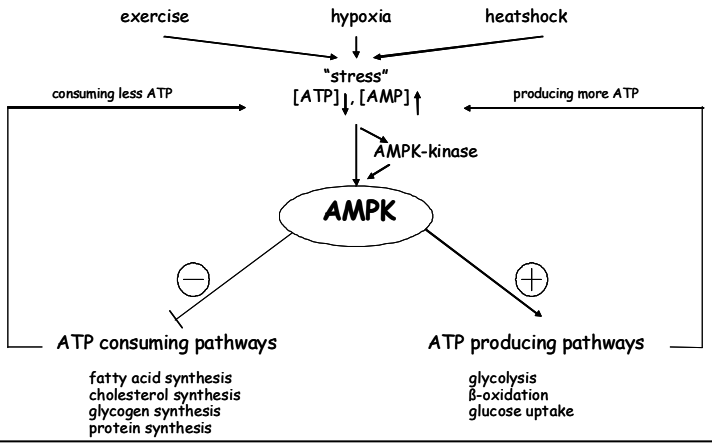


Figure 1. Model of the AMP-activated protein kinase (AMPK) cascade. Stressors such as exercise, hypoxia, or heat shock, lead to a decrease in cellular ATP and an increase in cellular AMP. This activates AMPK, which phosphorylates rate-limiting enzymes of all energy metabolism pathways. This leads to acceleration of all ATP-producing pathways and a deceleration of all ATP-consuming pathways, maintaining the cellular ATP concentration. From Frederich et al, 2009.

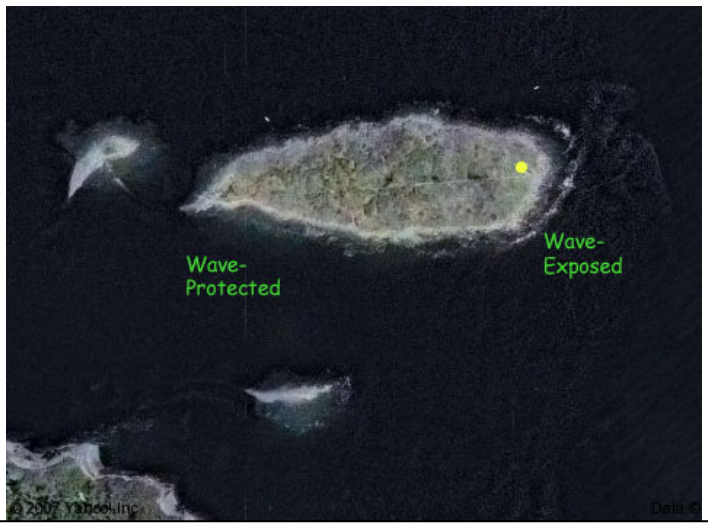


Figure 2. Aerial view of the two field sites on Wood Island, Biddeford Pool, Maine. The yellow dot represents the lighthouse.

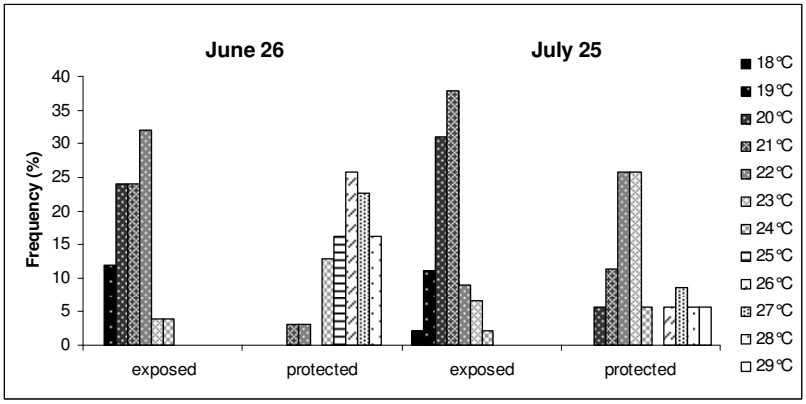


Figure 3. Distribution of whelk tissue temperatures on two summer days in 2007. Whelks on wave exposed shores (n = 25 to 45) typically have cooler body temperatures than whelks along wave-protected shores (n = 31 to 35).

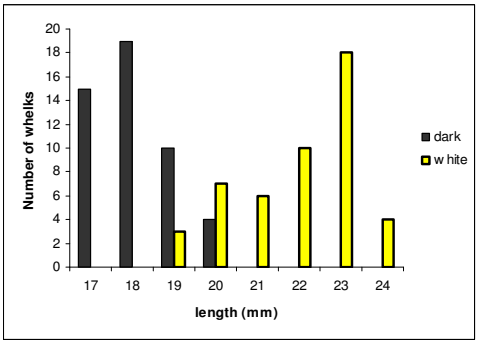


Figure 4. Distribution of shell lengths between dark and white whelks used in heating experiments. (n =48)

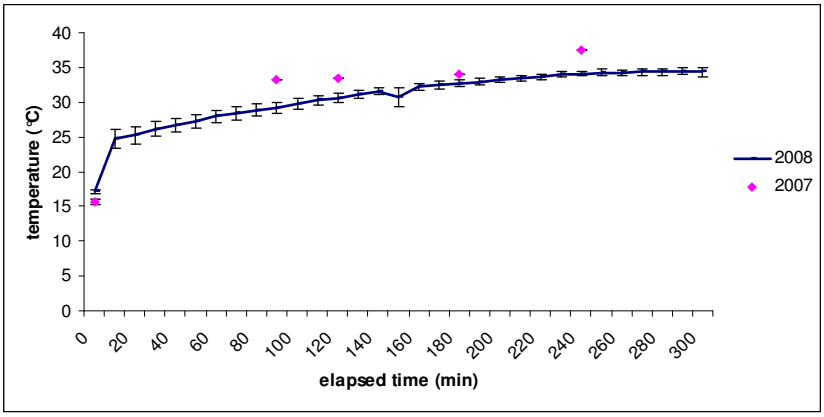


Figure 5. Whelk body temperature during exposure to heat lamp in laboratory-controlled environment. Mean heating time course for whelks during the 34°C lab experiments in 2007 (diamonds; n = 3) and 2008 (line; n = 2).

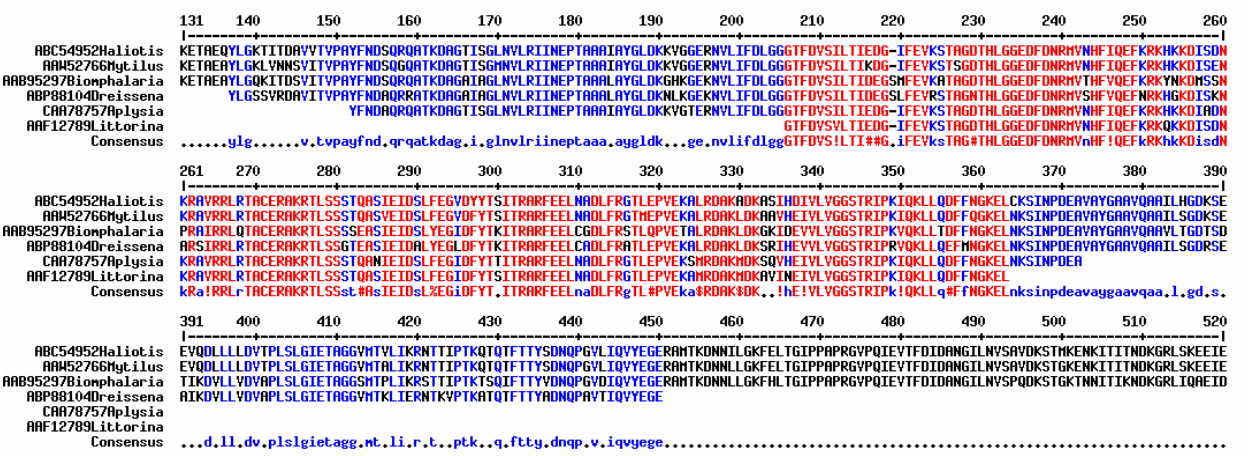


Figure 6. Alignment of Hsp70 amino acid sequences for invertebrate species. Degenerate primers were designed based on highly conserved parts in the aligned sequences, using *Littorina* sp. was used as the target sequence. Sequences were obtained from GenBank. Sequences conservation is indicated as: black, no conservation; blue, some conservation; and red, complete conservation among the compared species. Numbers preceding Genus name refer to GenBank accession numbers.

Target	Primer	Nucleotide sequence
Hsp70, degenerate	SNF1	5'- TAY TTY AAY GAY WSN CAR MGN CAR -3'
	SNF2	5'- TTY GAY GTN WSN GTN YTN ACN ATH GAR GAY -3'
	SNF3	5'- ATG GTN AAY CAY GGN ATH CAR GAR TTY -3'
	SNR1	5'- YTT RTC CAT YTT NGC RTC NCK CAT -3'
	SNR2	5'- YTT RTT NAR YTC YTT NCC RTT -3'
	SNR3	5'- YTC NCC YTC RTA NAC YTG DAT -3'
	SNF4	5'- TgY gTN ggN gTN TTY CAR CAY ggN AAR –3'
	SNF5	5'- gAR ATH ATH gC AAY gAY CAR ggN AAY –3'
	SNF6	5'- TAY gTN gC TTY ACN gAY ACN gAR –3'
	SNR4	5'- RTT RTT RTC YTT NgT CAT –3'
	SNR5	5'- RTC RAA NAT NAC YTC DAT YAT –3'
	SNR6	5'- DAT NCC RTT NgC RTC DAT RTC RAA –3'
Hsp70, specific	SNHF1	5'-TCTTTGCCGTTGAAGAAGTCCTGC-3'
	SNHR1	5'-ATCCATGAAATCGTCCTGGTGGGT-3'
18s, specific	18s F2	5'-gCC gCA CgA gAT TgA gCA ATA ACA-3'
	18s R1	5'-Agg ACA CgT TgA TCC CTT CAg TgT-3'

Table 1. Nucleotide sequence of *Nucella lapillus* primers used for amplification of Hsp70. The nucleotide sequence for 18s was designed for *Cancer irroratus* (Frederich et al. 2009). Nucleotide code: g, guanine; C, cytosine; A, adenine; T, thymine; Y=T or C; R=A or g; D=A or g or T; N (any nucleotide)=A, g, C or T.

TTGTTGAGTTCTTTGCCGTTGAAGAAGTCCTGCAAAAGCTTCTG
GATCTTGGGGATACGGGTGGATCCACCCACCAGGACGATTTCA
TGGATGGCGGGCTTGTCGATCTTGGCATCACGCATAGACTTCTC
CACTGGCTCCAAGGTGCCACGGAAGAGGTCAGCGTTCAGCTCC
TCAAACCTGGCACGAGTGATGCTGGTGTAAAAGTCAATCCCTTC
AAACAGTGAGTCGATCTCGATGCTGGCCTGCGTGGAGGAGGAC
AGAGTCCTCTTTGCACGCTCACAGGCGGTGCGCAGACGCCGCA
CGGCGCGCTTGTTCTCAGAGATGTCCTTCTTGTGCTTGCGCTTG
AACTCCTGTATNCCATGATTCACCATA

M	V	N	H	G	I	Q	E	F	K	R	K	H	K	K
D	I	S	E	N	K	R	A	V	R	R	L	R	T	A
C	E	R	A	K	R	T	L	S	S	S	T	Q	A	S
I	E	I	D	S	L	F	E	G	I	D	F	Y	T	S
I	T	R	A	R	F	E	E	L	N	A	D	L	F	R
G	T	L	E	P	V	E	K	S	M	R	D	A	K	I
D	K	P	A	I	H	E	I	V	L	V	G	G	S	T
R	I	P	K	I	Q	K	L	L	Q	D	F	F	N	G
K	E	L	N											

Figure 7. Top panel: partial Hsp70 DNA sequence for *Nucella lapillus*. Bottom panel: predicted amino acid sequence for Hsp70 in *N. lapillus.*

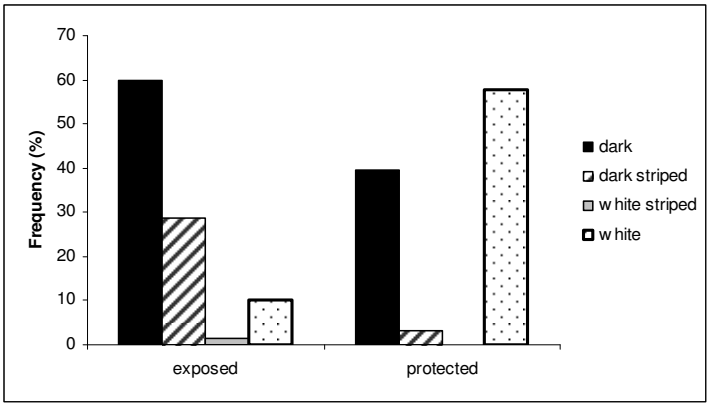
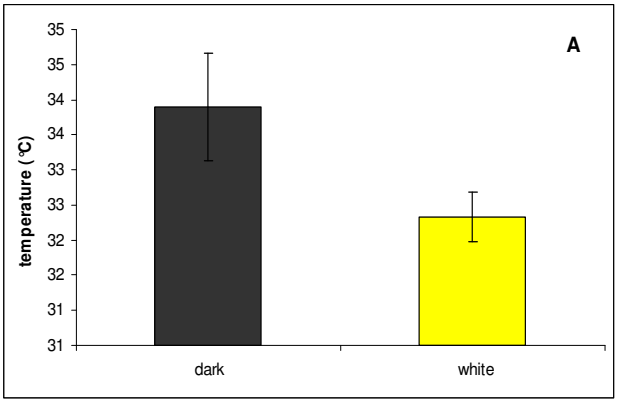
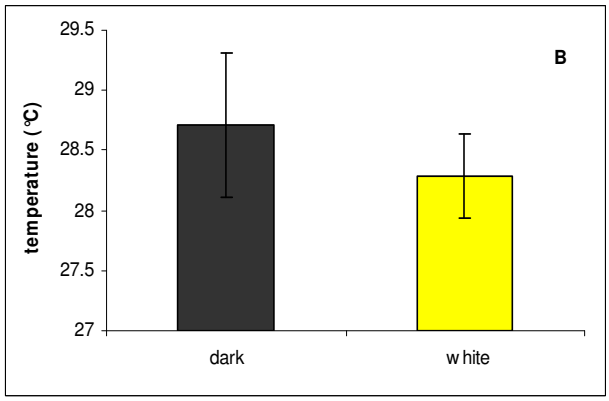


Figure 8. Distribution of whelk color morphs on two shores of Wood Island. The frequency of color morphs was determined on wave-protected (n = 66) and wave-exposed shores (n = 70). Darker morphs were more common on wave-exposed shores while lighter morphs predominated on wave-protected shores. Only the dark and white whelks were used for subsequent measurements.





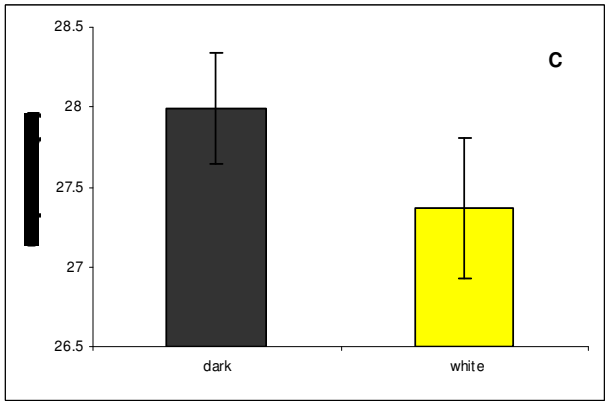


Figure 9. Mean whelk tissue temperature at collection. Panel A represents whelks from the first transplant in 2008. Panels B and C represent whelks from the second transplant in 2008, from the protected and exposed shores respectively.

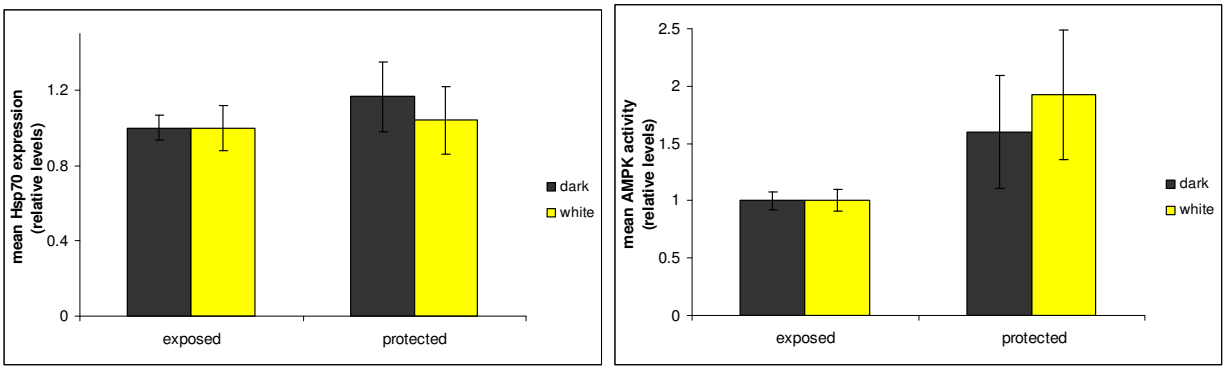


Figure 10. 2008 field reciprocal transplant results. The left panel represents the mean Hsp70 protein levels per treatment (n = 9 to 10); the right panel represents the mean AMPK activity per treatment (n = 9 to 11).

Source	df	SS	F	p
Color	1	0.038	0.181	0.673
Shore	1	0.098	0.472	0.497
Color*Shore	1	0.038	0.181	0.673
Error	34	7.064		

Source	df	SS	F	p
Color	1	0.248	0.153	0.698
Shore	1	5.462	3.377	0.075
Color*Shore	1	0.248	0.153	0.698
Error	34	54.994		

Table 2. Two-way analysis of variance to compare Hsp expression (left) and AMPK activity (right) in two gastropod color morphs at two intertidal locations.

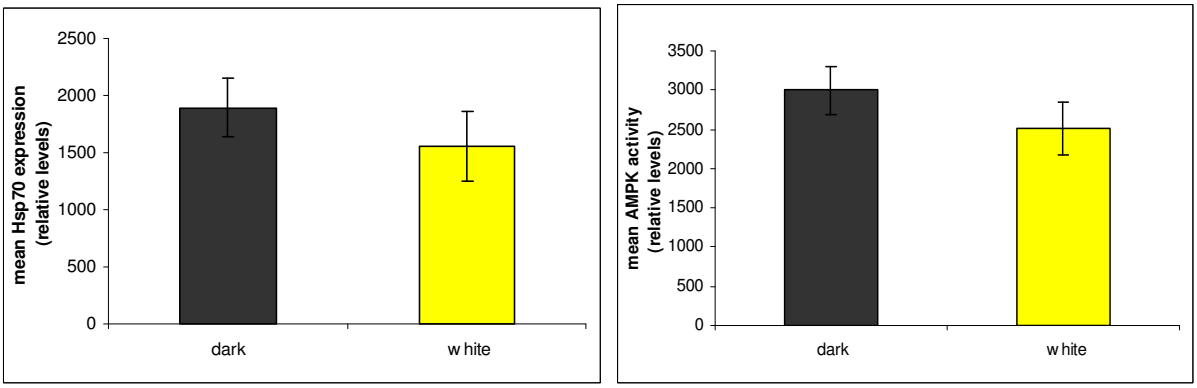


Figure 11. Mean Hsp70 protein levels and AMPK activity in control whelks with no heat exposure (n = 7). Seawater temperature was 15°C.

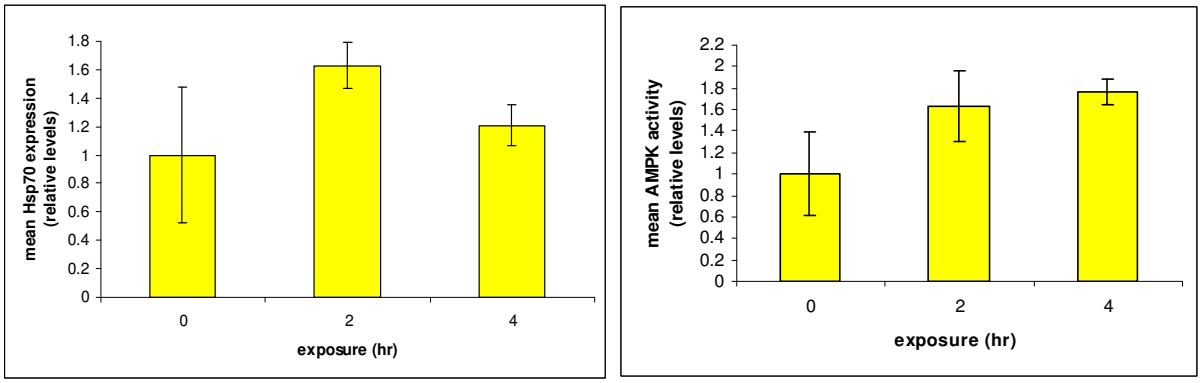


Figure 12. Cold-acclimated white whelks exposed to 24°C. The left panel represents mean Hsp70 protein levels; the right panel represents mean AMPK activity (n= 4 to 5).

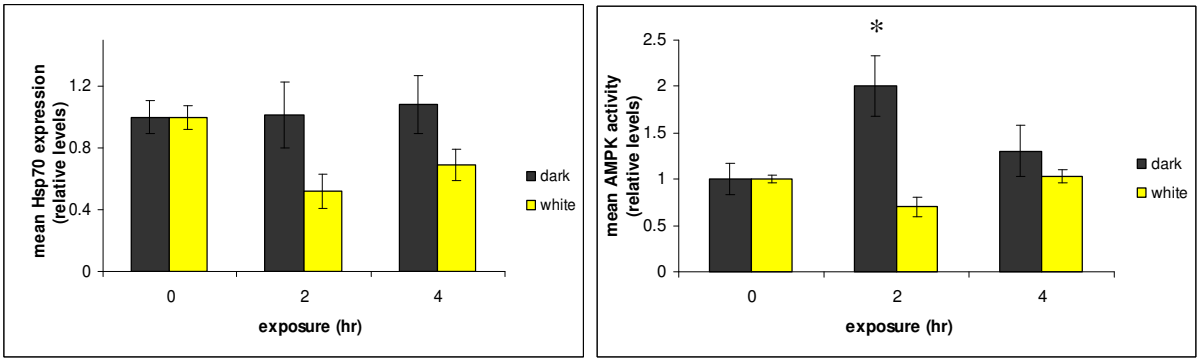


Figure 13. Dark and white whelks exposed to 28-30°C. The left panel represents mean Hsp70 protein levels; the right panel represents mean AMPK activity (n = 6). Asterisk (*) indicates significance, $p < 0.05$.

Source	df	SS	F	P
Color	1	0.787	6.496	0.016
Time	2	0.321	1.325	0.281
Color*Time	2	0.407	1.681	0.203
Error	30	3.633		

Source	df	SS	F	p
Color	1	2.495	11.024	0.002
Time	2	0.757	1.672	0.205
Color*Time	2	2.86	6.139	0.005
Error	30	6.79		

Table 3. Two-way analysis of variance to compare Hsp expression (left) and AMPK activity (right) in two gastropod color morphs at three time points exposure to 28-30°C.

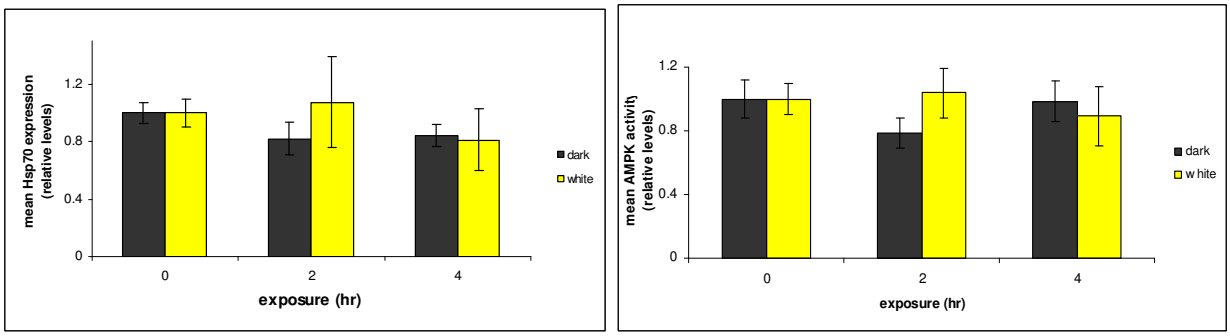


Figure 14. Left panel: Hsp70 protein levels during thermal stress at 28°C. Right panel: AMPK activity during thermal stress at 28°C. (n = 6).

Source	df	SS	F	P
Color	1	0.05	0.278	0.602
Time	2	0.19	0.531	0.594
Color*Time	2	0.145	0.404	0.671
Error	30	5.38		

Source	df	SS	F	p
Color	1	0.026	0.242	0.627
Time	2	0.049	0.233	0.794
Color*Time	2	0.189	0.89	0.421
Error	30	3.186		

Table 4. Two-way analysis of variance to compare Hsp expression (left) and AMPK activity (right) in two gastropod color morphs at three time points exposure to 28°C.

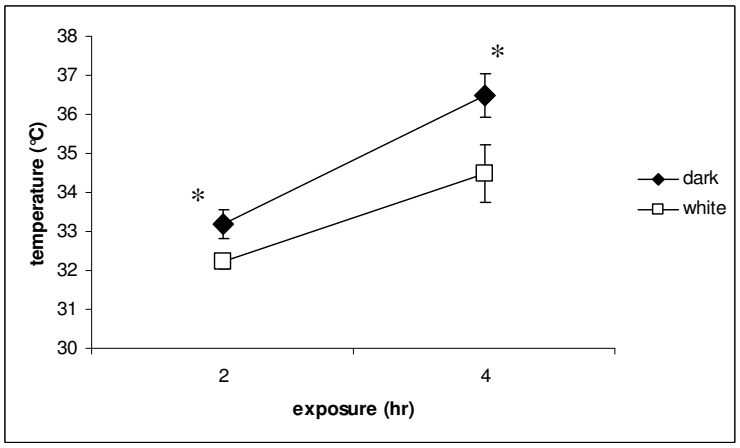


Figure 15. Mean whelk temperature at sampling times during a 34°C lab run (n = 6). Asterisks (*) indicate significance, $p < 0.05$.

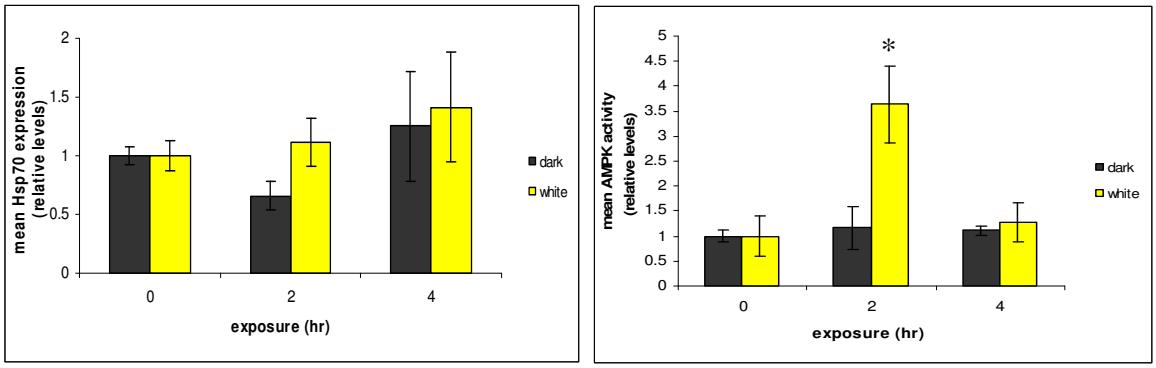


Figure 16. Left panel: Hsp70 protein levels during thermal stress at 34°C (n = 8 to 11). Right panel: AMPK activity during thermal stress at 34°C (n = 4 to 7). Asterisk (*) indicates significance, $p < 0.05$.

Source	df	SS	F	P
Color	1	0.594	0.683	0.413
Time	2	1.979	1.136	0.329
Color*Time	2	0.498	0.286	0.752
Error	51	44.403		

Source	df	SS	F	p
Color	1	5.892	3.977	0.056
Time	2	11.643	3.93	0.032
Color*Time	2	9.746	3.289	0.053
Error	27	40.001		

Table 5. Two-way analysis of variance to compare Hsp expression (left) and AMPK activity (right) in two gastropod color morphs at three time points exposure to 34°C.

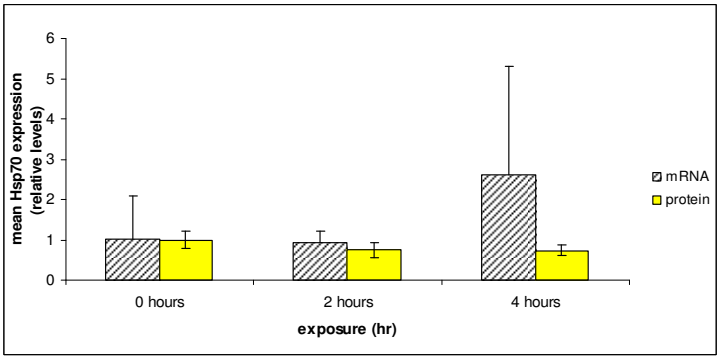


Figure 17. Hsp70 mRNA and protein expression levels during thermal stress at 34°C. Hsp70 protein (n = 3) and mRNA levels (normalized to 18s; n = 3) were measured after 0 hours, 2 hours and 4 hours of exposure to heat (n = 6).

Source	df	SS	F	p
Sample	1	2.811	1.369	0.255
Time	2	3.161	0.77	0.476
Sample*Time	2	4.28	1.042	0.37
Error	21	43.118		

Table 6. Two-way analysis of variance to compare Hsp mRNA levels and protein expression in white gastropod color morphs at three time points exposure to 34°C.