Behavioral, metabolic, and molecular stress responses of marine bivalve 
*Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature

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Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *Am J Physiol Regul Integr Comp Physiol* 293: R911–R921, 2007. First published May 23, 2007; doi:10.1152/ajpregu.00124.2007.—The present study aimed to determine the thermal response of the Mediterranean mussel *Mytilus galloprovincialis* by integrating information from various levels of biological organization including behavior, metabolic adjustments, heat shock protein expression, and protein kinase activity. Behavioral responses were determined by examining the effect of warming on valve closure and opening. Metabolic impacts were assessed by examining the activity of the key glycolytic enzyme pyruvate kinase (PK). Molecular responses were addressed through the expression of Hsp70 and Hsp90 and the phosphorylation of stress-activated protein kinases, p38 mitogen-activated protein kinase (p38 MAPK) and cJun-N-terminal kinases (JNKs). Mussels increased the duration of valve closure by about sixfold when acclimated to 24°C rather than to 17°C. As indicated by the activity of PK, such behavior caused metabolic depression and probably a shift from aerobic to anaerobic metabolism. Acclimation to temperatures higher than 24°C caused an increase in mortality and induced the expression of Hsp72. Increased phosphorylation of p38 MAPK and JNKs indicated activation of MAPK signaling cascades. The potential involvement of MAPKs in the induction of Hsp genes in the tissues of *M. galloprovincialis* is discussed. In conclusion, it seems that *M. galloprovincialis* lives close to its acclimation limits and incipient lethal temperature and that a small degree of warming will elicit stress responses at whole organism and molecular levels.


*In various phyla of marine invertebrates, the transition to internal (systemic) hypoxia was found to characterize the borders of the thermal tolerance window, in fully oxygenated waters. In general, anaerobic metabolism sets in at both cold and warm temperature extremes. These observations led to the development of the concept of oxygen- and capacity-limited thermal tolerance in animals (19, 20, 23, 24). This concept suggests that, at the low and high borders of the thermal envelope (defined as pejus thresholds, Tp), animals show a reduction in aerobic capacity. This reduction is not caused by falling levels of ambient oxygen but through limited capacity of oxygen supply mechanisms (ventilation, circulation) to cover an animal’s temperature-dependent oxygen demand. Thermal adaptation relies on the capacity of glycolytic and mitochondrial metabolism such as the respiratory chain and the tricarboxylic acid cycle. The thermal responses of such fundamental biochemical mechanisms contribute to defining performance levels, which are optimal only within a limited window of thermal tolerance. The latter defines how animals exert their ecological functions through their mode of life and behavioral traits (18–20). It is well known, however, that moderate levels of a stressful factor may only become effective during long-term exposure. Thus, long-term studies of the effect of ambient temperature on several levels of biological organization should give a realistic picture of its impact on the biology of animals in their habitats. Overall, the physiological mechanisms defining long-term tolerance and their interaction toward setting thermal limits of the intact organism are not yet sufficiently understood, whereas individual physiological mechanisms that respond to elevated temperature have been identified (25).

Thermal limits and adaptation have been studied in bivalves from rocky shores. These investigations have revealed a close relationship of heat shock protein (Hsp) expression with vertical zonation (33). The heat shock response is an energy-consuming process, during activation of transcription, synthesis of Hsps, and the ATP-dependent chaperoning by Hsps. Hawkins (10) has estimated that the cost of protein synthesis constitutes 20–25% of the energy budget of the Northern blue mussel, *Mytilus edulis*. A shift to anaerobiosis as a result of thermally induced hypoxia in marine bivalves, as it takes place in other marine animals (32, 34), will cause metabolic depression and consequently a reduction in ATP turnover. If this is the case, there might be an effect on the expression of Hsp, although this has not been experimentally shown. Furthermore, changes in the period that mussels keep their valves opened or closed will affect the amount of water they filter and consequently the amount of food they retain. It is not known how thermal stress affects valve opening vs. closure behaviors; however, exposure of blue mussel *M. edulis* to water temperatures higher than 25°C impairs their clearance rate (30) and consequently the amount of food available for energy turnover. Thus, during prolonged thermal stress, bivalves may readjust their metabolic profile and energy budget to meet the energy demand for Hsp synthesis. It has been proposed (5) that mitogen-activated protein kinase (MAPK) signaling might be...
involved in the regulation of Hsp expression in blue mussels. Evidence from mammalian cell systems also indicates that the activity of p38 MAPK and cJun-N-terminal kinases (JNKs) is essential for Hsp expression during various cell stresses (26, 31). However, to our knowledge, there is no evidence relating MAPK activation and Hsp expression in the tissues of marine bivalves during prolonged thermal stress.

Although major advances have been made to resolve the mechanisms through which marine animals respond to thermal stress, little is known about the long-term response to warming (33). This study attempts to integrate information from various levels of biological organization, including behavior (valve opening/closing), metabolic adjustments, heat shock protein expression, and protein kinase activity, into a coherent story of how heat stress may influence the Mediterranean mussel *M. galloprovincialis*. The impact of temperature on behavior was analyzed by examining the frequency and duration (ml/h) of valve closure, which relate to the rate of filtration and food assimilation, and thus the rate of energy (ATP) supply. Moreover, valve closure may induce tissue hypoxia even in oxygenated water (16). As an aspect of regulation in aerobic and anaerobic metabolism, we analyzed the effect of ambient temperature on the activation of the key glycolytic enzyme pyruvate kinase (PK). PK controls the flux of phosphoenolpyruvate (PEP) to succinate during anaerobiosis. Modification of the enzyme to a less-active form contributes to metabolic depression (4, 35). In addition, we determined whether the Hsp70 and Hsp90 response and metabolic readjustments occur concomitantly in the tissues of *M. galloprovincialis*. Given that the MAPK family may play an important role in coordinating gene responses to various stresses, we also examined the phosphorylation and hence activation of stress-activated protein kinases, p38 MAPK and JNKs, in the tissues of *M. galloprovincialis* during long-term acclimation at increasing temperature.

**MATERIALS AND METHODS**

**Animals**

Adult specimens of *M. galloprovincialis* (75–80 mm length) used for the present study were collected during spring (average sea water temperature 17°C) in the area of Halastra in the Thermaikos Gulf, and these were held in aquaria containing recirculating natural aerated seawater. Water temperature was controlled at 18 ± 0.5°C and salinity at 32 ± 3.5‰. Mussels were kept in aquaria under these conditions for 2 wk prior to experimentation. Seawater pH was 8.05 ± 0.02.

**Experimental Procedures**

1) **Effect of water temperature on mortality of mussels.** After 2 wk of acclimation to 18°C, mussels (30–40 animals) were introduced into six aquaria and brought to 18°C, 20°C, 24°C, 26°C, 28°C, or 30°C by warming of the water at a rate of 0.1°C per minute. Mussels were checked for mortality every day for 30 days. Mussels failing to close their shells in response to external stimuli were considered dead. The number of dead animals in each experiment was expressed as a percentage of the total number.

2) **Effect of water temperature on valve closing behavior.** Individual mussels were placed in one open 500-ml glass chamber each. One of the two valves was glued to the bottom of the chamber. One end of a thin wire was glued to the surface of the second valve while the other end was attached to an isotonic transducer. This arrangement permitted the recording of valve movements (closure or opening). The chambers were divided into eight groups of ten. Then each group of chambers was placed into eight separate aquaria containing sea water. Mussels were allowed to acclimate for 10 days to each temperature of 7°C, 14°C, 18°C, 20°C, 24°C, 26°C, 28°C, or 30°C, respectively. The periods and the mean fraction of time during which valves remained open or closed at each temperature of acclimation were estimated from recorded traces. The experiment was repeated five times.

3) **Effects of water temperature on the expression of Hsp70 and Hsp90 on the phosphorylation of p38 MAPK and JNKs and on the activity of PK.** **ANIMAL TREATMENTS.** Threshold temperatures for the expression of Hsp70 and Hsp90, for the phosphorylation of p38 MAPK and JNKs and for changes in the activity of PK, were determined in mussels placed into five aquaria, where they were left to acclimate to 18°C for 2 wk. Then water temperature was adjusted to 20°C, 24°C, 26°C, or 28°C, and mussels were left to acclimate for 30 days. Individuals were removed after 5, 10, 15, 20, or 30 days. Mantle tissue and posterior adductor muscle (PAM) were dissected, freeze-clamped between aluminum tongs cooled in liquid nitrogen, and ground under liquid nitrogen. Tissue powders were stored at −80°C. Animals kept at 18°C were used as controls.

**PREPARATION OF TISSUE SAMPLES FOR SDS-PAGE.** Tissue powders were homogenized in 3 ml/g of cold lysis buffer [20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM HEPES, 0.2 mM Na3VO4, 10 mM benzamidine, pH 7, supplemented with 200 μM leupeptin, 10 μM trans-epoxy-succinyl-l-leucylamido-(4-guanidino)butane, 5 mM dithiothreitol (DTT), 300 μM phenylmethylsulfonyl fluoride (PMSF)] (5).

**PROTEIN SEPARATION AND GEL ANALYSIS.** The homogenates were centrifuged at 13,000 g for 30 min at 4°C. The supernatants were then transferred to 1.5 ml tubes, and 100 μl of 2× sample buffer were added to each sample. The samples were boiled for 5 min and filtered through a 0.45-μm filter. Aliquots of 10 μl were then loaded on a 12% polyacrylamide gel, and Western blot analysis was performed using an enhanced chemiluminescent detecting system.

**Fig. 1.** A: effect of water temperature on the mortality of *Mytilus galloprovincialis* during 30 days of acclimation to different temperatures. B: mean periods of valve closure or opening during acclimation to different water temperatures. Values are means ± SE; n = 5.
fluoride (PMSF), 120 μM pepstatin, 1% vol/vol Triton X-100) and extracted on ice for 30 min. Samples were centrifuged (10,000 g, 10 min, 4°C), and the supernatants were boiled with 0.33 vol of SDS-PAGE sample buffer (330 mM Tris-HCl, pH 6.8, 13% vol/vol glycrol, 133 mM DTT, 10% wt/vol SDS, 0.2% wt/vol bromophenol blue). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

PREPARATION OF TISSUE SAMPLES FOR SDS-PAGE. Equal amounts of proteins (100 μg) were separated on 10% (wt/vol) acrylamide, 0.275% (wt/vol) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm; Schleicher and Schuell, Keene, NH). Nonspecific binding sites on the membranes were blocked with 5% (wt/vol) nonfat milk in TBST [20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (vol/vol) Tween 20] for 30 min at room temperature. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies. Antibodies used were as follows: monoclonal mouse anti-heat shock protein, 70 kDa, and monoclonal mouse anti-heat shock protein, 90 kDa (Sigma); monoclonal mouse anti-phospho-SAPK-JNK (Thr183-Tyr185) and polyclonal rabbit anti-phospho-p38 MAP kinase (Thr180-Tyr182) (Cell Signaling). After washing in TBST (3 periods, 5 min each time) the blots were incubated with horseradish peroxidase-linked secondary antibodies, washed again in TBST (3 periods, 5 min each time), and the bands were detected using enhanced chemiluminescence (Chemicon) with exposure to Fuji Medical X-ray films. Films were quantified by laser-scanning densitometry (GelPro Analyzer Software, GraphPad).

PREPARATION OF TISSUE HOMOGENATES FOR THE DETERMINATION OF PK ACTIVITY. For the determination of PK activity, samples of frozen tissue powders (200–500 mg) were rapidly weighed and homogenized (1:5, wt/vol) in ice-cold 50 mM imidazole-HCl (pH 7.0) containing 100 mM sodium fluoride, 10 mM EDTA, 10 mM EGTA, 30 mM 2-mercaptoethanol, 40% glycerol (vol/vol), and 0.1 mM PMSF added just prior to homogenization, using a Polytron PT10 homogenizer (3 periods, 20 s each time). After centrifugation (25,000 g, 20 min, 4°C), the supernatant was removed and passed through a 5-ml column of Sephadex G-25 equilibrated in 40 mM imidazole-HCl buffer (pH 7.0) containing 5 mM EDTA, 15 mM 2-mercaptoethanol, and 20% glycerol to remove metabolites of low molecular mass (11). The column was centrifuged in a desktop centrifuge at 2,000 g for 1 min, and the supernatant was used for the determination of enzyme activity. Standard assay conditions for PK were as follows: 50 mM imidazole-HCl buffer, 2 mM ADP, 0.15 mM NADH, 50 mM KCl, 5 mM MgCl₂, 2 IU dialyzed lactate dehydrogenase and PEP, either 2 mM for the determination of \( V_{\text{max}} \) (\( V_{2 \text{mM}} \)) or 0.05 mM for the determination of \( V_0 \) (\( V_{0.05 \text{mM}} \)). The ratio \( V_0/V_{\text{max}} \), which reflects the relative activity of PK, was then calculated to determine metabolic depression during acclimation to high temperature. The ratio \( V_0/V_{\text{max}} \) was found to decline in the adductor muscle and mantle of bivalve mollusks during anoxia indicating a shift of PK toward a less-active form during metabolic depression (15, 16). Assays for \( V_0 \) and \( V_{\text{max}} \) were conducted at 18°C and at each acclimation temperature.

Statistics

Changes over time were tested for significance at the 5% level by using one-way analysis of variance (ANOVA) and by performing Bonferroni post-hoc tests for group comparisons. Values are presented as means ± SE.

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Fig. 2. Levels of Hsp70 in the mantle tissue of submersed *M. galloprovincialis* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for Hsp70. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± SE; \( n = 5 \) preparations from different animals. Inducible isofrom Hsp72, open circles; constitutive isofrom Hsp73, closed circles. *\( P < 0.05 \) compared with the control (0 days).
RESULTS

Effect of Water Temperature on Mussel Mortality

Figure 1A shows the development of mortality over time during acclimation to different temperatures. Very few (about 1%) of the mussels died below 26°C, although some were closing their shells progressively at 26°C. Animals started to die as water temperature reached 26°C, leading to 5% mortality within 5 days and 20% after 30 days. Mussel mortality increased significantly in animals acclimated to 28°C, when almost 20% of the mussels died within the first 5 days and 30% after 30 days. Mussel mortality increased further at 30°C, when 80% of the animals died within 15 days of acclimation.

Effect of Water Temperature on Valve Opening vs. Closure

The effect of acclimation temperature on the mean periods of valve closure or opening is shown in Fig. 1B. The period of valve opening increased as the acclimation temperature increased from 7°C to 16°C. Thereafter, however, the mean period of valve opening declined, suggesting that further increases in acclimation temperature stimulated valve closure up to 24°C. Beyond 26°C, a trend to increase the period of valve opening was observed.

Effect of Acclimation Temperature on the Expression of Hsp70 and Hsp90

The expression of Hsps was monitored in mantle tissue and PAM. In the Hsp70 family two main bands were identified, Hsp72 (the inducible form) and Hsp73 (the constant form) during acclimation to any temperature. The levels of Hsp72 showed a gradual increase within 30 days of acclimation of mussels to 26°C. Increased Hsp72 expression was also observed during acclimation to 28°C (Fig. 2). A similar expression profile was observed in the PAM (Fig. 3).

In response to acclimation to various temperatures, Hsp90 displayed similar changes as Hsp72 (Figs. 4 and 5).

Effect of Acclimation Temperature on the Phosphorylation of the Kinases p38 MAPK and JNKs

Activation of MAPKs has been implicated in the stimulation of Hsp expression (26, 31). Thus, phosphorylation and hence activation of p38 MAPK and JNKs was determined in both mantle and PAM at different acclimation temperatures. One form of JNK was detected, which corresponds to the 46-kDa isoform of the mammalian enzyme (Figs. 6 and 7). Increased acclimation temperatures caused significant changes in the levels of the phosphorylated forms of JNK and p38 MAPK in both mantle (Fig. 6) and PAM (Fig. 7). In the mantle, phosphorylation levels of JNK doubled after 10 days of acclimation.
Fig. 4. Levels of Hsp90 in the mantle of submersed *M. galloprovincialis* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for Hsp90. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± SE; *n* = 5 preparations from different animals. *P* < 0.05 compared with the control (0 days).

Fig. 5. Levels of Hsp90 in the PAM of submersed *M. galloprovincialis* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for Hsp90. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± SE; *n* = 5 preparations from different animals. *P* < 0.05 compared with the control (0 days).
Fig. 6. Phosphorylation levels of cJun-N-terminal kinase (JNK) in the mantle tissue of submersed *M. galloprovincialis* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for the phosphorylated form of JNKs. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± SE; *n* = 5 preparations from different animals. *P < 0.05 compared with the control (0 days).

Fig. 7. Phosphorylation levels of JNK in the PAM of submersed *M. galloprovincialis* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for the phosphorylated form of JNKs. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± SE; *n* = 5 preparations from different animals. *P < 0.05 compared with the control (0 days).
Fig. 8. Phosphorylation levels of p38 mitogen-activated protein kinase (p38 MAPK) in the mantle of submersed *M. galloprovincialis* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for the phosphorylated form of p38 MAPK. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± SE; *n* = 5 preparations from different animals. *P* < 0.05 compared with the control (0 days).

Fig. 9. Phosphorylation levels of p38 MAPK in the PAM of submersed *M. galloprovincialis* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for the phosphorylated form of p38 MAPK. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± SE; *n* = 5 preparations from different animals. *P* < 0.05 compared with the control (0 days).
to 24°C and decreased thereafter to control levels. Warming to 26°C caused a fivefold increase in the levels of phosphorylated JNK after 20 days. After 30 days, phosphorylated JNK had decreased slightly but levels remained higher than those of controls. During acclimation of mussels to 28°C, the levels of phosphorylated JNK increased progressively (more than 4-fold) and remained elevated after 30 days. The level of phosphorylated p38 MAPK showed a significant increase in the mantle after 10 days of acclimation to 24°C and 26°C and remained elevated thereafter (Fig. 8). A more prominent increase in the phosphorylation of p38 MAPK was observed after 5 days of acclimation to 28°C. Similar patterns of changes in the phosphorylation levels of JNK and p38 MAPK were observed in the PAM (Figs. 7 and 9) after acclimation to 20°C, 24°C, and 26°C. A stronger response in the phosphorylation of p38 MAPK was observed in the PAM than in the mantle at 28°C.

Effects of Acclimation Temperature on the Activity of PK

Figures 10 and 11 summarize the patterns of PK activity in the mantle tissue and the PAM during acclimation of mussels to different water temperatures. No change in PK activity occurred at temperatures lower than 24°C. However, warming seemed to modify enzyme activity, with a decrease in the mantle at 24°C. The relative activity of the enzyme (V0/Vmax) decreased from 0.95 to 0.57 within 15 days of acclimation, indicating a shift of PK toward a less-active form (Fig. 10). A similar pattern was observed in the PAM. The relative activity of PK from the PAM decreased within the first 20 days of acclimation to 24°C and remained reduced thereafter. Further warming, however, seemed to modulate PK activity from mantle tissue or PAM in a way entirely different from the pattern observed at 24°C. Changes in enzymatic activity within the first 10 days of exposure to either 26°C or 28°C were similar to those observed when the mussels were acclimating to 24°C. Thereafter, however, the activity and activity status (V0/Vmax) of PK from mantle reached levels beyond control values, indicating activation of the enzyme (Fig. 10). Similar increase in PK activity was observed for PAM at 26°C (Fig. 11). However, at 28°C PK activity in the PAM recovered up to control levels.

Fig. 10. Activity of pyruvate kinase (PK) from the mantle of *M. galloprovincialis* during acclimation to different water temperatures. Activity (µmol/min per g wet wt) was determined at 2 mM (Vmax) and 0.05 mM of PEP (V0). Values are mean ± SE; n = 10 preparations from different animals. Vmax (closed circles) and V0 (open circles) are given at left, and the ratio V0/Vmax is given at the right.
DISCUSSION

The data obtained in the present study demonstrate that *M. galloprovincialis* cannot survive sea water temperatures of and beyond 26°C over extended periods of time (Fig. 1A). The mortality of mussels increased most drastically during warming to 30°C, and 45% of mussels died within the first 5 days. The increase in the mortality of mussels during exposure to temperatures higher than 24°C might be attributed to reduced ability to assimilate food and associated energy. When temperature exceeds 25°C, filtration falls significantly in *M. galloprovincialis* (Anestis A, Portner HO, Staikou A, Michaelidis B, unpublished data) and in *M. edulis* (1, 2, 9, 30). Under these conditions mussels would rely on stored fuels to replenish the consumed ATP. The latter might cause depletion of stores and might contribute to cell death, explaining partly the increase in mussel mortality in water temperatures beyond 24°C, which would only support time-limited tolerance. During summer and especially between the end of July and the middle of August, surface sea water temperatures in the habitat of *M. galloprovincialis* fluctuated between 26°C and 28°C (6). However, at night temperature may drop 0.5–1.5°C. According to unpublished observations, 30–55% of mussels die when day temperature of surface sea water rises up to 28°C, indicating that mussels live at the upper limit of their acclimation capacity. Similar to *M. galloprovincialis*, *M. edulis* displays an upper lethal temperature between 26°C and 28°C (27). At first sight, this appears puzzling since *M. galloprovincialis* displays a more southern distribution pattern and experiences environmental temperatures that are on average warmer than for *M. edulis* or a third congener, the Pacific blue mussel, *M. trossulus* (3). However, the Mediterranean is a basin, which has no tides, such that Mediterranean mussels live in the sublittoral zone and are not exposed to large diurnal temperature fluctuations. In contrast, *M. edulis* and *M. trossulus* live in the intertidal zone and they are thereby potentially exposed to much higher temperature extremes. This may explain the similarity in upper thermal limits despite different geographical distribution ranges.

*M. galloprovincialis* acclimated to 24°C displayed extended periods of valve closure compared with controls (Fig. 1B).
These behavioral changes were paralleled by a reduction in the PK activity status (Figs. 10 and 11), indicating low glycolytic rate and, thus, periodic reductions of energy turnover. These data are in line with previous reports indicating that metabolic depression accompanies valve closure in bivalves (7, 16). In addition, temperature-induced anaerobiosis has been repeatedly reported for marine invertebrates (8, 17, 21, 34). Overall, periodic metabolic depression may be suitable to balance the rise in energy demand during moderate warming; however, this is a passive strategy of thermal tolerance and would occur at the expense of reduced aerobic scope for activity (18–20).

Consistent with other reports (5, 12, 13) and in line with the model of oxygen- and capacity-limited thermal tolerance (18, 19), the present study shows that in the passive range of tolerance, activation of the heat shock response occurs in tissues of *M. galloprovincialis* during long-term exposure to thermal extremes beyond 24°C. The gradual increase in the inducible isoform Hsp70 in the PAM and mantle of *M. galloprovincialis* (Figs. 2 and 3) seem to be consistent with the suggestion of Buckley et al. (5) that the gradual accumulation of inducible Hsp70 might act as a buffer against subsequent heat stress and support increased thermotolerance in gradually warming environments. At the same time, PK activity changed in a biphasic way in PAM and mantle tissues (Figs. 10 and 11) during acclimation to 26°C and 28°C. Although PK activity decreased during the first 10 days of acclimation, reflecting metabolic depression, it increased later on, indicating a reactivation of metabolic rate. Hsp synthesis during prolonged thermal stress in mussels may thus be fueled by heat-induced activation of metabolism, possibly involving enhanced glycolytic production of ATP. Significant increases in enzymatic activities were also found for two other glycolytic enzymes, hexokinase and aldolase, during exposure of *M. galloprovincialis* to 26°C and 28°C (Anestis A, Portner HO, Saikou A, Michaelidis B, unpublished data). The reactivation of metabolic rate beyond 26°C is consistent with the extended gaping observed at high temperatures (Fig. 1B), which might reflect enhanced oxygen demand in parallel to a stimulation of glycolysis. In fact, an increase in the rate of oxygen consumption has been reported for *M. edulis* acclimated at temperatures higher than 24°C (38).

It has been proposed that the upregulation of Hsp genes in warm acclimatized mussels, such as *M. trossulus*, takes place in two steps. During the first step, the increased levels of Hsp70 and potentially Hsp90 maintain HSF1 in an inactive state. Only when temperatures reach the high threshold temperature, HSF1 is released to transactivate Hsp genes. According to recent evidence, this might involve either protein-protein interactions with other transcriptional effector molecules, such as the heat-shock factor binding protein (HSBP1) (28, 29), and/or regulation via phosphorylation of specific serine residues on HSF1 that precedes Hsp gene transactivation (39). The signaling pathways responsible for the phosphorylation of HSF1 have not been fully elucidated. However, p38 MAPK and JNKs may be involved in this mechanism (26, 31, 36). In fact, the results obtained in the present work reveal a marked increase in the levels of the phosphorylated form of p38 MAPK and JNK in PAM and mantle from *M. galloprovincialis* exposed to temperatures beyond 24°C (Figs. 6–9). The increased phosphorylation of kinases parallels the increased expression of Hsp. These data strongly support the involvement of MAPK signal cascading in the induction of Hsp genes in the tissues of *M. galloprovincialis* during thermal stress.

Taking seasonal changes in temperature into account, we find the present data suggest that, especially during July and August, Mediterranean mussels (*M. galloprovincialis*) live near their incipient lethal temperature since they regularly encounter water temperatures higher than 25°C. The metabolic changes that occur between 22°C and 24°C, before molecular markers respond to thermal stress, will negatively affect the growth of mussels and probably impair several other physiological processes such as gamete production and reproduction rates. Changes in population dynamics may, thus, set in independent of and before biochemical stress indicators are affected. This conclusion is further supported by recent reports, according to which the population size appears to decline as a result of lowered scope for growth and reproduction, rather than heat-induced death per se (22, 37). In areas of “thermal pollution,” e.g., in overheated areas of the intertidal zone, sessile organisms like mussels will more affected by temperature extremes. Here, environmental temperatures likely exceed those eliciting oxygen limitations, thereby affecting individual survival unless it successfully uses strategies of passive tolerance. It is evident from the obtained results that, although Hsp expression reflects exposure to extreme temperatures, studies of other physiological processes such as metabolism are most useful in estimating the earliest effects of ambient temperature on organisms.

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