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Metabolic and molecular stress responses of sublittoral bearded horse mussel *Modiolus barbatus* to warming sea water: implications for vertical zonation

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SUMMARY

The present study set out to investigate the thermal limits of the Mediterranean bivalve *Modiolus barbatus*, acclimated to various temperatures, and includes a comparison of laboratory determined limits with its temperature-dependent restriction to deeper water layers in its natural habitat. Thermal responses and limits were determined by integrating information from various levels of biological organization, including the expression of Hsp70 and Hsp90, the phosphorylation of stress-activated protein kinases, p38 mitogen-activated protein kinase (p38 MAPK) and cJun-N-terminal kinases (JNKs) as well as metabolic adjustments. The latter were assessed by examining temperature effects on the activity of the key glycolytic enzyme pyruvate kinase (PK). The expression of Hsp70 and Hsp90 was activated when mussels were acclimated to temperatures above 20°C. Increased phosphorylation of p38 MAPK and JNKs at about the same temperatures indicate activation of MAPK signaling cascades and their potential involvement in the induction of Hsp genes. As indicated by the activity of PK, *Modiolus barbatus* maintains some aerobic capacity when acclimated to temperatures up to 24°C, while further warming probably caused metabolic depression and a shift from aerobic to anaerobic metabolism. An increase in mortality occurred in parallel, during acclimation to temperatures above 24°C. Our results indicate that both the biochemical stress indicators and metabolic status respond in parallel once hypoxemia becomes extreme. Comparison with our previous study of thermal limits and vertical distribution in *M. galloprovincialis* dwelling in shallow waters emphasizes the relevance of maintained aerobic scope over that of passive tolerance for permanent vertical zonation at higher temperatures in the field. These findings and conclusions are in line with the concept of oxygen and capacity limited thermal tolerance and the associated systemic to molecular hierarchy of thermal limitation.

Key words: bivalves, temperature, mortality, heat shock proteins, mitogen-activated protein kinases, pyruvate kinase, aerobic scope.

INTRODUCTION

Environmental temperature has a pervasive impact on ectothermic organisms, and influences function at all levels of biological organization from the whole organism to the molecular level (Hochachka and Somero, 2002). As a result, environmental temperature impacts ecosystem level processes such as biogeographical distribution patterns (Brown, 1984; Brown et al., 1996; Gaston, 2003) or species interactions (Sanford, 1999). Thermal biology has been at the center of research examining small or large scale physiological patterns in natural populations of marine organisms along latitudinal or intertidal clines (e.g. Sommer et al., 1997; Sommer and Pörtner, 1999; Sommer and Pörtner, 2002; Helmuth et al., 2002; Sokolova and Pörtner, 2003; Sorte and Hofmann, 2004). Evidence that links the physiological mechanisms to the interacting forces that are drivers for small or large-scale ecosystem patterns is only just emerging with a focus on temperature (Pörtner and Knust, 2007).

Physiological traits and underlying biochemical mechanisms are thus very important in setting thermal limits and species boundaries, however, the systemic to molecular hierarchy of thermal limitation still has to be elaborated in more detail (Pörtner, 2002a). For example, the expression of heat shock proteins (Hsps) and on their role in animal thermotolerance has attracted much attention during the last decade. Hsps are now well known from a variety of taxa and are described as molecular chaperones that refold proteins denatured from a variety of insults including thermal stress (Feder and Hofmannn, 1999). Because Hsps prevent the aggregation of heat-damaged proteins and facilitate their renaturation following a heat shock, they are likely to play an important role in thermotolerance (Parsell et al., 1993; Parsell and Lindquist, 1994). From an ecological perspective the threshold temperatures inducing expression of Hsps may determine extreme thermal limits and it has been hypothesized that this threshold is related to the species boundaries in an ecosystem (Somero, 2002; Hofmann, 2005).

According to the concept of oxygen and capacity limited thermal tolerance, thermal limits and distribution boundaries of a species are first of all determined by their ability to maintain aerobic capacity, before biochemical stress indicators such as Hsps become important (Pörtner, 2001; Pörtner, 2002a). In fact, abundance of fish (eelpout) populations decreased once warming passed the borders of the window set by aerobic scope (Pörtner and Knust, 2007). Accordingly, metabolic adaptations to temperature setting aerobic scope, including the capacities of glycolytic and mitochondrial pathways, are relevant in thermal acclimation and adaptation. The thermal responses of such fundamental biochemical mechanisms contribute to defining performance levels, which are optimal only within a limited window of thermal tolerance. In turn, thermal tolerance defines an animal's ecology through its mode of life and behavioral traits (for reviews, see Pörtner, 2001; Pörtner, 2002a; Pörtner, 2002b). At the low and high borders of the thermal envelope

(defined as pejus thresholds, T_p), animals show a reduction in their aerobic capacity. This reduction is not caused by falling of ambient oxygen levels but through limited capacity of oxygen supply mechanisms (ventilation, circulation) to cover an animal's temperature-dependent oxygen demand.

It is well known 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 the integration and relative importance of impacted functions for the fitness and survival of animals in their habitats. How the physiological and biochemical mechanisms responding to thermal stress define long-term tolerance and their interactions in setting thermal limits of the intact organism has been insufficiently explored (Pörtner et al., 2005). Laboratory experiments can identify thermal limits and the capacity of marine animals to acclimate to temperature change so that responses to thermal changes in the natural environment can be explained (Pörtner and Knust, 2007) or predicted.

Thermal limits and adaptation have been studied in congeneric bivalves and gastropods from rocky shores. These intertidal organisms exploit the passive range of tolerance beyond the optimum of aerobic scope (c.f. Pörtner, 2002a; Pörtner, 2002b). These investigations have revealed a close relationship of Hsp expression with vertical zonation (Hofmann and Somero, 1996; Tomanek and Somero, 1999; Tomanek and Somero, 2002) (see also Somero, 2002). Differences in expression of Hsps among species may be relevant in shaping passive tolerance. In this context, the regulatory background of the heat shock response requires further investigation.

The intertidal zone is small in the Mediterranean, however, submerged bivalve species display vertical zonation and are, accordingly, exposed to different temperature regimes. Mussels (*Mytilus galloprovincialis*) from upper water layers (including the intertidal) are exposed to diurnal temperature extremes during summer, whereas bearded horse mussels *Modiolus barbatus* are found at lower depths and more stable temperatures. For an analysis of the influence of temperature on vertical zonation, we studied the thermal limits and adaptation of the two bivalves. Results for *M. galloprovincialis* were presented by Anestis et al. (Anestis et al., 2007), and those for *M. barbatus* by the present study.

The horse mussel occurs in the lower-eulittoral–sublittoral fringe, which extends down to depths of 110 m. They are found attached to rocky substrata by strong byssus threads. The species is found around the British Isles and further South to Mauritania, West Africa as well as in the Mediterranean (Poppe and Gotto, 2000). Around Greece, *M. barbatus* is distributed in coastal marine environments at a depth of 8–25 m. To determine when thermal stress is initiated in the tissues of acclimating *M. barbatus* we studied the expression of Hsp70 and Hsp90 during long-term acclimation (30 days) at different water temperatures. As shown recently, mitogen-activated protein kinase (MAPK) signaling might be involved in the regulation of Hsp expression in *M. galloprovincialis* (Anestis et al., 2007). Thus, we also examined the phosphorylation, and hence activation, of stress-activated protein kinases, p38 MAPK and JNKs, in the tissues of *M. barbatus* during long-term acclimation to warmer temperature. Moreover, study of thermal acclimation in *M. galloprovincialis* had demonstrated that mussels reorganize metabolism by inducing anaerobic components of intermediate metabolism (Anestis et al., 2007). We therefore analyzed the activation of the key glycolytic enzyme pyruvate kinase (PK) during warming. PK controls the flux of phosphoenolpyruvate (PEP) to succinate during anaerobiosis. Modification of the enzyme to a less-active form contributes to metabolic depression (Brooks and Storey, 1997; Storey and Storey, 1990). In an attempt to relate our data on the molecular and biochemical responses of *M. barbatus* to temperature, to the vertical distribution of the mussel in the sea, we also measured water temperature in the field.

MATERIALS AND METHODS

Animals

Adult specimens of *Modiolus barbatus* (Linnaeus 1758) (55–60 mm long) used for the present study were collected during the spring of 2006 (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 weeks prior to experimentation. Seawater pH was 8.05±0.02.

Experimental procedures

Mortality

After 2 weeks of acclimation at 18°C, bearded horse 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 the percentage of the total number.

Biochemical indicators

Animal treatments

Threshold temperatures for the expression of Hsp70 and Hsp90 as well as for the phosphorylation of p38 MAPK and JNKs were determined in mussels placed into five aquaria, where they were left to acclimate to 18°C for 2 weeks. Then water temperature was adjusted to 20°C, 24°C, 26°C, 28°C or 30°C, and mussels were left to acclimate for 5, 10, 15, 20 or 30 days. Following acclimation, 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. For changes in the activity of PK, powdered tissue of animals

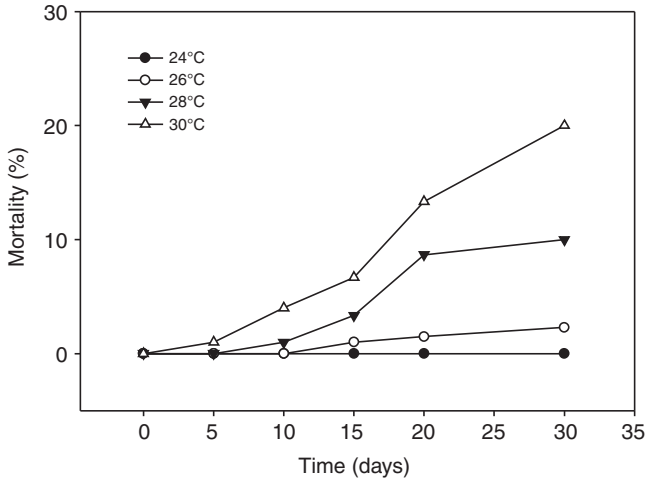

Fig. 1. Effect of water temperature on the mortality of *M. barbatus* during 30 days of acclimation to different temperatures.

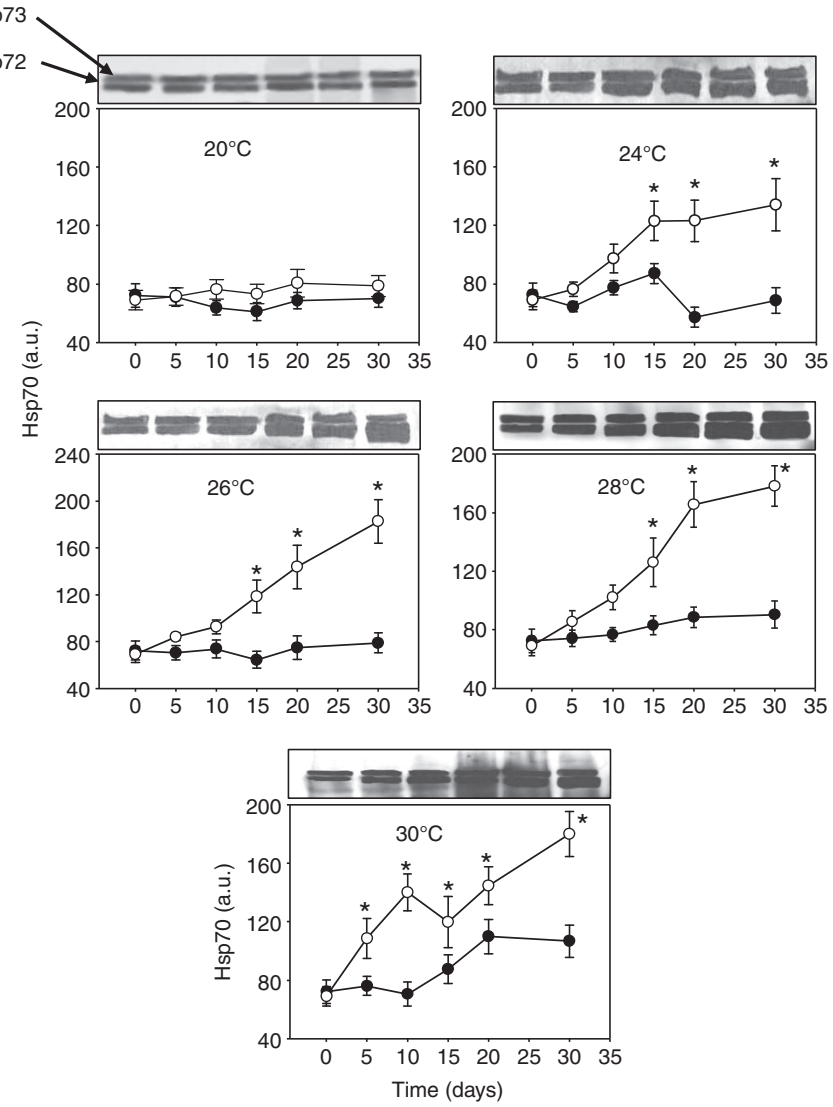

Fig. 2. Levels of Hsp70 (a.u., arbitrary units) in the mantle tissue of submersed *M. barbatus* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for Hsp70. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± s.e.m.; N=5 preparations from different animals. Hsp72 (inducible isoform), open circles; Hsp73 (constitutive isoform), filled circles. *P<0.05 compared with the control (0 days).

acclimated at 18°C, 24°C, 26°C and 30°C was used. Animals kept at 18°C were used as controls.

SDS-PAGE and immunoblot analysis

Tissue powders were homogenized in 3 ml g^{-1} of cold lysis buffer [20 mmol l^{-1} β-glycerophosphate, 50 mmol l^{-1} NaF, 2 mmol l^{-1} EDTA, 20 mmol l^{-1} Hepes, 0.2 mmol l^{-1} Na_3VO_4, 10 mmol l^{-1} benzamidine, pH 7, supplemented with 200 μmol l^{-1} leupeptin, 10 μmol l^{-1} *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, 5 mmol l^{-1} dithiothreitol (DTT), 300 μmol l^{-1} phenylmethylsulfonyl fluoride (PMSF), 120 μmol l^{-1} 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 mmol l^{-1} Tris–HCl, pH 6.8, 13% vol/vol glycerol, 133 mmol l^{-1} 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, USA).

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, USA). Non-specific binding sites on the membranes were blocked with 5% (wt/vol) nonfat milk in TBST [20 mmol l^{-1} Tris–HCl, pH 7.5, 137 mmol l^{-1} 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 Chemical Co., St Louis, MO, USA); monoclonal mouse anti-phospho-SAPK-JNK (Thr183–Tyr185) and polyclonal rabbit anti-phospho-p38 MAP kinase (Thr180–Tyr182; Cell Signaling, Beverly, MA, USA). After washing in TBST (three times, 5 min each) the blots were incubated with horseradish peroxidase-linked secondary antibodies, washed again in TBST (three times, 5 min each), and the bands were detected using enhanced chemiluminescence (Chemicon International, Inc, Temecula, CA, USA) with exposure to Fuji Medical X-ray films and quantified by laser-scanning densitometry (GraphPad, GelPro Analyzer Software, San Diego, CA, USA).

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 mmol l^{-1} imidazole–HCl (pH 7.0) containing

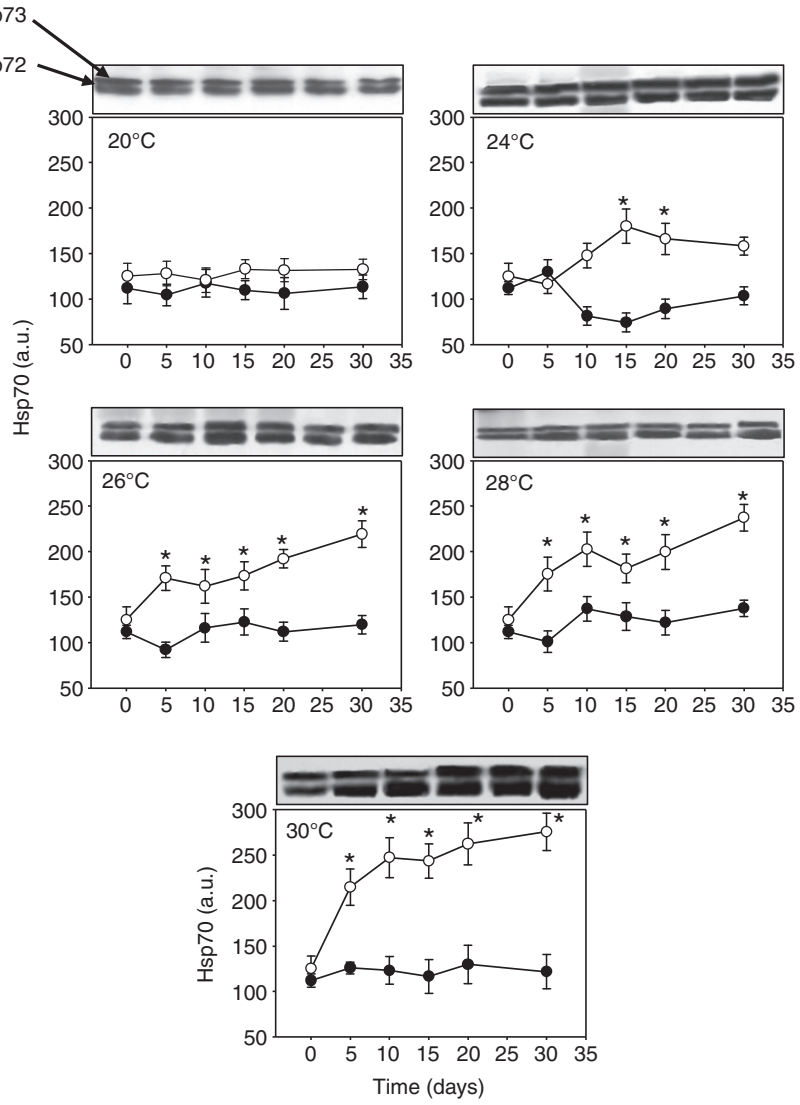

Fig. 3. Levels of Hsp70 in the posterior adductor muscle (PAM) of submersed *M. barbatus* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for Hsp70. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± s.e.m.; *N*=5 preparations from different animals. Hsp72 (inducible isoform), open circles; Hsp73 (constitutive isoform), filled circles. **P*<0.05 compared with the control (0 days).

100 mmol l^{-1} sodium fluoride, 10 mmol l^{-1} EDTA, 10 mmol l^{-1} EGTA, 30 mmol l^{-1} 2-mercaptoethanol, 40% glycerol (vol/vol), and 0.1 mmol l^{-1} PMSF added just prior to homogenization, using a Polytron PT10 homogenizer (three times, 20 s each). 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 mmol l^{-1} imidazole–HCl buffer (pH 7.0) containing 5 mmol l^{-1} EDTA, 15 mmol l^{-1} 2-mercaptoethanol, and 20% glycerol to remove metabolites of low molecular mass (Helmerhorst and Strokes, 1980). The column was centrifuged in a benchtop centrifuge at 2000 ***g*** for 1 min, and the supernatant was used for the determination of enzyme activity. Standard assay conditions for PK were as follows: 50 mmol l^{-1} imidazole–HCl buffer, 2 mmol l^{-1} ADP, 0.15 mmol l^{-1} NADH, 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 2 i.u. dialyzed lactate dehydrogenase and PEP, either 2 mmol l^{-1} for the determination of V_{max} ($V_{2\ mmol\ l^{-1}}$) or 0.05 mmol l^{-1} for the determination of V_o ($V_{0.05\ mmol\ l^{-1}}$). The ratio V_o/V_{max}, which reflects the relative activity of PK, was then calculated to determine metabolic depression during acclimation to high temperature. The ratio V_o/V_{max} was found to decline in the adductor muscle and mantle of bivalve molluscs during anoxia indicating a shift of PK toward a less-active form during metabolic depression (Holwerda et al., 1984; Holwerda et al., 1989). Assays for V_o and V_{max} were conducted at 18°C and at each acclimation temperature.

Measurements of water temperature in the field

Water temperature in the field was measured at noon at regular intervals between 1/1/2007 and 31/9/2007. Temperature was measured vertically once every meter to a depth of 20 m using a Multiparameter Water Quality Meter (Model WQC-24, DKK-TOA company).

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 ± s.e.m.

RESULTS

Effect of water temperature on mussel mortality

Fig. 1 shows the mortality over time during acclimation to different temperatures. No mortality of mussels was observed during acclimation at 24°C, while about 3% of the mussels died when acclimated at 26°C. Mussel mortality increased significantly as water

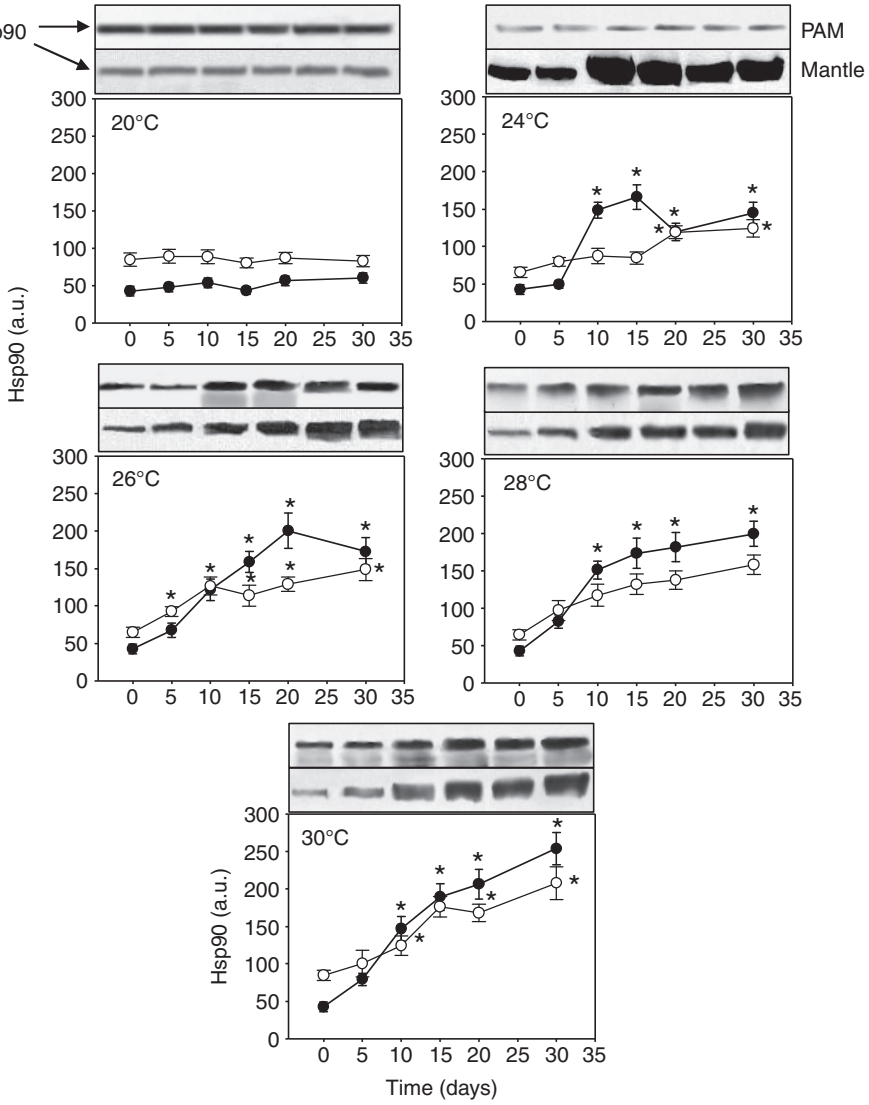

Fig. 4. Levels of Hsp90 in the mantle (filled cycles) and posterior adductor muscle (PAM; opened cycles) of submersed *M. barbatus* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for Hsp90. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± s.e.m.; N=5 preparations from different animals. *P<0.05 compared with the control (0 days).

temperature reached 28°C and 30°C, leading to 10% and 20% mortality after 30 days, respectively.

Effect of acclimation temperature on the expression of Hsp70 and Hsp90

The expression of Hsps was monitored in mantle tissue and the PAM. Two members of the Hsp70 family were found to be prominent, Hsp72 (the inducible form) and Hsp73 (the constitutive form), during acclimation to all temperatures. The expression of Hsp72 began when *M. barbatus* were acclimated at 24°C and the levels of Hsp72 showed a gradual increase within 30 days of acclimation. Increased Hsp72 expression was also observed during acclimation to 26°C, 28°C and 30°C (Fig. 2). A similar expression profile was observed in the PAM (Fig. 3). In response to acclimation at various temperatures, Hsp90 showed similar changes to Hsp72 (Fig. 4).

Effect of acclimation temperature on the phosphorylation of the kinases JNKs and p38 MAPK

Phosphorylation and hence activation of JNKs and p38 MAPK was determined in both the mantle and PAM after acclimation at different temperatures. One form of JNK was detected, which corresponds to the 46 kDa isoform of the mammalian enzyme (Figs 5 and 6). An increase in acclimation temperatures caused significant changes in the levels of the phosphorylated form of JNK in both the mantle and PAM (Fig. 5). Specifically, the levels of phosphorylated JNK in the mantle increased slightly during acclimation at 24°C, whereas it doubled progressively after 30 days regardless of whether acclimation occurred to 26°C, 28°C or 30°C. A similar pattern was observed in the PAM during acclimation at different temperatures (Fig. 5). Similar to JNK, increasing temperatures caused increases in the levels of phosphorylated p38 MAPK in the mantle and PAM. However, in this case the resulting levels of phosphorylated p38 were temperature dependent. As shown in Fig. 6 the increase in the levels of phosphorylated p38 in the mantle and PAM was initiated at 26°C and increased further with increases in the acclimation temperatures.

Effects of acclimation temperature on the activity of PK

The values of V_o, V_{max} and their ratio V_o/V_{max}, as determined for PK from mantle and PAM of *M. barbatus* at 20°C, are given in Table 1. Fig. 7 summarizes the pattern of PK activity in mantle tissue and PAM during acclimation of *M. barbatus* to different water temperatures. No change in PK activity occurred at

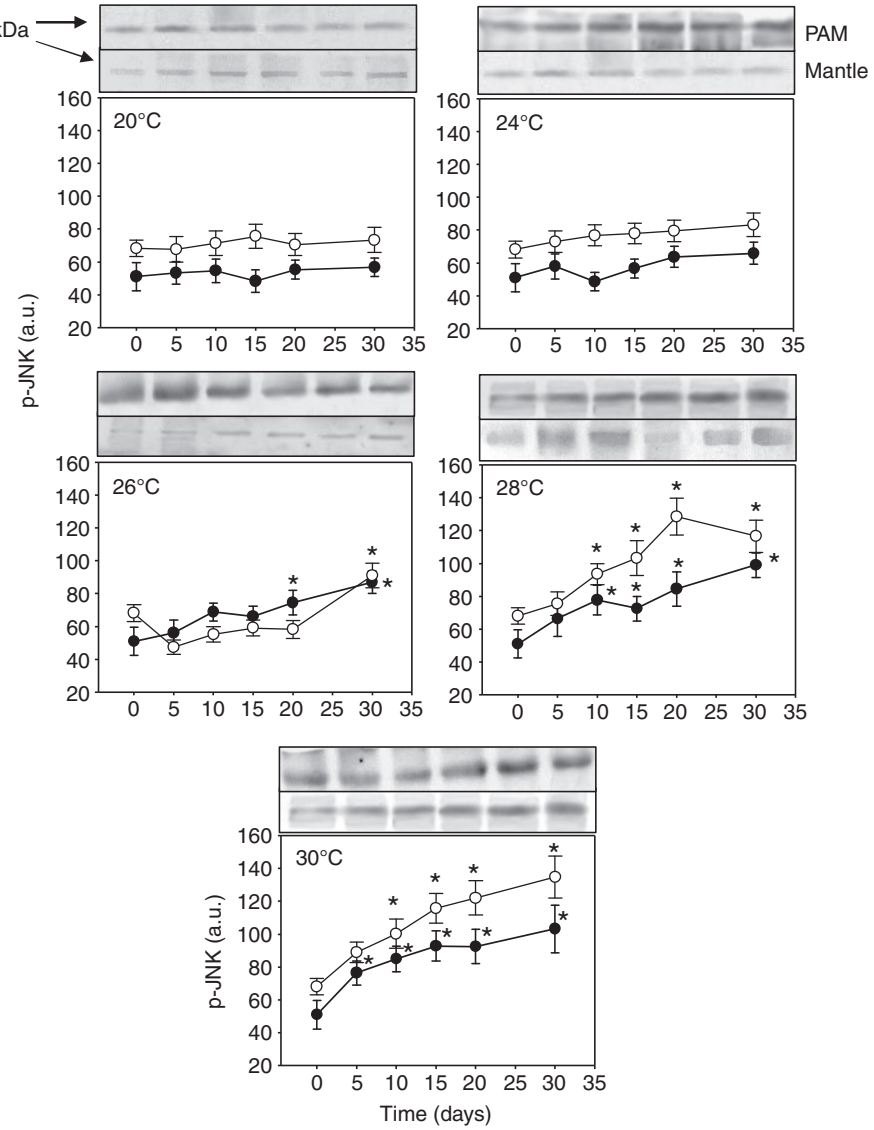

Fig. 5. Phosphorylation levels of cJun-N-terminal kinase (JNK) in the mantle tissue (filled cycles) and in the posterior adductor muscle (PAM; opened cycles) of submersed *M. barbatus* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for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

temperatures lower than 26°C. Further warming, however, modified enzyme activity, causing a significant decrease in both mantle and PAM at 30°C (Fig. 7). In the mantle, the relative activity of the enzyme (V_o/V_{max}) remained at the same levels during acclimation of *M. barbatus* at 24°C and 26°C, whereas it decreased markedly from 0.51 to 0.054 within 20 days of acclimation at 30°C, indicating a shift of PK towards a less-active form (Fig. 8). A similar pattern was observed in the PAM. The relative activity of PK from the PAM decreased within the first 15 days of acclimation to 30°C and still remained reduced after 30 days of acclimation.

DISCUSSION

The data obtained in the present study demonstrate that *M. barbatus* cannot survive sea water temperatures beyond 26°C over extended periods of time (Fig. 1). At 26°C only a small fraction (about 3%) of the mussels died within 30 days of acclimation. Mussel mortality increased most drastically during warming to 28°C and 30°C and reached 10% and 20%, respectively, after 30 days. Similar to *M. barbatus*, mortality of *M. galloprovincialis* began when mussels were acclimated at 26°C. In contrast to *M. barbatus*, however, the mortality of *M. galloprovincialis* was significantly, about 20-, 40- and 85-fold higher after 30 days at 26, 28 and 30°C, respectively (Anestis et al., 2007). These data indicate a higher tolerance of *M. barbatus* to elevated sea water temperature.

Another characteristic difference between *M. barbatus* and *M. galloprovincials* is the temperature of sea water inducing the expression of Hsp70 and Hsp90. As shown in Figs 2–4 the onset temperature of enhanced synthesis of Hsps (T_{on}) in the tissues of *M. barbatus* is beyond 22–23°C, whereas in *M. galloprovincialis*, as in other species of the genus *Mytilus*, it is beyond 25°C (Anestis et al., 2007; Hofmann and Somero, 1996; Buckley et al., 2001). These data indicate that the tissues of *M. barbatus* may be more responsive to temperature change than tissues in *M. galloprovincialis*. As reported elsewhere, the activation of the heat-shock response is indicative of thermal denaturation of proteins during periods of heat stress (Feder and Hofmann, 1999). Vertical zonation and expression of Hsps has been well studied in congeneric molluscs and there is a close relationship between these two parameters (Somero, 2002; Hofmann, 2005). Studies of physiological and biochemical responses of three congeneric blue mussels of the genus *Mytilus* showed that, in line with their vertical distribution, *M. galloprovincialis* is able to cope better with higher

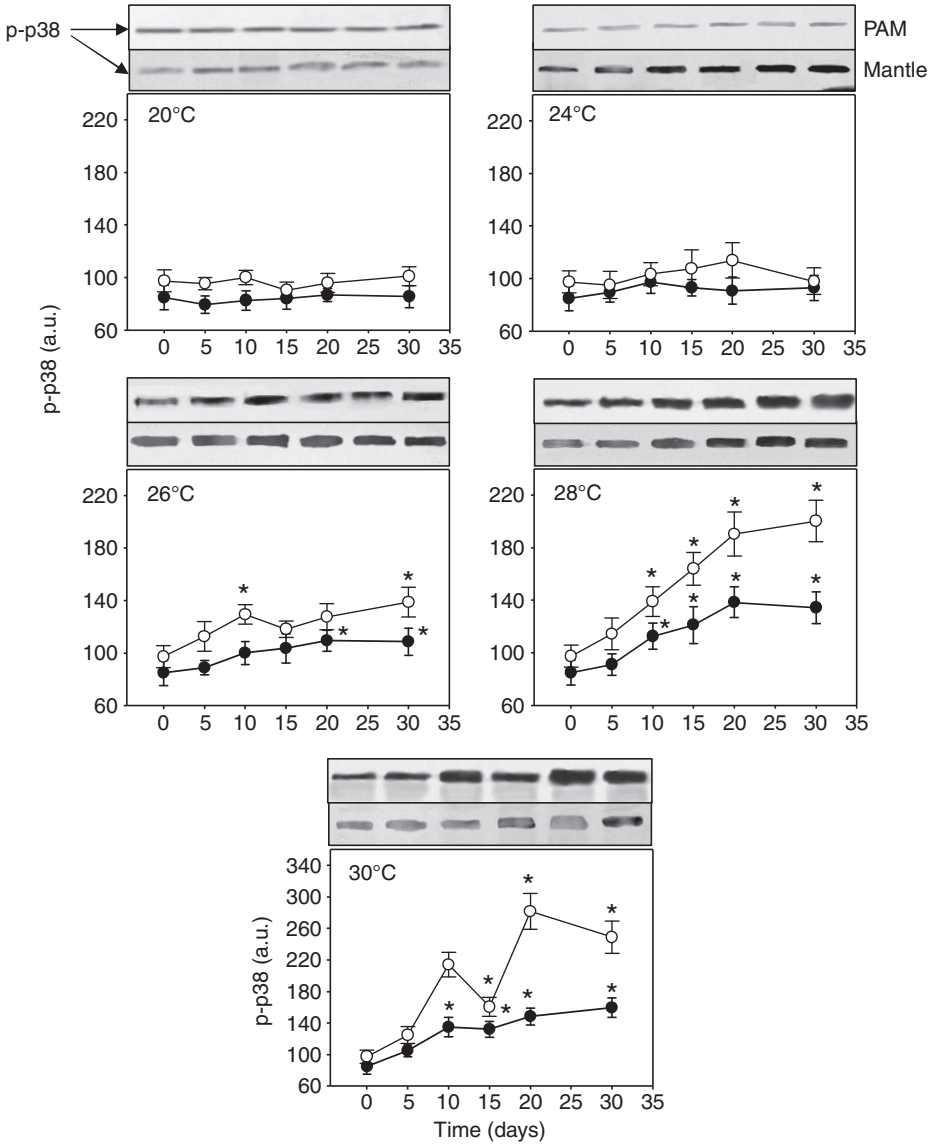

Fig. 6. Phosphorylation levels of p38 mitogen-activated protein kinase (MAPK) in the mantle tissue (filled cycles) and in the posterior adductor muscle (PAM; opened cycles) of submersed *M. barbatus* during acclimation to different water temperatures. Tissue extracts were subjected to SDS-PAGE and immunoblotted for the phosphorylated form of JNKs. Representative immunoblots are shown for each acclimation temperature. Blots were quantified by laser-scanning densitometry. Values are means ± s.e.m.; N=5 preparations from different animals. *P<0.05 compared with the control (0 days).

temperatures than *M. trossulus* and *M. edulis*. Accordingly, *Mytilus* congeners display different tolerances at high temperature. Furthermore, thermal tolerance changes with acclimation/acclimatization history (Hofmann and Somero, 1996; Roberts et al., 1997; Buckley et al., 2001; Brady and Somero, 2006).

The gradual increase in the inducible isoform of Hsp70 in the PAM and mantle of *M. barbatus* (Figs 2 and 3) seems to be consistent with the suggestion by Buckley et al. (Buckley et al., 2001), that the gradual accumulation of inducible Hsp70 might act as a buffer against subsequent heat stress and support increased thermoprotection in gradually warming environments. It has been proposed that the upregulation of Hsp genes in mussels such as *M. trossulus* exposed to increased temperatures, 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 a high threshold temperature is surpassed, is HSF1 released to transactivate Hsp genes. According to recent evidence, this might involve either protein–protein interactions with other

Table 1. Comparison of relative activity of pyruvate kinase in the tissues of *M. barbatus* and *M. galloprovincialis*

	Modiolus barbatus			*Mytilus galloprovincialis**		
	V_o	V_{max}	V_o/V_{max}	V_o	V_{max}	V_o/V_{max}
Tissue	(0.05 mmol l^{-1})	(2 mmol l^{-1})		(0.05 mmol l^{-1})	(2 mmol l^{-1})	
Mantle	1.66±0.14	2.82±0.29	0.55	0.24±0.0022	1.72±0.12	0.13
PAM	2.97±0.23	6.45±0.72	0.46	1.49±0.23	4.87±0.54	0.30

*Data from Anestis et al. (Anestis et al., 2007).
PAM, posterior adductor muscle.
Numbers in parentheses indicate the concentration of substrate used during assay of pyruvate kinase (PK) activity at 20°C.

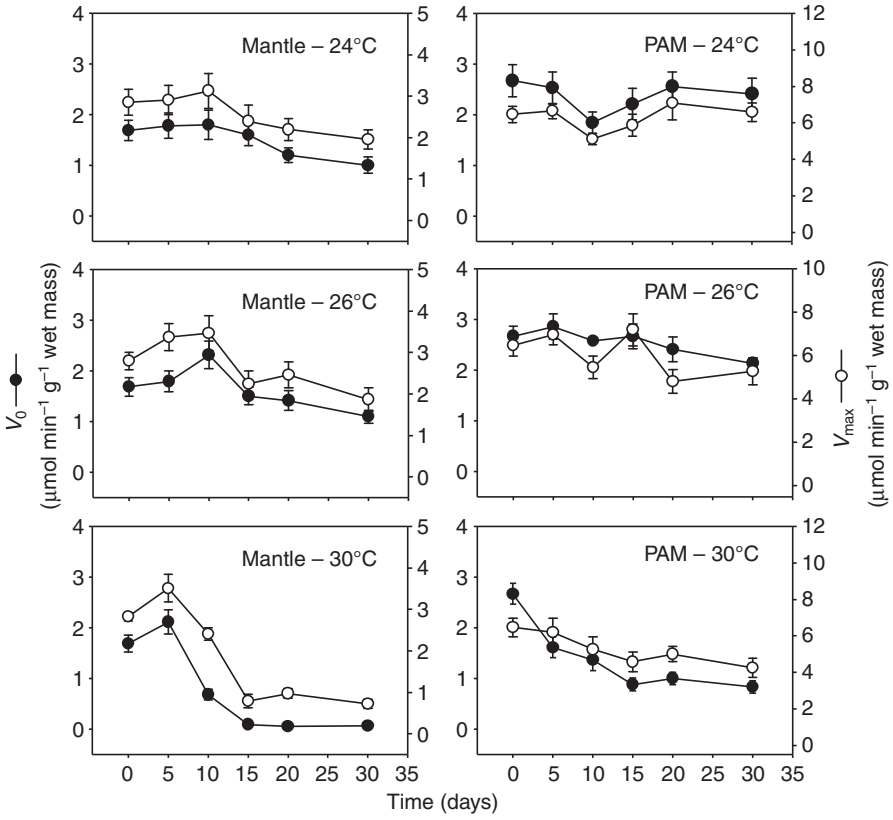

Fig. 7. Activity of pyruvate kinase (PK) from the mantle and posterior adductor muscle (PAM) of *M. barbatus* during acclimation to different water temperatures. Activity (μmol min^{-1} g^{-1} wet mass) was determined at 2 mmol l^{-1} (V_{max}; open circles) and 0.05 mmol l^{-1} of phosphoenolpyruvate (PEP; V_o; filled circles). Values are means ± s.e.m.; N=10 preparations from different animals.

transcriptional effector molecules, such as the heatshock factor binding protein (HSBP1) (Satyal et al., 1998; Sarge et al., 1993), and/or regulation *via* phosphorylation of specific serine residues on HSF1 that precedes Hsp gene transactivation (Xia and Voellmy, 1997). 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 (Rafie et al., 2003; Sheikh-Hamad et al., 1998; Uehara et al., 1999). 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. barbatus* exposed to temperatures beyond 24°C (Figs 5 and 6). These data are in line with an involvement of the MAPK signalling cascade in the induction of Hsp genes in the tissues of *M. barbatus* during thermal stress.

At first sight it is puzzling that, in contrast to *M. galloprovincialis* (Anestis et al., 2007), in the tissues of *M. barbatus* the expression of Hsps (Figs 2–4) is induced at temperatures at least 2°C lower than that inducing mortality (Fig. 1). This observation may well explain the better passive survival of extreme temperatures by *M. barbatus*. This observation also emphasizes the importance of refolding of denaturated proteins for extreme thermotolerance.

However, the ability of organisms to survive thermal stress is not only a matter of Hsp functioning, but also of the organism's ability to meet the energy demand for protein repair (Somero, 2002; Hofmann, 2005). Energy is required at several steps of the heat-shock response, including the activation of transcription of heat-shock genes, the synthesis of Hsps, and the ATP-requiring chaperoning by Hsps. Furthermore, energy is required if proteins are irreversibly denatured and need to be replaced. Hawkins (Hawkins, 1985) estimated that the cost of protein synthesis constitutes 20–25% of the energy budget of the northern blue mussel, *M. edulis*.

Such aspects of oxygen and energy homeostasis need to be considered within the concept of oxygen and capacity limited thermal tolerance, which leads to an understanding of temperature dependent functional optima (Pörtner, 2002a; Pörtner et al., 2005). Enhanced thermal limitation occurs progressively through the loss of aerobic scope beyond T_p, the consecutive onset of anaerobic metabolism at T_c (critical temperature) and, at even more extreme temperatures, of molecular denaturation at T_d (denaturation temperature) (Pörtner, 2001; Pörtner, 2002a). Energy demand is easily met at optimum temperatures, whereas only basal metabolism is supported at T_c. Additional anaerobic energy is required beyond T_c.

The activity of the key glycolytic enzyme PK reveals the metabolic rate and status in the tissues of molluscs. According to our results, PK activity indicates a twofold higher glycolytic capacity in *M. barbatus* than in *M. galloprovincialis* (Table 1). Furthermore, PK controls the flux of phosphoenolpyruvate (PEP) to succinate during anaerobiosis. In response to anoxia the relative activity of PK (V_o/V_{max}) declines in the adductor muscle and mantle of bivalve molluscs, indicating a reduction of PK capacity and a shift towards a less active form during metabolic depression (Holwerda et al., 1984; Holwerda et al., 1989). In addition, low levels of relative PK activity mirror low rates of pyruvate supply to aerobic metabolism and, thus, low rates of overall energy turnover. To begin with, the higher ratio of V_o/V_{max} determined for *M. barbatus* thus indicates a higher glycolytic rate and a lower degree of metabolic depression compared to *M. galloprovincialis*. The onset temperature causing the reduction in V_o/V_{max} was significantly higher (26°C) in *M. barbatus* than in *M. galloprovincialis* (24°C) (Anestis et al., 2007). These observations emphasize the importance of, firstly, maintained aerobic capacity in extended thermal tolerance as in *M. barbatus* and, secondly, the time-limited nature of metabolic depression in passive tolerance to thermal

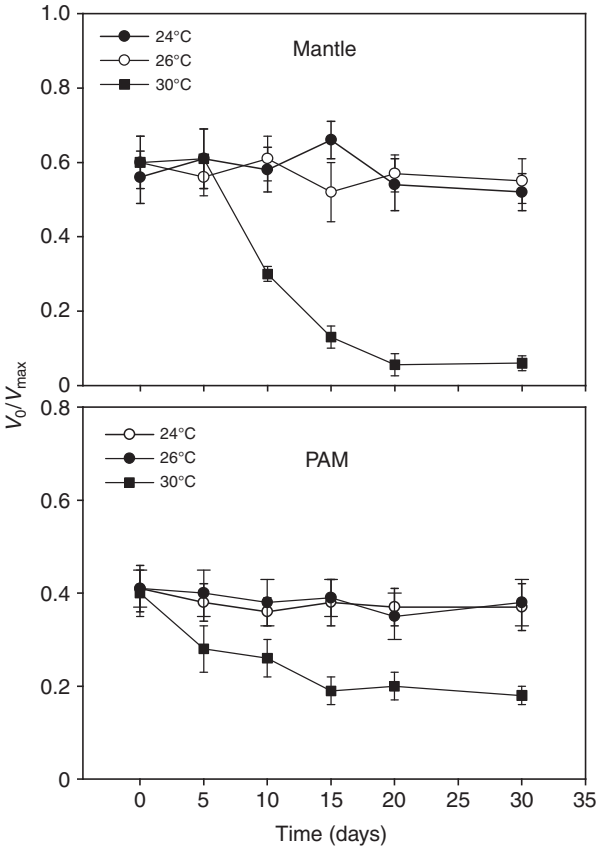

Fig. 8. The V_o/V_{max} ratio in the mantle and posterior adductor muscle (PAM) during exposure of *M. barbatus* to different water temperatures. Values are means ± s.e.m., *N*=10 preparations from different animals.

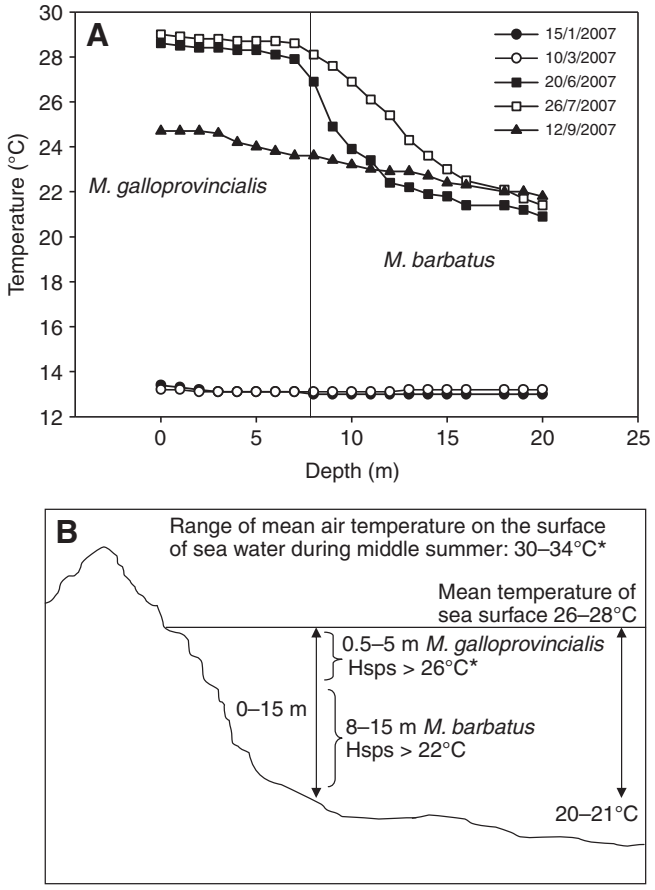

Fig. 9. Correlation between the vertical zonation of *M. galloprovincialis* and *M. barbatus* and the annual patterns of sea water temperatures and the expression of Hsps in the tissues. (*Anestis et al., 2007.)

extremes as in *M. galloprovincialis* (c.f. Pörtner, 2002). These considerations are again in line with a better passive heat protection of the bearded mussel, *M. barbatus*.

The question that finally arises is, what is the ecological relevance of the different metabolic patterns and molecular stress responses in the two bivalves species? In the Thermaikos gulf *M. galloprovincialis* is found at shallower depth (from 1–7 m depth) and experiences larger temperature fluctuations than *M. barbatus*. Specifically, during mid summer *M. galloprovincialis* could experience temperatures ranging from 24°C up to 28°C (Fig. 9A,B). Long-term warming beyond 24°C, however, is lethal for *M. galloprovincialis*. Beyond 25°C, filtration falls significantly in *M. galloprovincialis* (A.A., H.O.P., A. Staikou and B.M., unpublished data) as well as in other *Mytilus* species (Bayne et al., 1976; Gonzalez and Yevich, 1976; Schulte, 1975), preventing food uptake and long-term survival. These observations emphasize the importance but time-limited nature of metabolic depression in passive heat tolerance.

According to the thermal modulation of PK activity, some metabolic depression also occurs in *M. barbatus* when they are acclimating at temperatures beyond 26°C (Figs 7 and 8), a temperature which causes slightly enhanced mortality during long-term exposure (Fig. 1). However, *M. barbatus* faces temperatures above a maximum of 22–23°C at the borders of its distribution for short period only (Fig. 9). These observations are in line with the conclusion that maintenance of full aerobic scope is crucial for long-term thermal tolerance in the field (c.f. Pörtner and Knust, 2007). Our findings would suggest an earlier limitation of aerobic scope but a wider range of more passive tolerance supported by reduced aerobic scope (pejus range) in *M. barbatus* than in *M. galloprovincialis*. This earlier limitation is reflected in the earlier onset of the heat shock response which then improves passive heat tolerance at higher temperatures. However, with the data at hand it is currently unclear whether the earlier onset of a decrease in aerobic scope and of the heat shock response go hand in hand in this species.

Taking seasonal changes in temperature into account, 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. Their extended aerobic range combined with their delayed and limited (compared to *M. barbatus*) depression of metabolic rate may support survival during short-term extreme exposures. While *M. barbatus* seems to be more capable of passively surviving extreme temperatures than *M. galloprovincialis,* long-term exposures to temperatures beyond 23°C, associated with constrained aerobic scope, may impair relevant physiological processes such as growth, gamete production and reproduction rates. It has recently been reported that the size of fish population begins to decline as soon as scope for growth is reduced, and does not involve heat-induced death *per se* (Pörtner and Knust, 2007; Wang and Overgaard, 2007).

The earlier onset of Hsp formation in *M. barbatus* than in *M. galloprovincialis* in fact indicates earlier onset of thermal limitation, with an as yet unexplored physiological background. Hypoxemia may also set in early and contribute to eliciting the heat shock response (Pörtner, 2002a). Overall, the present data as well as the ones presented by Pörtner and Knust (Pörtner and Knust, 2007), clearly show that laboratory data on tolerance need to be interpreted against a background of field data in order to evaluate their relevance in the natural environment.

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