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Field studies and projections of climate change effects on the bearded horse mussel *Modiolus barbatus* in the Gulf of Thermaikos, Greece

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ABSTRACT: The role of thermal stress phenomena in setting stratified distribution limits was investigated in the bearded horse mussel *Modiolus barbatus* in response to the seasonal temperature regime in the field. Mussels were transplanted from their natural depth range at ca. 20 m to 3 m depth, where they then experienced enhanced variability of ambient conditions. In specimens from both depths, thermal stress was assessed from the inducible heat shock response (HSR), the accumulation of irreversibly damaged proteins, and from metabolic characters including the putative shift from aerobic to anaerobic metabolism. During both winter and summer, the HSR became involved more at shallow depths than at 20 m depth. The accumulation of succinate during summer indicates transition to anaerobiosis. The results suggest that the development of anaerobic conditions and the exploitation of the HSR are closely intertwined. The field data corroborate that glycolytic capacity, the level of energy turnover, and also protection from protein damage play a role in setting passive tolerance to extremes in environmental temperature. We suggest that limits to vertical distribution *of M. barbatus* are set by the time and degree of exploitation of the mechanisms sustaining passive thermal tolerance and the avoidance of protein damage in the warmth.

KEY WORDS: Bivalves · *Modiolus barbatus* · Environmental warming · Physiological patterns · Heat shock response · HSR

INTRODUCTION

There is strong consensus that coastal marine ecosystems, along with the goods and services they provide, are threatened by anthropogenic global climate change (IPCC 2007, Harley et al. 2006). Despite recent progress in climate change science, the responses of marine ecosystems to the complex effects of climate change remain poorly understood. Drivers of climate-related change in marine systems include ocean acidification, changes in water temperature, solar radiation, oceanographic variables (e.g. currents, wind speed, wave action), sea level rise, and the frequency or intensity of extreme events and precipitation (IPCC 2007). These changes in environmental variables might directly impact the physiology, behaviour, growth, reproductive capacity, mortality, and biogeography of marine organisms. They might also impact organisms indirectly via changes in the food chain that alter predation levels and the productivity, structure, and composition of the ecosystem. All of these effects bear consequences for the biogeography of species as well as for biogeochemical cycles and ecosystem services. These changes will create operational changes in the use of marine resources by human societies (e.g. species selection, site selection, sea cage technology). As a unifying principle, the ultimate effects of global

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warming at ecosystem level will build on species-specific responses (Pörtner 2001, 2002, 2010, Helmuth et al. 2010, Somero 2010). For reliable projections of future changes it is important to develop a mechanism-based cause-and-effect understanding. The latter must be integrative and encompass effects of climate change at various levels of biological organization, from molecular, cellular, organismal, population, and community-ecosystem.

Many laboratory studies have defined the thermal limits and thermotolerance of bivalves by studying the expression of heat shock proteins (Hsps) (Hofmann & Somero 1995, Buckley et al. 2001). Hsps may co-define extreme thermal limits, and it has been hypothesized that they are related to species boundaries in intertidal ecosystems (Hofmann 2005, Tomanek 2010). Moreover, physiological processes such as heart functioning or metabolic adaptations setting aerobic scope through regulated capacities of glycolytic and mitochondrial metabolism, including the respiratory chain and the tricarboxylic acid cycle, appear equally important and may even take priority in determining the thermal windows of species, and thereby biogeographical limits. Their limited capacity at thermal limits may also be crucial in initiating the heat shock response (HSR) (Pörtner 2001, 2002, 2010, Braby & Somero 2006, Anestis et al. 2007, 2008, Somero 2010). Following the principles of the concept of oxygen and capacity limited thermal tolerance (OCLTT), the complex integration of such biochemical and physiological mechanisms shapes the thermally limited performance capacity of the whole organism (Pörtner 2002, 2010, Pörtner & Knust 2007, Eliason et al. 2011). Such interpretation is in line with recent investigations of the transcriptomic responses of marine mollusks to heat stress (Lockwood et al. 2010, Tomanek & Zuzow 2010).

With several factors co-varying due to climate change, the challenge of predicting the effects of climate warming on marine organisms in their natural environment is high because the combined effects of 2 or more variables cannot be predicted from the individual effects of each factor, as the impact of one factor may be either strengthened (synergistic effects) or weakened (antagonistic effects) by the variation in another factor (cf. Pörtner 2010). For reliable predictions of the possible impacts of global warming on marine animals, field studies are therefore required to validate findings obtained in the laboratory and to verify a role for thermal tolerance limits of marine organisms in shaping their response to climate change in the natural environment (Pörtner & Knust 2007, Pörtner & Farrell 2008, Helmuth 2009, Fuller et al. 2010, Helmuth et al. 2010, Hofmann & Todgham 2010).

Mediterranean coasts are very vulnerable systems (Jeftic et al. 1992, Nicholls & Hoozemans 1996). Summer conditions are characterized by high temperatures and low food availability. Increases in the frequency or intensity of such warm conditions may detrimentally affect marine organisms, as indicated by mass mortalities observed in the northwestern Mediterranean (Coma et al. 2009, Gambaiani et al. 2009). Based on various low (B1) or high (A1F1) emission scenarios, IPCC (2007) has predicted atmospheric warming by 1.8 or 4°C, respectively, for the year 2100. Consequently, the oceans are also expected to warm, causing strong impacts on marine systems (Austin & Rehfisch 2005, Parmesan 2006).

The present study was undertaken to monitor indicators of thermal stress and physiological performance in bearded horse mussels *Modiolus barbatus*, in an effort to bring together mechanisms at molecular and metabolic levels to a coherent picture of effects under field conditions and during long-term seasonal temperature fluctuations. For an in-depth analysis of the specific role of temperature in shaping the vertical zonation of this species, and in order to assess the role of temperature variability with values reaching beyond the species' thermal niche, we transplanted specimens from ca. 20 m to 3 m depth and examined how the HSR and metabolic patterns varied over a 7 mo period. Tissues such as mantle and posterior adductor muscle (PAM) were sampled and analyzed for the expression of Hsp70, Hsp90, and for the levels of irreversibly damaged or ubiquinated proteins. The role of metabolic capacities and of the putative shift from aerobic to anaerobic metabolism was assessed by determining the activities of key glycolytic enzymes and the levels of the anaerobic end product succinate. Moreover, data on productivity over 10 yr were analyzed to illustrate the role of seasonal changes in temperature and food availability at local scales. For an assessment of unifying principles the data are compared with those of an earlier study of *Mytilus galloprovincialis* (Ioannou et al. 2009) in the Thermaikos Gulf.

MATERIALS AND METHODS

Animals, study site, and experimental design

Experiments were conducted in the Thermaikos Gulf near Halastra (40° 32′ 35″ N, 22° 46′ 02″ E) (Fig. 1A). Only adult *Modiolus barbatus* (6.31 ±

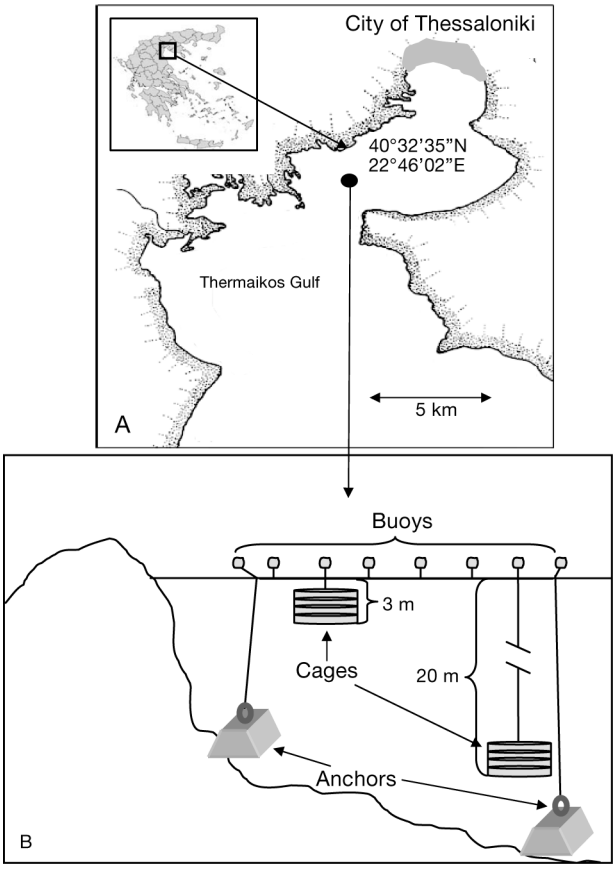

Fig. 1. (A) Thermaikos Gulf where *Modiolus barbatus* were studied. (B) Schematic presentation of suspended cages at 3 m and 20 m depths. See text for further explanation

0.42 g, mean ± SD; shell length 4.02 ± 1.4 cm; height 2.36 ± 0.11 cm; width 1.44 ± 0.15 cm) were used for the field studies. Mussels were collected in late October 2008 from a wild population at 23 to 25 m depth and were placed in 2 plastic cages, each cage containing about 400 individuals. The cages were suspended from horizontal ropes stretched out and maintained at 1 m distance from the water surface with buoys. The first cage was suspended from horizontal ropes at 3 m distance from the water surface and the second one at 20 m depth and at least 2 to 3 m above the bottom (Fig. 1B).

Mussels were sampled from the cages every month starting in November 2008 and ending in July 2009. During each sampling period, 15 individuals were dissected on ice, and the mantle and PAM were frozen in liquid nitrogen. Tissue samples were transported back to the laboratory on dry ice and stored in liquid nitrogen for an analysis of the expression of Hsp70, Hsp90, ubiquitin conjugates, activities of glycolytic enzymes, and levels of succinate.

Preparation of tissue samples for SDS/PAGE

Tissue powders were homogenized in 3 ml (per gram of tissue) 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-epoxy succinyl-L-leucylamido-[4-guanidino]butane, 5 mmol l^{-1} dithiothreitol, 300 µmol l^{-1} phenyl methyl sulfonyl fluoride [PMSF], 120 µmol l^{-1} pepstatin, 1% v/v 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 when combined with 0.33 volumes of SDS/PAGE sample buffer (330 mmol l^{-1} Tris-HCl, pH 6.8, 13% v/v glycerol, 133 mmol l^{-1} DTT, 10% w/v SDS, 0.2% w/v bromophenol blue). Protein concentrations were determined by the method of Bradford (1976).

SDS/PAGE and immunoblot analysis

Equal amounts of proteins (100 µg) were separated on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 µm; Schleicher & Schuell). Non-specific binding sites on the membranes were blocked with 5% (w/v) non-fat milk in TBST (20 mmol l^{-1} Tris-HCl, pH 7.5, 137 mmol l^{-1} NaCl, 0.1% (v/v) Tween 20) for 30 min at room temperature. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies. Antibodies used were: monoclonal mouse anti-Hsp 70kDa and monoclonal mouse anti-Hsp 90kDa (Sigma). After washing in TBST (3 × 5 min) the blots were incubated with horseradish peroxidase-linked secondary antibodies, washed again in TBST (3 × 5 min), 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).

Quantitative immunochemical assay for ubiquitin conjugates

The amount of ubiquitinated protein in the mantle and PAM was quantified using a solid-phase immunochemical assay as described by Hofmann & Somero (1995). Samples were diluted to a concentration of 5 µg ml^{-1} in a saline solution; 100 µl volumes

were loaded in triplicate onto a presoaked nitrocellulose membrane (0.45 µm) in a dot blot vacuum apparatus (BioRad), and gravity-fed through the membrane. The membrane was blocked, incubated for 1.5 h with a polyclonal rabbit anti-ubiquitin conjugate (Cell Signalling) primary antibody diluted 1:2500, and then incubated for 1 h with protein-A horseradish peroxidase conjugate diluted 1:5000 (Vector Laboratories, PI-1000). Visual detection and analysis were identical to methods outlined in 'SDS/PAGE and immunoblot analysis' above.

Preparation of tissue homogenates for the determination of enzymatic activities

Frozen tissue powder (200 to 500 mg) was homogenized (1:5, wt/vol) in ice-cold 50 mmol l^{-1} imidazole-HCl (pH 7.0) containing 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. 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. The column was centrifuged (2000 × *g*, 1 min, 5°C), and the supernatant was used for the determination of enzyme activity. All enzymes were assayed for 10 min in 50 mmol l^{-1} imidazole–HCl pH 7.0 in a final assay volume of 1 ml. Specific assay conditions were as follows:

(1) Hexokinase (HK) (ATP:D-hexose-6-phosphotransferase; E.C. 2.7.1.1): 10 mmol l^{-1} $MgCl_2$, 0.15 mmol l^{-1} NAD^+, 5 U ml^{-1} NAD^+-dependent glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), 10 mmol l^{-1} glucose. The assay was started by the addition of 1 mmol l^{-1} adenosine 5′-triphosphate (ATP). In samples from gill tissue, HK activity was calculated by subtraction of baseline activity catalyzed by endogenous glucose dehydrogenase.

(2) Phosphofructokinase (PFK) (ATP:D-fructose-6-phosphate 1-phospho-transferase; E.C. 2.7.1.11): 1 mmol l^{-1} dithiothreitol, 0.15 mmol l^{-1} NADH, 5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 5 U ml^{-1} aldolase, 50 U ml^{-1} triose phosphate isomerase, 5 U ml^{-1} glycerol-3-P dehydrogenase, 2.5 mmol l^{-1} ATP. The assay was started by the addition of 2 mmol l^{-1} fructose-6-phosphate (F-6-P).

(3) Aldolase (Ald) (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase; E.C. 4.1.2.13): 0.15 mmol l^{-1} NADH, 50 U ml^{-1} triose phosphate isomerase, 5 U ml^{-1} α-glycerophosphate dehydrogenase. The assay was started by the addition of 3 mmol l^{-1} fructose-1,6-bisphosphate (F-1,6-P2).

(4) Pyruvate kinase (PK) (E.C. 2.7.1.40): 2 mmol l^{-1} ADP, 0.15 mmol l^{-1} NADH, 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 2 IU dialyzed lactate dehydrogenase and 2 mmol l^{-1} phosphoenolpyruvate (PEP). Assays for maximum activity (V_{max}) were conducted at 18°C.

Determination of succinate in tissues

Tissue succinate was measured using commercial kits from Boehringer Mannheim/R-Biopharm Enzymatic BioAnalysis/Food Analysis (Cat. No. 10 176 281 035).

Analysis of sea water physicochemical parameters and chlorophyll *a* (chl *a*)

Measurements of salinity, oxygen partial pressure (pO_2), sea water temperature, and conductivity were carried out at 3 m and 20 m depths, at 12:00 h between 25 November 2008 and 20 November 2009 using a multiparameter water quality meter (Model WQC-24, DKK-TOA). Integrated samples were collected monthly from 2000 to 2009 by using a hose approx. 20 m long, matching water column depth. Chl *a* was determined twice per month following the method of Lorenzen (1967), Koukaras & Nikolaidis (2004), and Nikolaidis et al. (2006) and was expressed in µg l^{-1}.

Statistics

Changes over time were tested for significance at the 5% level by using 1-way repeated analysis of variance (repeated ANOVA) and by performing Bonferroni post-hoc tests for group comparisons. Individuals collected on 25 November 2008 comprised the control group. Values are means ± SD.

RESULTS

Seasonal profile of physicochemical parameters

As shown in Fig. 2A, the lowest temperatures at 3 m depth were recorded between mid-January and February, at 10.4 and 10.2°C, respectively. Water temperature rose to 11.7°C in early March, and the highest values were measured in mid-July (28.2 to

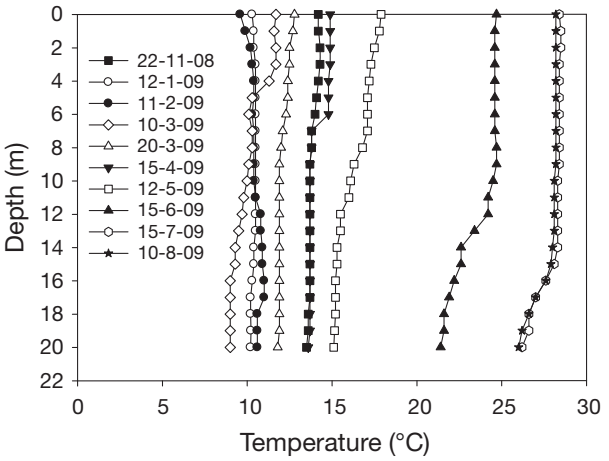

Fig. 2. Seasonal fluctuations of sea water temperature between 3 and 20 m depths recorded between November 2008 and August 2009

28.6°C). At 20 m depth, the lowest temperatures of 9.6 and 10.3°C were recorded between January and March. When compared to the surface, warming at depth during spring was thus delayed by 1 mo. The highest value recorded in mid-July at 20 m was about 4°C lower than at 3 m depth. There were no seasonal differences in salinity, pO_2, and conductivity of the sea water (data not shown).

Seasonal profiles of heat-shock-protein levels

The induction of Hsp70 and Hsp90 was monitored in mantle and PAM tissues of experimental field *Modiolus barbatus* between November 2008 and July 2009 (Figs. 3 & 4, respectively). In both tissues, 2 main bands of the Hsp70 family were identified, the inducible Hsp72 and the constitutive Hsp73. At 3 m

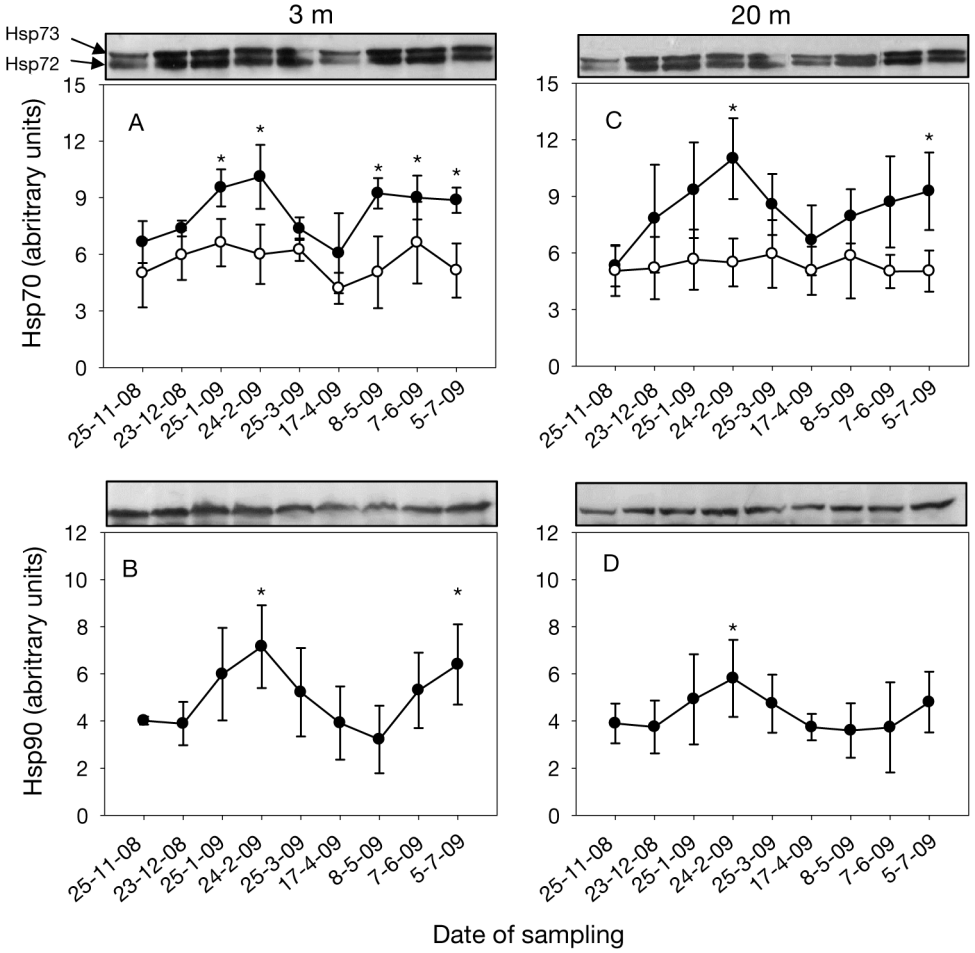

Fig. 3. Seasonal changes in the levels of inducible Hsp72 (●) and constitutive Hsp73 (○) and Hsp90 in the mantle of *Modiolus barbatus* field-collected at 3 m (A,B) and 20 m (C,D) depths. Representative immunoblots are shown for each acclimatization temperature. Values are means ± SD; n = 5 preparations from different mussels. *$p < 0.05$ compared with individuals collected on 25 November 2008. Hsp: heat shock protein

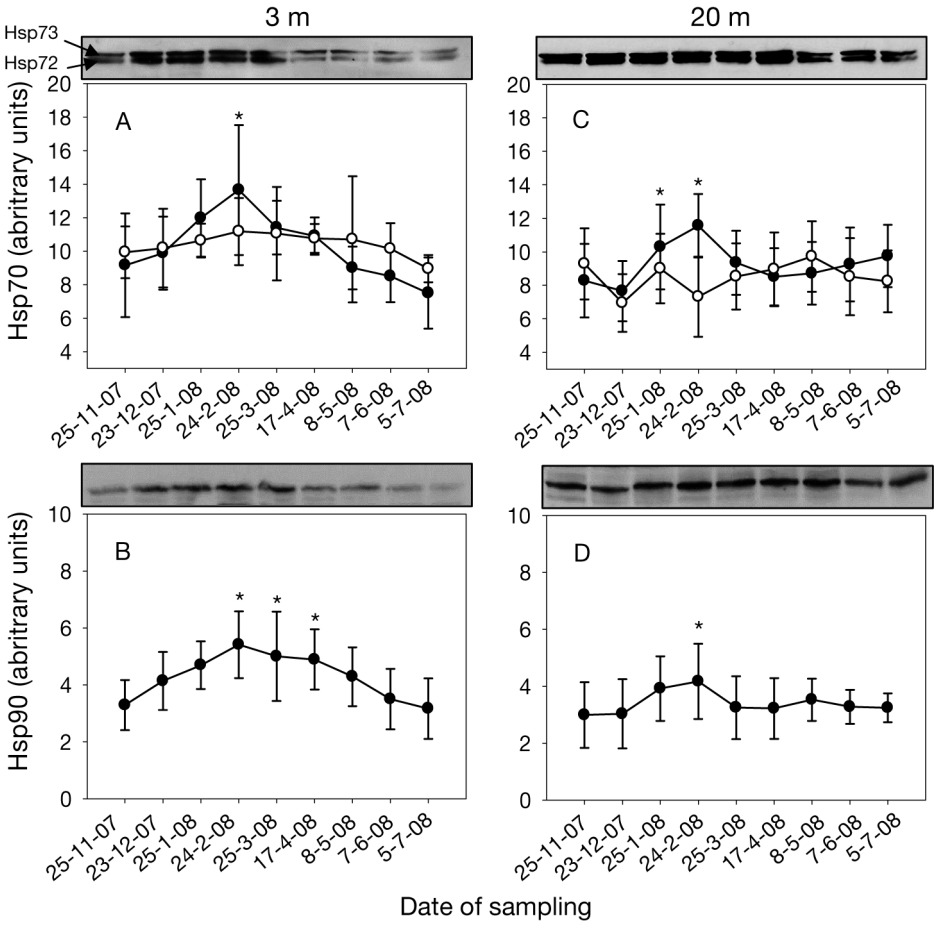

Fig. 4. Seasonal changes in the levels of inducible Hsp72 (●) and constitutive Hsp73 (○) and Hsp90 in the posterior adductor muscle of *Modiolus barbatus* field collected at 3 m (A,B) and 20 m (C,D) depth. Representative immunoblots are shown for each acclimatization temperature. See Fig. 3 for further explanations

depth in the mantle, Hsp72 expression was found to be a factor of significant variance between the samplings ($p < 0.0001$), while this was not observed for Hsp73, either at 3 m ($p = 0.2638$) or at 20 m ($p = 0.1$). More specifically, the expression of Hsp72 at 3 m in this tissue increased progressively after mid-December 2008, leading to the highest levels at the end of February 2009. Thereafter, the expression of Hsp72 decreased but was induced again in early June, and remained at high levels by mid-July (Fig. 3A). At 20 m depth, the changes in Hsp72 levels in the mantle of *M. barbatus* during winter were similar to those observed at 3 m ($p = 0.0082$). However, at 20 m a statistically significant induction of Hsp72 occurred only in mid-July (Fig. 3C). In the PAM a significant induction in Hsp72 was observed mainly during winter at both 3 and 20 m depths ($p = 0.0012$) and $p < 0.0001$, respectively) (Fig. 4). In contrast to the mantle, however, no induction of Hsp72 was observed during summer at both depths.

The seasonal expression of Hsp90 was similar to the expression patterns of Hsp70 in mantle and PAM at both water depths (Figs. 3 & 4, respectively). The statistical analysis revealed that the expression of Hsp90 causes significant variation between the samplings in all cases.

Seasonal changes in the levels of ubiquitin conjugates

The seasonal patterns in the levels of ubiquitin conjugates in mantle and PAM are shown in Fig. 5. There were no significant changes in ubiquitin conjugate levels in the mantle of *Modiolus barbatus* at 20 m and in PAM at both 3 and 20 m. In these cases the p values reported were 0.12, 0.21, and 0.96, respectively. However, irreversibly damaged proteins were detected in the mantle at 3 m, but only after mid-June (Fig. 5A, $p < 0.0001$).

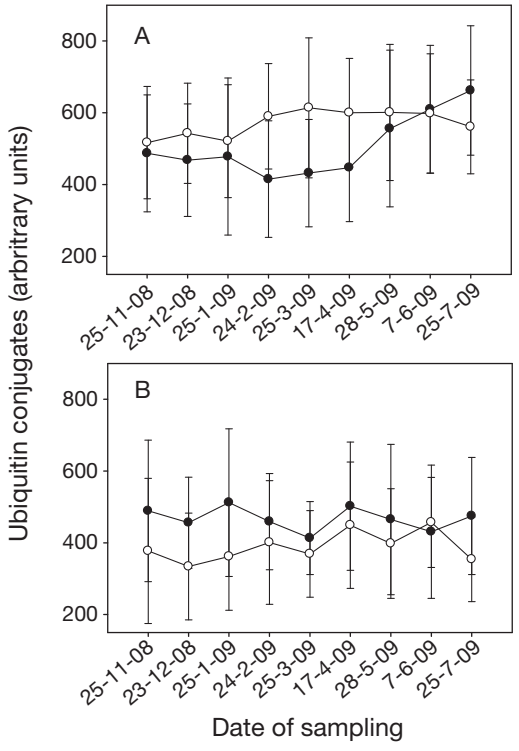

Fig. 5. Seasonal changes in the levels of ubiquitin conjugates in the (A) mantle and (B) posterior adductor muscle of *Modiolus barbatus* at 3 m (●) and 20 m (○) depth. See Fig. 3 for further explanations

Seasonal changes in metabolic indicators

The V_{max} of HK and aldolase in the mantle of *Modiolus barbatus* remained unchanged during all seasons and depths (Fig. 6). In contrast, the V_{max} of PFK displayed a progressive increase after the end of May (Fig. 6C) at both depths, and the V_{max} of PK displayed 2 peaks, one in winter and a second one in the middle of July (Fig. 6G). The p values accompanying the repeated ANOVA revealed that both PFK and PK maximal activities are factors of significant variation between the different samplings ($p < 0.0001$ in all cases).

In PAM, the V_{max} of HK, PFK, and PK increased significantly during winter at both depths (Fig. 6B,D,H, respectively, $p < 0.0001$ in all cases) and decreased progressively during spring. No activation of enzymes was observed during seasonal warming.

The seasonal changes in the levels of succinate in the mantle and PAM are given in Fig. 7. In the mantle, succinate accumulated at the end of May at both depths. Further accumulation of succinate was recorded during mid-summer (Fig. 7A). The p values for both depths ($p < 0.0001$) indicate the significance of succinate concentration as a factor of variation between different samplings. In PAM ($p < 0.001$), such an accumulation took place mainly during July at both depths (Fig. 7B). In general, succinate levels increased more markedly in the mantle than in PAM during mid-summer (Fig. 7).

The pattern of changes in the content of chl *a* in the Thermaikos Gulf for the period 2000–2009 is shown in Fig. 8. Chl *a* was highest between February and April, and lowest during the warm summer months.

DISCUSSION

The HSR in *Modiolus barbatus* displayed a peak in February, when the species experienced low values of ambient temperature in the Thermaikos Gulf (Fig. 2), supporting the need for chaperoning. However, the levels of ubiquitin conjugates did not indicate increased amounts of irreversibly damaged proteins in the tissues of *M. barbatus* (Fig. 5), suggesting that *M. barbatus* may be more tolerant to low temperatures than other bivalves such as *M. galloprovincialis* (Dutton & Hofmann 2008). It has been reported that cold-shock resistance differs among acclimated European mussel populations and might depend on their genetic backround and thermal history (Jansen et al. 2007). Low succinate levels during winter (Fig. 7) indicate that *M. barbatus* does not reach the low critical temperatures (T_c) during the coldest period of year (cf. Pörtner 2001, 2002). It remains to be explored whether the HSR induced by the low temperatures in *M. barbatus* is elicited by hypoxic conditions (Kassahn et al. 2009) developing between lower pejus and critical temperatures during winter. In general we interpret our present findings within the framework of the concept of OCLTT. This concept is the only integrative concept currently available, which would allow us to link our biochemical findings to ecosystem-level observations, across levels of biological organisation. The concept is presently supported by findings in species from various phyla; it helps to explain ecosystem level phenomena from a physiological angle, and thus provides a suitable framework for our present interpretations.

Seasonal cooling caused an increase in the activity of the key glycolytic enzyme PK (Fig. 6G) indicating that *Modiolus barbatus* might compensate for the exposure to low temperatures by increasing the glycolytic potential. This metabolic response is in line with that observed in the congeneric *Modiolus modiolus* (Lesser & Kruse 2004) and in the mussel *Mytilus galloprovincialis* (Ibarguren et al. 1990, Ioannou et al. 2009), and with the proliferation of mitochon-

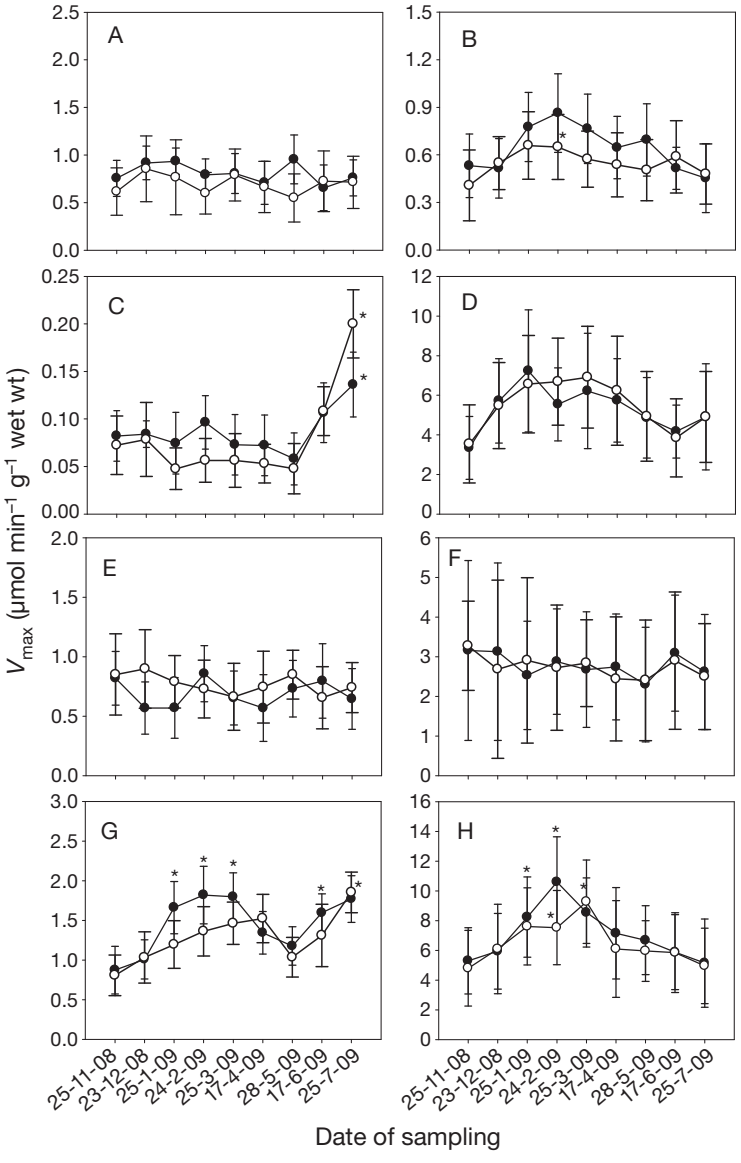

Fig. 6. Seasonal changes in the V_{max} (µmol min^{-1} g^{-1} wet wt) of (A,B) the glycolytic enzymes hexokinase, (C,D) phosphofructokinase, (E,F) aldolase, and (G,H) pyruvate kinase from mantle (left column of graphs) and posterior adductor muscle (right column of graphs) of *Modiolus barbatus* field-collected at 3 m (●) and 20 m depths (○). See Fig. 3 for further explanations

dria found in tissues of cold-acclimated active marine invertebrates (Sommer & Pörtner 2002). During cold-induced hypoxia, the level of oxidative stress may rise, causing a compensatory expression of antioxidant enzymes (Viarengo et al. 1995, Abele et al. 2002, Lesser & Kruse 2004, Lurman et al. 2010). These processes are known to stimulate the expression of Hsps (Kassahn et al. 2009).

A physiological process that has been associated with the HSR during winter is the reproductive activity of mussels (Meistertzheim et al. 2009). In general the reproduction in bivalve molluscs follows a seasonal cycle. Gametogenesis and energy storage occur in the mantle tissue during spring and summer, when a large shift in cell types is evident. The period between January and February is characterized by sexual repose in *Modiolus barbatus*. Early and late stages of gametogenesis were found between March and May, and spawning peaked from June until August (Mladineo et al. 2007).

The second HSR, initiated in the mantle of *Modiolus barbatus* at both 3 and 20 m depth in early May (8 May 2010), reached a maximum by mid-July (Fig. 3A,C, respectively). The water temperature in early May was 17.4°C at 3 m and 15.2°C at 20 m depth, and increased to 28.4°C at 3 m and 26.3°C at 20 m by mid-July (Fig. 2). The specimens transplanted to 3 m depth experienced temperatures approximately 2°C higher than those in their natural habitat (20 m), and surpassed the upper thermal threshold of HSR induction. Under these field conditions the inducible forms Hsp72 and Hsp90 reached peak expression in the mantle in mid-summer. Moreover, the accumulation of ubiquitin conjugates in the mantle of transplanted individuals after mid-June demonstrates that irreversible protein damage also occurred (Fig. 7), emphasizing the severity of the heat stress, which was uncompensated for despite the onset of the HSR in the mantle of *M. barbatus* in early May. While a previous laboratory study indicated a heat-shock induction temperature (T_{on}) between 22 to 24°C (Anestis et al. 2008), the present field study indicates that the HSR sets in at mean temperatures of ≤22°C in the field. The principles of OCLTT suggest that this may be related to the effect of elevated metabolic rate, inducing anaerobiosis and reducing thermal tolerance in the warmth (cf. Pörtner et al. 2010).

A close relationship may exist between gametogenesis, metabolic pattern and chaperoning. Guderley & Pörtner (2010) recently discussed that the enhanced oxygen demand of reproductive organs during gonadal development, and that gametogenesis might reduce oxygen availability to tissues, leading to a fall of residual mitochondrial oxidative capacity and of metabolic capacity in somatic tissues, and thereby to a reduction in the limits of heat tolerance associated with an earlier (i.e. at lower temperatures) onset of anaerobic metabolism. In support of this con-

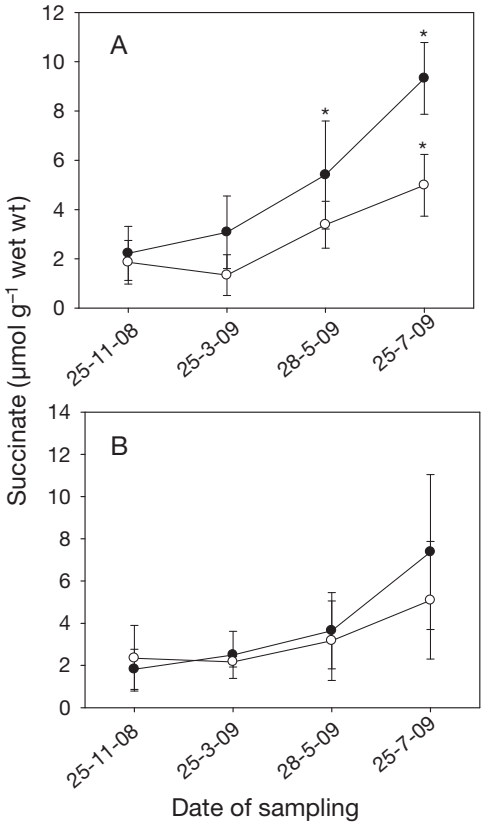

Fig. 7. Seasonal changes in the levels of succinate in the (A) mantle and (B) posterior adductor muscle of *Modiolus barbatus* field-collected at 3 m (●) and 20 m (○) depth. See Fig. 3 for further explanations

clusion, the levels of succinate increased significantly in the mantle of *Modiolus barbatus* at both 3 and 20 m in early summer (Fig. 7A) during the time the reproductive cycle was initiated. Similarly, Zandee et al. (1980) showed that succinate production is seasonally stimulated in *Mytilus edulis.* Ahmad & Chaplin (1979) reported that incorporation of $^{14}CO_2$ into succinate peaked in the middle of May. The authors suggested that the seasonal onset of anaerobic metabolism cannot be due to the effect of temperature and that it might be related to the energy demand of reproductive activity. During this period the glycogen content of tissues is high, and glycolytic rate is elevated. Our findings of increased activities of rate limiting glycolytic enzymes such as PFK and PK (Fig. 6C,G, respectively) are in line with this conclusion.

According to OCLTT, the shortening in oxygen supply caused by the elevated energy demand of reproduction would lead to lower limits of heat tolerance, reflected in an earlier onset of the hypoxic conditions that induce HSR and anaerobic metabolism. This conclusion is in line with recent publications (Gracey et al. 2008, Anestis et al. 2010) describing a close relationship between the onset of anaerobiosis and the HSR in bivalves. These relationships would also explain the findings by Rossi et al. (2006) that the marked reduction in the content of carbohydrates during gametogenesis (by enhanced glycolytic activity) parallels the induction of Hsp70 and Hsp90. The fact that the levels of succinate during mid-summer were twice as high in the mantle of *Modiolus barbatus* at 3 m than in those at 20 m depths might emphasize a close link between ambient temperature (limits), reproduction induced energy demand, reduction in aerobic capacity and the onset of the HSR, as well as the onset and exploitation of anaerobic metabolism. Moreover, the increase in the levels of Hsp90 mainly during mid-summer indicates a greater need for chaperoning during warming and coincides well with the accumulation of ubiquitin conjugates in the mantle (Figs. 3 & 5, respectively).

In contrast to findings in the mantle, the accumulation of succinate in PAM observed during mid-summer (Fig. 4) was not paralleled by induction of Hsps (Fig. 4). In line with our results, Lesser & Kruse (2004) reported lower levels of Hsp72 in the PAM of summer acclimatized *Modiolus modiolus* than in those acclimatized in winter. The data obtained in the present study contrast those obtained from earlier laboratory studies where PAM in *M. barbatus* exposed beyond 26°C displayed a potent induction of Hsp72 and Hsp90 (Anestis et al. 2008). Such differences between the effects of field acclimatization and laboratory thermal acclimation have previously been

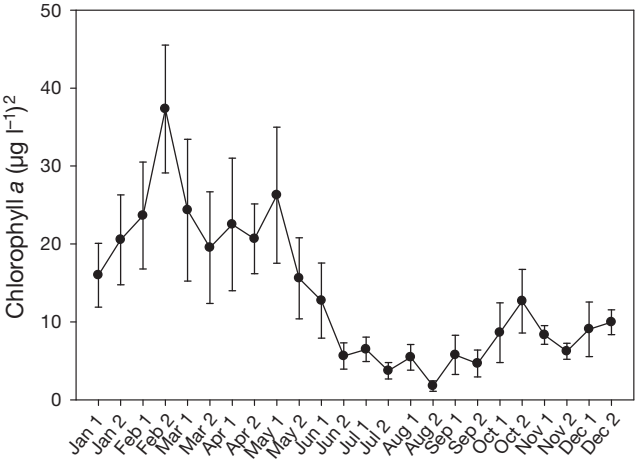

Fig. 8. Annual variation of chlorophyll *a* (chl *a*) concentrations (µg l^{-1}); mean square (x^2) chl *a* values and SD at 1 sampling station in the inner part of Thermaikos Gulf (NW Aegean Sea) at twice-monthly intervals from 10 yr data (2000–2009)

reported (Roberts et al. 1997, Somero 2010) and may well be related to the differential dynamics of thermal limits at tissue and whole organism levels.

A hypothesis that might explain the different phenomena in mantle and PAM in the field is the reallocation of energy from PAM with a low energy turnover to reproductive organs with a high energy turnover, especially during periods of low availability of food. Furthermore, tissues with reduced energy turnover may have a lower sensitivity to reduced oxygen supply than those with elevated energy demand. Synthesis of stress proteins is expensive in terms of cellular energy budget (Creighton 1991, Parsell & Lindquist 1993). Hawkins (1991) has estimated the costs of protein synthesis to constitute 20 to 25% of the energy budget of the blue mussel *Mytilus edulis*. Mobilization of carbohydrate reserves from the adductor muscle to support gonadal production during gametogenesis has been reported for several scallop species (Barber & Blake 1981, Epp et al. 1988, Brokordt et al. 2000a,b, Martínez et al. 2000).

Food availability (chl *a*) in the Thermaikos Gulf is similar to the rest of the Mediterranean Sea (Coma et al. 2009). As reported above, early and late stages of gametogenesis were found between March and May, and spawning peaked from June until August (Mladineo et al. 2007). Reproductive activity of *Modiolus barbatus* results in the depletion of carbohydrates and lipids such that gametogenesis may rely, for the most part, directly on the energy of ingested food (Mladineo et al. 2007). Consequently, *M. barbatus* has to face both the reduction in food and the gradual elevation of ambient temperature from spring to summer. Both may lead to constraints in energy allocation to reproductive organs and a further exacerbation of heat stress. Spawning activity at high temperature, in combination with reduced food availability, may thus be the main reason for the observed mass mortalities of mussels during summer (Worrall & Widdows 1984, Myrand et al. 2000). Interestingly, high activities of lysosomes are related to mass mortalities of mussels during summer (Tremblay et al. 1998), and have also been found in *M. barbatus* at 28°C (Dimitriadis et al. 2012). The latter coincides well with a reduction in PK activity in the PAM, and a reduced ability to gain energy from ingested food at temperatures beyond 26°C (Anestis et al. 2008, Ezgeta-Bali et al. 2010).

When integrating the molecular, metabolic, and physiological data for *Modiolus barbatus,* it becomes clear that the physiological performance of this species in its natural habitat peaks at about 26°C (Ezgeta-Bali et al. 2010), and this peak probably defines its upper limit of thermal tolerance. Nevertheless, we suggest that the availability of food at temperatures beyond 26°C would co-determine the biogeographical boundaries of *M. barbatus* in the Thermaikos Gulf by influencing the capacity of heat resistance. As reported elsewhere, energy demand and the allocation of energy to growth and reproduction may contribute equally to determining the distribution limits of mussels (Hofmann & Somero 1995, Pörtner et al. 2006). Consequently, the temperature tolerance range of an organism can be defined as the range of temperatures where body growth is positive and where the optimum temperature is defined as the temperature at which growth is maximal (Willmer et al. 2000). The optimum temperature is thought to be a reflection of a steady increase of metabolism with temperature on the one hand and a stabilisation or decline in ingestion rate at high temperatures on the other hand (Van der Veer et al. 2009).

The analysis of meteorological data since 1950 indicates increasing air temperatures in the Themaikos Gulf, especially after 1980 (Fig. 9). Moreover, records of sea temperatures for 10 yr show that during summer, mussels increasingly face stressfully high temperatures (Fig. 2). Although the relationship between weather and oceanographic conditions is not straightforward, long-term studies on seawater temperature conditions already show a clear-cut warming trend of coastal waters worldwide including the Mediterranean Sea (Levitus et al. 2000, Vargas-Yáñez et al. 2008, 2010). Consequently, our present data suggest that prolonged exposure of *Modiolus barbatus* to sublethal temperatures will occur and act directly via causing physiological stress. On the other hand, energy shortage will be involved in causing even higher rates of mortality as proposed for several benthic species (Coma et al. 2002) including clams (Weitere et al. 2009).

The energy shortage phenomenon supports an integrative view to understand how anomalous climatic conditions may have induced the mass mortality of some benthic species (Coma & Ribes 2003, Coma et al. 2009) (Fig. 10). The low availability of food associated with a loss in aerobic scope likely involves oxidative stress as indicated by field studies (Roméo et al. 2003, Regoli et al. 2004, Lesser et al. 2010) and transcriptomic analyses (Lockwood et al. 2010, Tomanek & Zuzow 2010). Such a cellular response might impair the ability of stressed organisms to grow and reproduce (Petes et al. 2007, 2008, Fearman & Moltschaniwskyja 2010), resulting in changes of species community structure and richness (Menge et al. 1997, 2008, MacLeod et al. 2004, Smith et al. 2006).

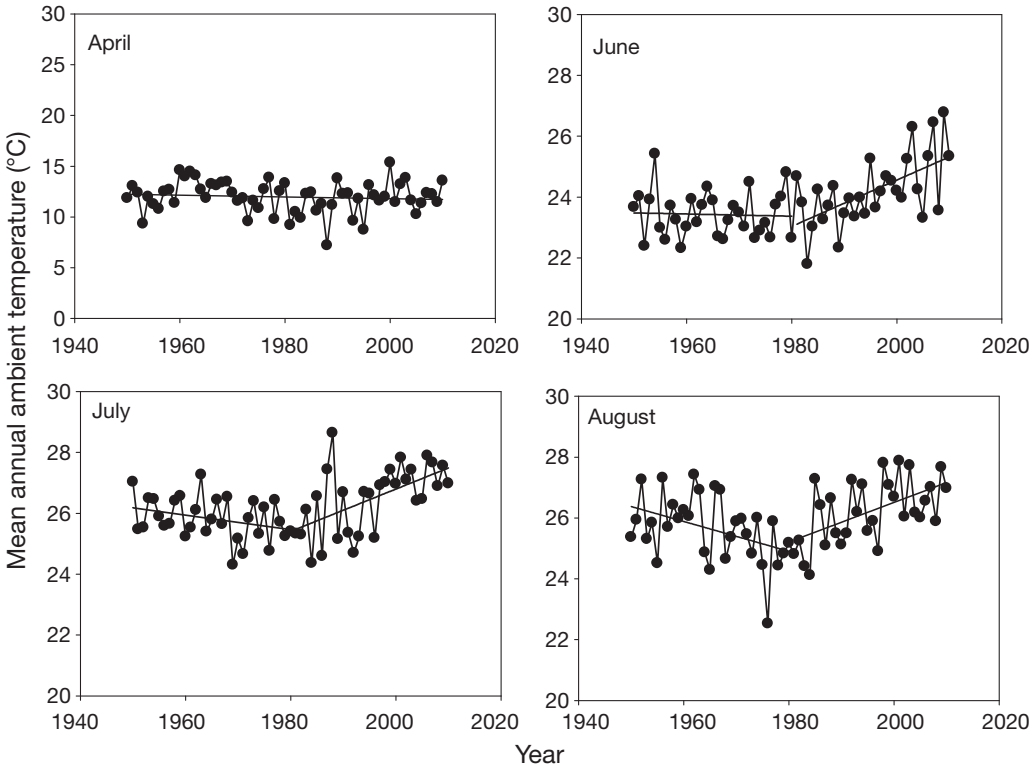

Fig. 9. Annual variation in ambient air temperature in the area of Thermaikos Gulf

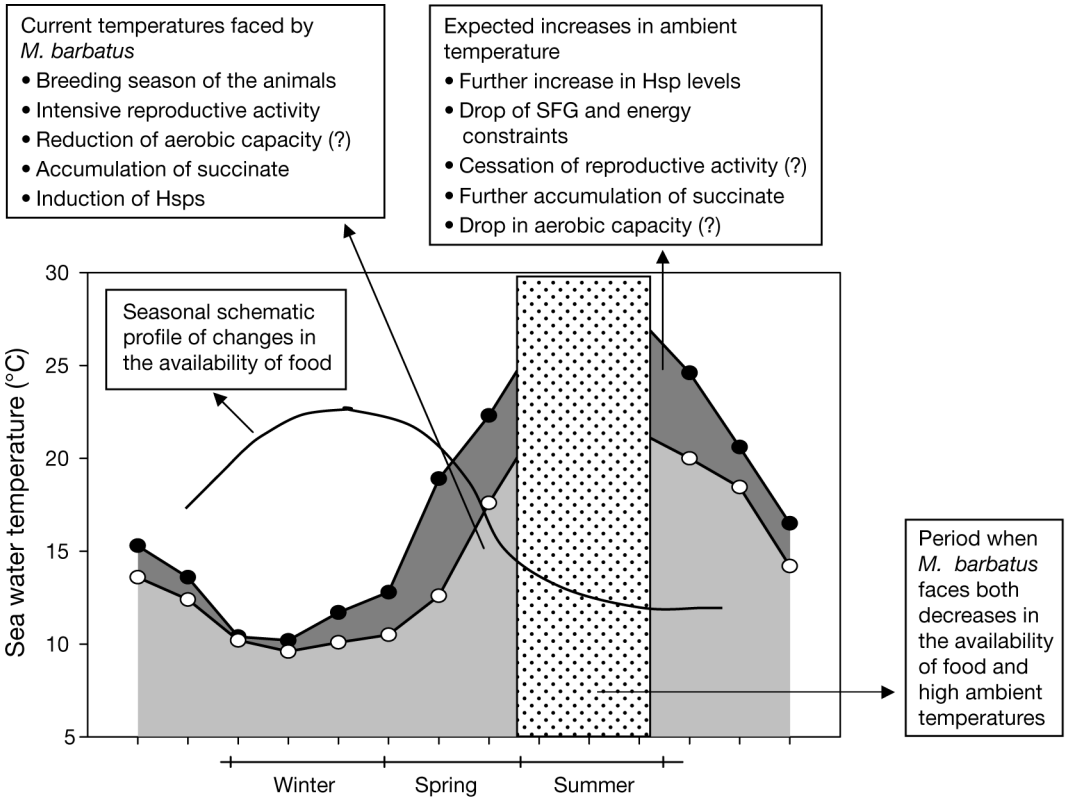

Fig. 10. Schematic presentation of expected impacts of global warming on the physiological performance of *Modiolus barbatus*. Depth: (●) 3 m; (○) 20 m. SFG: scope for growth

Quantifying the energetic costs and their role in establishing an energetics-based limitation on the highest position of a species in the intertidal zone is of great importance (Somero 2002) and represents an important challenge for future investigations. The OCLTT concept provides a matrix on which the impact of various stressors, including food limitation, on temperature-dependent aerobic scope and performance can be integrated (Pörtner 2010). Overall, the data provide a perspective of how the stresses experienced at various levels of biological organisation combine to reduce performance and enhance disturbance in energy budgeting and availability to life-sustaining processes, and, thereby, describe the climate sensitivity of life from an integrative point of view (Fig. 10).

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