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Temperature effects on key metabolic enzymes in *Littorina saxatilis* and *L. obtusata* from different latitudes and shore levels

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Abstract Effects of temperature on activities of key metabolic enzymes, citrate synthase (CS), NADP-dependent isocitrate dehydrogenase (NADP-IDH), aspartate aminotransferase (AAT), pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK), were studied in high and low shore *Littorina saxatilis* and in low shore *L. obtusata* from the temperate North Sea and the sub-arctic White Sea. It was found that adaptation of *L. saxatilis* and *L. obtusata* to life at different latitudes and/or shore levels involves constitutive changes in enzymatic activities, so that animals naturally adapted to (micro-) environments with lower mean temperatures (i.e. in the White Sea or at low shore levels) tend to have higher enzyme activities as compared to their counterparts from warmer habitats (e.g. the North Sea or high shore levels, respectively). This suggests metabolic temperature compensation in different populations/subpopulational groups of this eurythermal species. Activities of all five studied enzymes were modulated by adaptations to high shore life in *L. saxatilis*, whereas only NADP-IDH, PK and PEPCK had different activities in animals from different latitudes. Adaptation to high shore life also involved an enhanced potential for anaerobic energy production via the succinate pathway in *Littorina* spp. (measured as the ratio of PEPCK and PK activities), which increased in the order: low shore *L. obtusata* ≤ low shore *L. saxatilis* < high shore *L. saxatilis*. Possibly, an increased anaerobic capacity is advantageous for snails at high shore levels, where they may experience limited oxygen access during prolonged air exposure. In contrast, the activation energies of the respective enzymes did not reveal a consistent pattern of variation and were similar in animals from different latitudes and shore levels. Arrhenius breakpoint temperatures (ABT) of the studied enzymes characterising a change in the thermal properties of the protein were found well within the average highs of ambient temperatures (20–35°C). Denaturation temperatures (T_d), indicating heat inactivation of the protein, were close to the environmental extremes experienced by *L. saxatilis* during summer low tide. These findings suggest that the metabolic machinery of this eurythermal species may function close to its physiological limits during summer low tide, especially at high shore levels.

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Introduction

Temperature is an important environmental factor influencing all life functions of an organism through changes in the rates of biochemical and physiological processes and in the stability of biomolecules (reviews: Hochachka and Somero 1973; Hoffmann 1983; Prosser 1991). Adaptation to the thermal environment is recognised as one of the evolutionary mainstreams and comprises the organism's ability to adjust metabolism on both short-term and evolutionary time scales (Hochachka and Somero 1973; Prosser 1991; Clarke 1998). Changes in the kinetic characteristics of enzymes reflect differences in metabolic regulation and are inevitably involved in adaptation and acclimation to ambient temperature (Somero 1975, 1978, 1995; Lagerspetz 1987; Vetter and Buchholz 1998). Hence, the analysis of temperature-dependent changes in enzyme structure and function is traditionally used as a powerful tool in studies of temperature acclimation and adapta-

tion of animals, particularly ectotherms, which are especially prone to direct temperature effects of their environment.

There is a continuously growing body of data concerning metabolic adaptations on the molecular (including enzymatic) level in different ectotherms, especially in those inhabiting extreme environments (reviews: Clarke 1998; Somero 1998; Pörtner et al. 1999, in press). However, the frequent absence of closely related species at high and low latitudes (Eastman 1993) makes comparative work on metabolic adaptations in cold- and warm-adapted organisms very difficult. As a result, temperature effects per se are often confounded with interspecific differences in other traits, which may play much stronger roles in governing metabolic rates in general and enzymatic activities in particular than the one played by temperature (Pierce and Crawford 1997a,b; Somero 1998). To overcome this problem, one should ideally choose a group of closely related species with known phylogeny or a single species with a wide geographical distribution. Such studies have been quite rare (e.g. Pierce and Crawford 1997a,b; Sommer et al. 1997; van Dijk et al. 1999) due to difficulties with finding suitable animal models. The intertidal gastropod *Littorina saxatilis* chosen for the present study is an example of a uniquely convenient object in this respect. Its distribution area covers $>50°$ by latitude, ranging from North Africa up to Svalbard (Reid 1996). This allows comparison of warm- and cold-adapted populations within an ecologically, morphologically and genetically cohesive unit such as a single species. Moreover, *L. saxatilis* is characterised by an exceptionally wide vertical distribution. It occupies the range from the upper subtidal to the supratidal splash zone and thus encounters environments with greatly differing temperature and humidity regimes even within a single population (Sokolova et al. 2000b).

The aim of the present investigation was to study the effects of temperature on the activity of key metabolic enzymes in *L. saxatilis* from different shore levels from the sub-arctic White Sea and the temperate North Sea. These two study areas differ greatly with respect to their climatic conditions, especially temperature regime. Subarctic White Sea populations of *L. saxatilis* experience extreme seasonal variations of temperature, from freezing (below –1.5°C in brackish seawater to temperatures of from –10°C to +15°C in air) in late autumn and winter to highs of from +15°C to +20°C and more in summer (Babkov 1998; Sokolova et al. 2000b). In contrast, both temperature fluctuations and temperature extremes are much less pronounced in the North Sea and range from 3–6°C in winter to 15–18°C in summer (Hickel et al. 1997; R. Scharek et al., unpublished data available at http://www.pangaea.de). Additionally, environmental parameters change strongly along the vertical shore gradient of the intertidal within a single population of *L. saxatilis*. Generally, high shore levels are characterised by high temperature fluctuations and temperature extremes and by prolonged periods of desiccation and oxygen deprivation during low tide, whereas environmental conditions are milder and more stable at low shore levels (Sokolova et al. 2000b). Thus, comparison of temperature effects on enzyme activities in high versus low shore and White versus North Sea (sub-) populations of the periwinkles provides a useful model allowing to identify potentially adaptive changes in enzymatic function in *Littorina* from different thermal environments on vertical and latitudinal scales.

For the present study, five enzymes representative of different metabolic pathways were chosen: citrate synthase (CS), NADP-dependent isocitrate dehydrogenase (NADP-IDH), aspartate aminotransferase (AAT), pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK). Citrate synthase is a key regulatory enzyme in the tricarbonic acid (TCA) cycle (Krebs and Johnson 1937) and was chosen as an indicator of aerobic capacity of animals. NADP-dependent isocitrate dehydrogenase is suggested to adopt a key role in a futile mitochondrial substrate cycle which is likely to be relevant to the control of respiration and mitochondrial proton leakage (Sazanov and Jackson 1994; Pörtner et al. 1999, in press). Pyruvate kinase and phosphoenolpyruvate carboxykinase are assumed to compete for a common substrate, phosphoenolpyruvate, channelling it to the TCA cycle via pyruvate (PK) and acetyl-CoA or to mitochondrial anaerobic pathways via oxaloacetate (PEPCK) and malate (Saz 1971; Bryant 1975). Thus, a ratio of specific activities of PK and PEPCK is considered as a good indicator of the potential anaerobic capacity of an animal (de Vooys 1980; Bowen 1984; Simpendörfer et al. 1995). Aspartate aminotransferase is involved in amino acid metabolism and suggested to play an important role during early anaerobiosis, together with alanine aminotransferase, by channelling aspartate to the mitochondrial production of succinate (de Zwaan 1991), as well as during post-anaerobic recovery, by supplying aspartate for the regeneration of AMP from IMP (Mommsen and Hochachka 1988). Thus, the chosen set of enzymes should give a good overview of aerobic and anaerobic capacities of *L. saxatilis*. For comparative purposes, PK and PEPCK activities were measured in a closely related species, *L. obtusata* from the North and White Seas, which covers a slightly more restricted latitudinal range and occupies only mid- to low shore levels (Reid 1996).

For the five studied enzymes, maximal activities (V_{max}) were measured at different temperatures in *L. saxatilis* and *L. obtusata* from different latitudes and/or shore levels. V_{max} was chosen because it is a mechanistically and physiologically important parameter setting upper limits to flux at specific steps in a biochemical pathway (Pette and Hofer 1980; Suarez et al. 1996). The aims of this study were:

1. to compare maximum enzyme activities in animals naturally adapted to different temperature regimes, from the viewpoint of the potential temperature compensation of enzymatic activities;

2. to analyse the relationships between Arrhenius breakpoint temperatures (ABT) and denaturation temperatures (T_d), characterising temperature-induced changes in the kinetic properties of the respective enzymes and the onset of enzymatic dysfunction, respectively, and to compare ABT and T_d values in snails from different thermal environments in order to recognise whether temperature adaptation involved changes in the thermal resistance of key metabolic enzymes;
3. to estimate the effects of temperature adaptation on activation energy (E_a) of reactions catalysed by key metabolic enzymes in the periwinkles;
4. to assess the potential role of increased enzymatic anaerobic potential (estimated as the ratio of PK over PEPCK activities) in the adaptation of *L. saxatilis* to life at high shore levels, where the animals may experience prolonged periods of oxygen deprivation during low tide.

The present study is part of a broader project addressing the physiological, biochemical and populational mechanisms of adaptation of *L. saxatilis* to environmental stresses (temperature, salinity, oxygen deficiency) and investigating the basis of the extremely eurybiont mode of life of this species.

Materials and methods

Collection sites and animals

Periwinkles (*Littorina* spp.) were collected in the intertidal zones of the Kandalaksha Bay of the White Sea (66°20′N; 33°39′E) in September 1999 and of Helgoland Island in the North Sea (54°11′N; 7°53′E) in October 1999 and April 2000. Most experiments were performed on animals collected in autumn 1999. Samples collected in spring 2000 were used for an examination of the heat resistance of NADP-IDH and CS during acute temperature stress in vivo (see below).

The two study areas are separated by $>10°$ latitude and differ greatly in their climatic conditions. Average monthly temperatures of the surface waters range between –0.9°C and 13.8°C (extremes of –1.5°C and 19.3°C, annual average 4.3°C) in the sub-arctic White Sea area (Babkov 1998) and between 4.3°C and 17.9°C (extremes of 3.4°C and 19.0°C, annual average 10.4°C) at the temperate North Sea site (Hickel et al. 1997; R. Scharek et al., unpublished data available at http://www.pangaea.de). The monthly average temperature of surface water never drops below zero, and no ice cover is formed at the North Sea site. In contrast, at the White Sea the ice cover may persist for ca. 6 months $year^{-1}$, and the monthly average temperature of surface water is negative for 5–6 months (Babkov 1998). Hence, the periwinkles from the sub-arctic White Sea could be considered cold-adapted compared to their North Sea counterparts. During autumn sampling, the surface water temperature was 10–12°C both at the White Sea and North Sea sites. In April 2000 the surface water temperature ranged between 4.5°C and 6.5°C at the North Sea site. The tidal range is 1.8–2.2 m at the White Sea sampling site and 1.6–2.8 m at the North Sea one.

In each study area, adult *L. saxatilis* (6–11 mm shell diameter) were collected from two contrasting shore levels: (1) low shore – from small stones and gravel patches in the low intertidal, within the brown macrophyte belt (*Ascophyllum nodosum* and *Fucus vesiculosus* in the White Sea and *F. vesiculosus* and *F. serratus* in the North Sea); (2) high shore – in the narrow belt along the upper limit of *L. saxatilis* distribution on large boulders and/or rocks. Adult *L. obtusata* (10–15 mm shell diameter) were collected in September–October 1999 at the low shore level in the White and North Seas. This species is absent from high shore levels in these areas.

It has previously been shown that high and low shore habitats of White Sea *L. saxatilis* differ greatly in their environmental conditions. The high shore level is characterised by prolonged air exposure (75–90% of the total time), high temperature fluctuations and temperature extremes of up to 47°C (body temperature, mean 30–35°C) during summer low tides (Sokolova et al. 2000b). In contrast, the low shore is only exposed for 10–40% of the time. During summer low tides, their body temperature is on average 10–15°C lower than in their high shore counterparts and usually does not exceed 35°C (Sokolova et al. 2000b). Temperature fluctuations experienced by snails are also smaller at low shore levels compared to the high shore in the White Sea (Sokolova et al. 2000b).

Although high and low shore habitats at the North Sea sampling site also differ greatly with respect to the duration of air exposure (I. Sokolova, personal observations), differences in temperature fluctuations and extremes between the two shore levels are probably less pronounced than at the White Sea, because the high shore level at the North Sea sampling site is never exposed to direct sunlight due to overshadowing by steep rocks (Janke 1997).

It has been previously shown that despite slight shifts in distribution depending on the weather, wave action, etc., the periwinkles maintain their vertical position in the intertidal during normal foraging behaviour, as well as after natural or artificial displacement (Smith and Newell 1955; Newell 1958; Johannesson et al. 1995a; Erlandsson et al. 1998). Thus, we assume that the studied subpopulations of *L. saxatilis* from different shore levels are at least partially isolated due to their behavioural preferences.

Snails were transported alive to the Alfred-Wegener-Institute (AWI) in Bremerhaven, Germany, and acclimated in aquaria with recirculated seawater, set to the salinity of the respective sampling sites (33.2–33.4‰ for the North Sea and 24.6–24.7‰ for the White Sea) for a minimum of 3 (usually 4–6) weeks prior to experimentation. Acclimation temperature was chosen close to the respective field temperature at the time of collection and was 12.5–13.0°C for autumn samples and 4.9–5.1°C for spring samples. Water was changed once every 2 weeks. Brown macroalgae (*F. vesiculosus*) from Helgoland Island were added as a food source ad libitum. No mortality was detected during transportation, and only minimum mortality ($<5\%$) was detected during laboratory acclimation.

Survey of maximal activities and heat stability of enzymes

For the determination of the temperature dependence of enzyme activities, animals collected in autumn 1999 at different shore levels of the White and North Seas were used. To avoid the possible influence of body size on enzyme activities within each population, only animals of similar size were chosen (shell diameter of 8.5–9.5 mm and 6–7 mm from the North and the White Sea, respectively). Animals were taken from the acclimation aquaria, quickly dissected and checked for trematode infestation. Infested animals were discarded, and foot muscle of seven to ten uninfested ones was ectomised, blotted dry and immediately homogenised in 1:10 (w/v) of the respective homogenisation buffer (see below) using tight-fit glass homogenisers (Glas-Col, Terre Haute, USA) continuously kept on ice. After homogenisation extracts were sonicated for 2 min at 0°C (Branson sonifier, Heinemann, Germany) and centrifuged for 10 min at 16,000 *g* and 2°C (Eppendorf 5402 centrifuge, Eppendorf-Netheler-Hinz, Germany). The supernatant was collected and used for the determination of enzyme activities. Enzyme activities were measured by recording changes in absorbance at 412 nm (CS) or 339 nm (other enzymes) with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Sweden) equipped with a thermostatted cuvette holder connected to a Haake DC thermostat (Haake, Germany). The temperature of the assay mixture was additionally controlled by a thermometer inserted in the cuvette. Activities of CS, NADP-IDH and AAT were determined in a

temperature range between 0°C and 50–60°C, until considerable heat inactivation of the enzyme was observed. PK and PEPCK activities were determined at 10, 20 and 30°C. At higher temperatures (>30°C) the time used to bring the assay mixture to the desired temperature was standardised (20 min) to reduce potential variation due to variable exposure times at sub-denaturating and denaturating temperatures.

For an examination of the heat resistance of the key mitochondrial enzymes CS and NADP-IDH during acute thermal stress in vivo, North Sea *L. saxatilis* collected in spring 2000 were used. Subsamples of 10–15 snails (8.5–9.5 mm shell diameter) from high and low shore levels were randomly placed into perforated plastic cages. Cages were put in a jar with natural seawater of 34‰ at room temperature (20–25°C) for 25–30 min. The jar was then mounted in a water bath (Haake DC, Haake, Germany) with a manually controlled heater, and the temperature was raised in steps of 1°C per 5 min (±2 s and ±0.1°C) (McMahon and Britton 1985; Sokolova et al. 2000b). The jar water was vigorously aerated to maintain temperature uniformity, and the temperature was continuously monitored by a thermometer inserted into the experimental jar. Two cages (one for each shore level) were withdrawn once the desired temperature of 45°C was reached. Homogenates were prepared from foot muscles of individual periwinkles as described above and used for the determination of the maximal activities of CS or NADP-IDH at 20°C. Three periwinkles from each shore level were used for homogenates, and the rest of the experimental animals was transferred into plastic jars with seawater at room temperature and allowed to recover for 2 h before scoring for mortalities. Animals were considered alive if they resumed activity and/or the contractility of foot muscle in response to mechanical stimuli. Control specimens were taken directly from the acclimation aquaria, and tissue extracts were prepared as described above. The experiment was repeated until enzyme activities were determined for a sufficient number of experimental animals (at least five individuals from each shore level).

Activities of citrate synthase (CS, EC 4.1.3.7), NADP-dependent isocitrate dehydrogenase (NADP-IDH, EC 1.1.1.42), aspartate aminotransferase (AAT, EC 2.6.1.1), pyruvate kinase (PK, EC 2.7.1.40) and phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.31) were measured spectrophotometrically in crude extracts of *L. saxatilis* and *L. obtusata* foot muscles according to the following studies (CS: Sidell et al. 1987; NADP-IDH: Goldberg and Ellis 1987; AAT: Rej and Horder 1987; PK and PEPCK: Simpendörfer et al. 1995). All substrate concentrations were specifically adjusted for the determination of maximum activities. Artifactual breakpoints in Arrhenius plots of enzyme maximal activities versus temperature might arise from temperature-dependent changes in the Michaelis constant (K_m) (Silvius et al. 1978). Therefore, substrate concentrations were adjusted at low, intermediate and high temperatures, and optimal substrate concentrations ensuring V_{max} were chosen.

All chemicals were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany), Merck (Darmstadt, Germany) or Hoffmann-La Roche (Grenzach-Wyhlen, Germany) and were of analytical grade.

Derived parameters and statistics

Enzyme activities and the ratio of PK to PEPCK activities in periwinkles from different seas and shore levels were compared by mixed-model ANOVAs. Factors "Shore level" and "Sea" were treated as fixed, factor "Temperature" was treated as random. Prior to analysis, assumptions of normal distribution of the studied variables and homogeneity of variances were tested. If any of the assumptions was violated, the data were log-transformed; this resulted in significantly improved normality and homogeneity of variances. As the first step of an analysis, three-way ANOVAs were performed estimating effects of the factors "Sea", "Shore level" and "Temperature". For CS, NADP-IDH and PEPCK, three-way ANOVA detected significant effects of "Sea"×"Temperature" or "Sea"×"Shore level" interactions on activities of the respective enzymes, thus preventing direct between-sea comparisons in a single ANOVA design. For these enzymes, two-way mixed-model ANOVAs were employed to analyse effects of factors "Sea" and "Temperature" for animals from the same shore level or "Shore level" and "Temperature" for snails from the same sea. Tukey's honest significant deviance (HSD) test for unequal n was used as a method of post hoc comparison, and the least squares method was employed for planned comparisons (Sokal and Rohlf 1995). Based on graphs and multiple comparisons, the temperature of heat inactivation (i.e. denaturation temperature, T_d) was determined for each enzyme as the temperature point beyond which an increase in temperature leads to a significant decrease in enzyme activity. It should be kept in mind that this method gives only a rough approximation of T_d due to large temperature steps (5°C).

The energy of activation (E_a) was determined from an Arrhenius plot, i.e. $\log V_{max}$ versus $1/T$ (K^{-1}). The apparent E_a value was calculated from the slope of the plot obtained by the least squares linear regression (Sokal and Rohlf 1995). Arrhenius breakpoint temperature (ABT) at which a significant change in the slope (i.e. in E_a) occurs, was determined in a non-denaturing temperature range (e.g. at the temperatures below T_d) using an algorithm for fitting two-segmented linear regressions as described by Yeager and Ultsch (1989). Slopes of regression lines were compared according to Zar (1996). Fisher's exact test was used for comparison of mortality levels in high and low shore periwinkles during acute thermal stress (Sokal and Rohlf 1995). Data are presented as means ± standard errors if not indicated otherwise.

Results

Heat stability of key metabolic enzymes in *Littorina saxatilis*

In the three enzymes analysed, denaturation temperature (T_d) was 40°C or higher. Heat inactivation of CS and NADP-IDH were observed at similar temperatures (40–45°C) (Figs. 1, 2), whereas the thermal stability of AAT was considerably greater, and significant thermal inactivation of this enzyme occurred only above 50–55°C (Fig. 3). Activities of PK and PEPCK were measured in a temperature range between 10°C and 30°C and were found to increase significantly with temperature ($P < 0.001$), with no signs of thermal inactivation.

Denaturation temperatures of CS and AAT did not differ considerably between animals from different latitudes, but NADP-IDH was more heat stable in White Sea animals than in their North Sea counterparts ($T_d = 45$°C and 40°C, respectively). For CS and NADP-IDH in *L. saxatilis* from different shore levels, no significant differences were found in T_d or in residual enzyme activity at denaturing temperatures. In contrast, residual AAT activity at denaturing temperatures (55–60°C) was significantly higher in high shore periwinkles than in their low shore counterparts, from both the White Sea and the North Sea ($P < 0.02$), suggesting that this enzyme was thermally more stable in high shore snails.

Thermal inactivation of two key mitochondrial enzymes, CS and NADP-IDH (40–45°C), occurred close to the acute lethal limits of *L. saxatilis* determined in our previous studies (Sokolova et al. 2000b). This suggested that dysfunction of mitochondrial enzymes may elicit mortality in *L. saxatilis* under conditions of rapid acute heating, which may be expected during summer low tide in the intertidal (Sokolova et al. 2000b). In order to

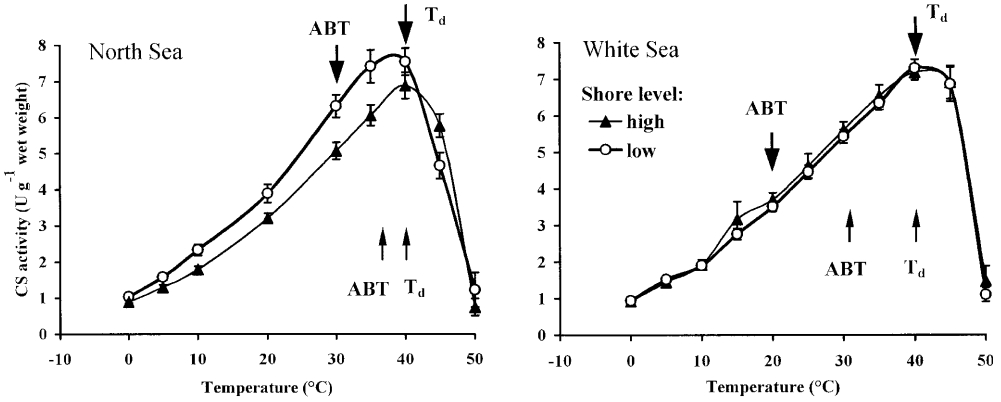


Fig. 1 *Littorina saxatilis*. Temperature dependence of CS activity in *L. saxatilis* from different latitudes and shore levels. *Vertical bars* represent standard errors; *absence of vertical bars* means that standard error is smaller than symbol size. For each temperature, $n=6$ (North Sea snails) or $n=5$–9 (White Sea ones), except 15°C and 25°C ($n=3$). Temperatures of heat denaturation (T_d) and Arrhenius breakpoint temperatures (*ABT*) are indicated by arrows (*thick arrows* high shore animals; *thin arrows* low shore ones)

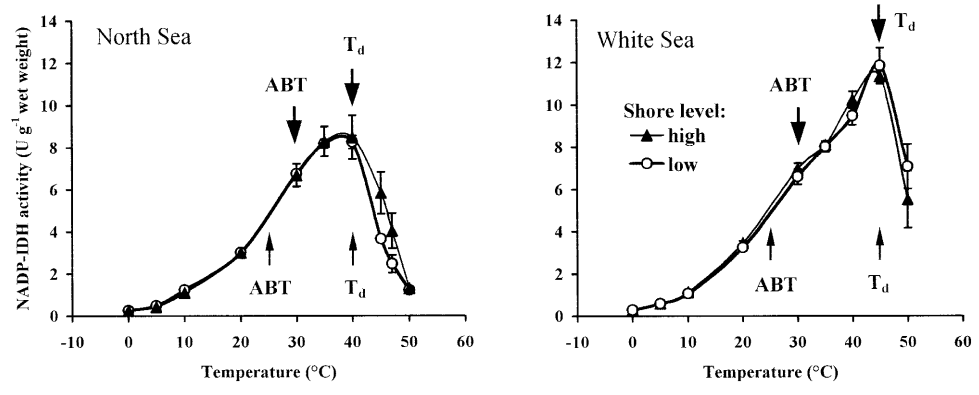
check whether enzyme activities are indeed impaired during heat stress in vivo, we compared activities of CS and NADP-IDH in North Sea animals collected in spring and acclimated to 5°C under control conditions and after acute heating to 45°C (at a rate of 1°C per 5 min). Our data show that heating to 45°C in vivo resulted in a significant decrease of CS activity in high and low shore snails ($F_{1,32}=18.76$, $P<0.001$), but only in a slight and insignificant decrease of NADP-IDH activity ($F_{1,23}=1.51$, $P=0.230$) (Fig. 4). As a result, the ratio of CS and NADP-IDH activities declined by 37–40% during acute heat stress. The decrease in CS activity coincided with an onset of mass mortality in the experimental animals (90% and 10% in low and high shore animals, respectively, $n=30$, Fisher's exact test: $P<0.001$).

Maximum activities of key metabolic enzymes in periwinkles from different latitudes and shore levels

Comparison of maximum enzyme activities was performed in a non-denaturing temperature range (below T_d) in snails from different latitudes and shore levels. It was found that maximum activities of key metabolic enzymes varied significantly, both on vertical and latitudinal scales, even after prolonged laboratory acclimation under the same conditions.

In general, White Sea animals tended to have higher activities of many key metabolic enzymes compared to their North Sea counterparts. Thus, V_{max} of PK, PEPCK and NADP-IDH were higher in White Sea *L. saxatilis* than in their conspecifics from the North Sea (PK: $F_{1,96}=165.6$, $P<0.0001$; PEPCK: $F_{1,96}=483.9$, $P<0.0001$; NADP-IDH: $F_{1,79}=8.95$, $P=0.004$ and $F_{1,89}=13.53$, $P=0.0004$ for low and high shore snails, respectively) (Figs. 2, 5). In contrast, maximum activities of CS and AAT did not differ significantly in White Sea and North Sea *L. saxatilis* (Figs. 1, 3). In *L. obtusata*, no significant differences in PK activity were found between animals from the White and the North Seas ($F_{1,33}=0.28$, $P=0.594$), whereas PEPCK activity was found considerably higher in White Sea *L. obtusata* than in their conspecifics from the North Sea ($F_{1,33}=32.8$, $P<0.0001$) (Fig. 6).

Fig. 2 *Littorina saxatilis*. Temperature dependence of NADP-IDH activity in *L. saxatilis* from different latitudes and shore levels. *Vertical bars* represent standard errors; *absence of vertical bars* means that standard error is smaller than symbol size. For each temperature, $n=9$ (North Sea snails) or $n=4$–6 (White Sea ones). Temperatures of heat denaturation (T_d) and Arrhenius breakpoint temperatures (*ABT*) are indicated by arrows (*thick arrows* high shore animals; *thin arrows* low shore ones)



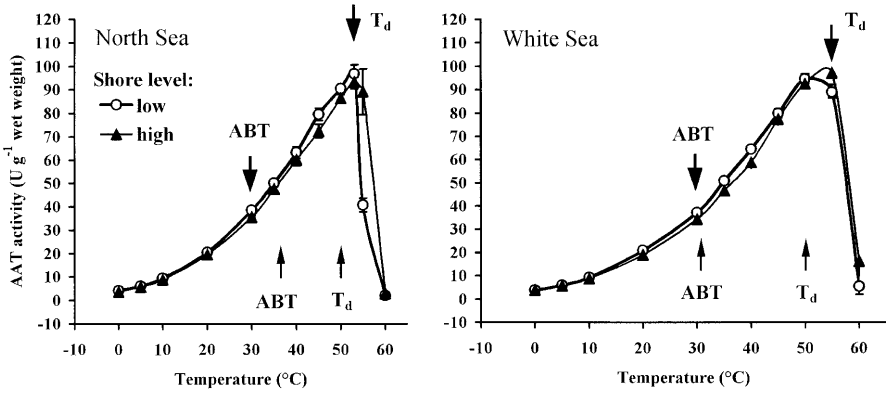


Fig. 3 *Littorina saxatilis*. Temperature dependence of AAT activity in *L. saxatilis* from different latitudes and shore levels. *Vertical bars* represent standard errors; *absence of vertical bars* means that standard error is smaller than symbol size. For each temperature, $n=6$. Temperatures of heat denaturation (T_d) and Arrhenius breakpoint temperatures (*ABT*) are indicated by arrows (*thick arrows* high shore animals; *thin arrows* low shore ones)

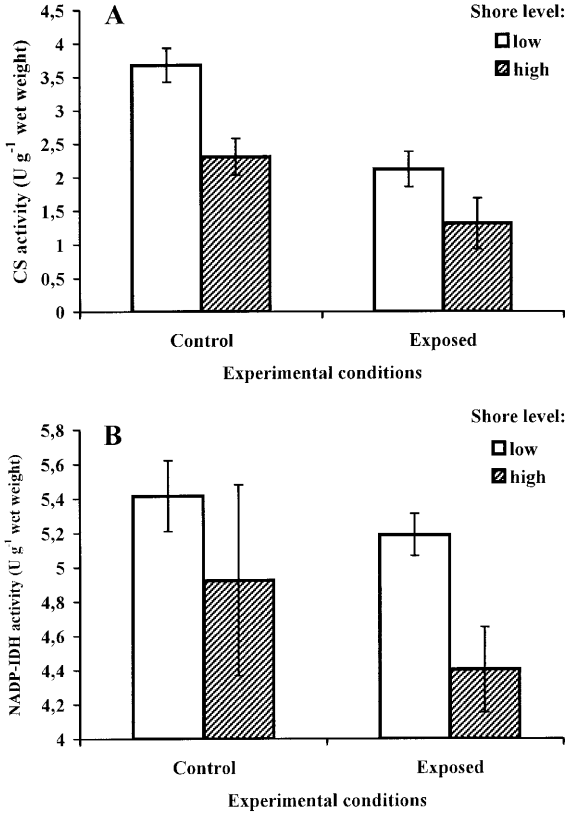


Fig. 4A, B *Littorina saxatilis*. Effect of acute heat stress in vivo on activity of key mitochondrial enzymes, CS (**A**) and NADP-IDH (**B**), in North Sea *L. saxatilis* from different shore levels. Animals were heated to 45°C at a rate of 1°C per 5 min. Enzyme activities were measured at 20°C. *Vertical bars* represent standard errors. At each temperature, $n=9$ for CS activities and $n=5$–10 for NADP-IDH activities

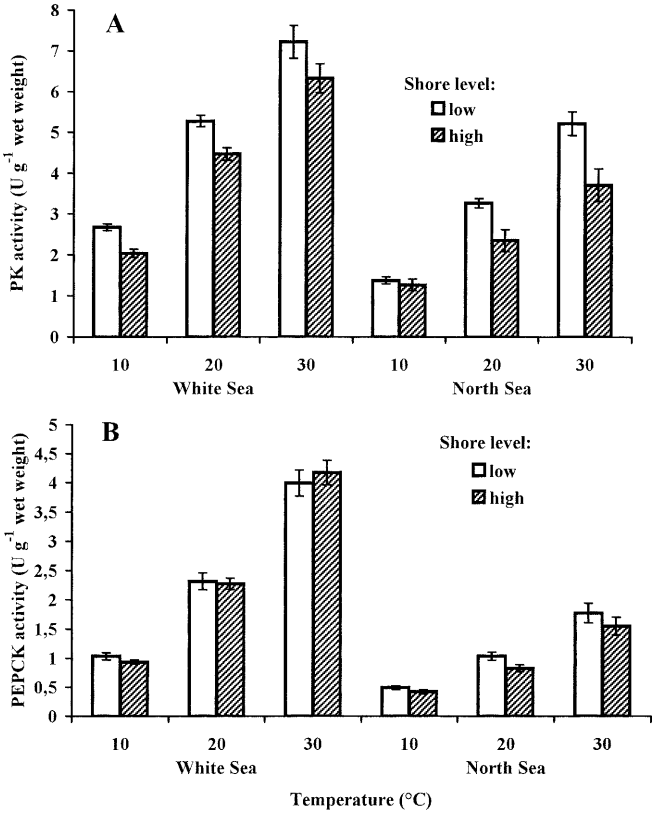


Fig. 5A, B *Littorina saxatilis*. Temperature dependence of PK (**A**) and PEPCK (**B**) activities in *L. saxatilis* from different latitudes and shore levels. *Vertical bars* represent standard errors. For each temperature, $n=9$

Comparisons between *L. saxatilis* subpopulations from different shore levels showed that V_{max} of key metabolic enzymes was usually higher in low shore animals than in their high shore counterparts at the respective temperatures. Thus, maximum activities of AAT and PK were significantly higher in low shore periwinkles as compared to high shore ones in the White and North Seas ($F_{1,8}=117.2$, $P<0.001$ and $F_{1,96}=33.1$, $P<0.001$, respectively). Maximum activities of CS and PEPCK were higher in low shore animals from the North ($F_{1,6}=84.39$, $P<0.001$ and $F_{1,48}=7.1$, $P=0.011$, respectively) although not from the White Sea ($F_{1,9}=2.11$, $P=0.18$ and $F_{1,48}=0.16$, $P=0.694$, respectively). For NADP-IDH, no effect of shore level on V_{max} was detected in North Sea animals ($F_{1,5}=4.92$, $P=0.077$). In White Sea periwinkles ANOVA suggested a significant effect of shore level on NADP-IDH activity

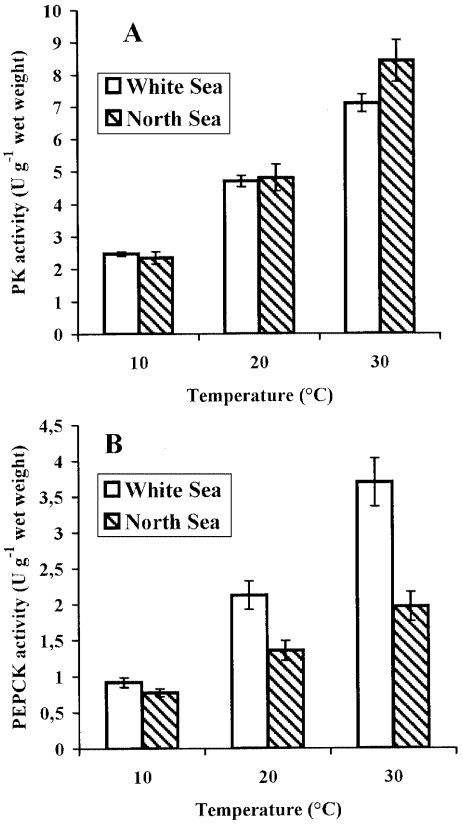


Fig. 6A, B *Littorina obtusata*. Temperature dependence of PK (**A**) and PEPCK (**B**) activities in *L. obtusata* from different latitudes. *Vertical bars* represent standard errors. For each temperature, $n = 6$ (White Sea animals) or $n = 7$ (North Sea ones)

($F_{1,7} = 8.31$, $P = 0.023$), though no consistent pattern of between-horizon differences could be seen (Fig. 2).

The comparison of spring to autumn samples of North Sea *L. saxatilis* revealed a significant effect of seasonal cold acclimation on activity of NADP-IDH but not on that of CS. Maximum activity of CS measured at 20°C was similar in spring animals (acclimated to 5°C) and in the autumn ones (acclimated to 12–13°C) ($F_{1,26} = 1.33$, $P = 0.259$; Figs. 1, 4). In contrast, NADP-IDH activity was nearly two times higher in cold-acclimated spring animals than in warm-acclimated autumn ones when measured at 20°C ($F_{1,21} = 24.72$, $P < 0.0001$; Figs. 2, 4). It should be noted that in a spring sample from the North Sea activities of CS and NADP-IDH were significantly higher in low shore periwinkles than in their high shore counterparts ($F_{1,32} = 13.57$, $P < 0.001$ and $F_{1,23} = 4.43$, $P = 0.046$ for CS and NADP-IDH, respectively).

Arrhenius breakpoint temperatures and activation energies

Breakpoint regression analysis showed that even within a non-denaturing temperature range there were significant changes in enzyme properties as indicated by significant changes in activation energy E_a above ABTs for CS, NADP-IDH and AAT (Table 1; Figs. 1, 2, 3). For the three enzymes studied with respect to ABT, changes in kinetic properties occurred in a relatively narrow temperature range – between 25°C and 35°C, but mostly at 30°C (Table 1). The activation energy of the respective enzymatic reactions significantly decreased beyond ABTs, by ca. 30, 40 and 70% for AAT, CS and NADP-IDH, respectively ($P < 0.05$ in each case). It is worth noting that the ABT of the most thermally resistant enzyme, AAT, was very similar to the ABTs of less resistant CS and NADP-IDH, suggesting that the change of enzyme properties beyond ABT is probably not related to heat denaturation of the respective enzyme. For PK and PEPCK activities, Arrhenius plots of log V_{max} versus $1/T$ (K^{-1}) were linear over the studied temperature range (10–30°C), and no ABTs were detected.

No consistent pattern could be revealed with respect to the variation in the ABTs of CS, NADP-IDH or AAT in animals from different latitudes or shore levels (Figs. 1, 2, 3; Table 1). For the CS reaction, the ABT was higher in the periwinkles of the temperate North Sea compared to their counterparts from the respective shore levels of the sub-arctic White Sea population. The same was observed for the ABT of the AAT reaction, but only for snails from low shore levels. At the same time, the ABTs for NADP-IDH were equal in animals from the White and North Seas. Similarly, between-shore comparisons of ABTs showed a variable pattern. The ABTs for the CS reaction tended to be higher in the periwinkles from low shore levels than in their high shore counterparts (30°C versus 20°C and 35°C versus 30°C in the White and the North Sea animals, respectively). In contrast, the ABT for NADP-IDH was 30°C for high shore periwinkles and 24.9°C for low shore ones (Table 1). The ABT of the AAT reaction was 30–35°C irrespective of shore level (Table 1).

Activation energies of the respective enzymatic reactions were compared in animals from different latitudes and shore levels in a temperature range between 0°C and the respective ABT. In most cases, E_a was similar in periwinkles from different latitudes (Table 1). Thus, E_a for CS (41.0–50.3 kJ mol^{-1}), NADP-IDH (78.6–83.4 kJ mol^{-1}) and PEPCK (45.0–53.0 kJ mol^{-1}) reactions did not differ significantly between White and North Sea *L. saxatilis* (Table 1). Similarly, activation energies for AAT (52.5–55.4 kJ mol^{-1}) and PK (38.7–40.5 kJ mol^{-1}) reactions did not differ in high shore animals from the White and the North Sea. However, in low shore samples, E_a was either significantly higher (for AAT) or lower (for PK) in White Sea *L. saxatilis* than in their conspecifics from the North Sea (55.7 kJ mol^{-1} versus 51.3 kJ mol^{-1} and 35.2 kJ mol^{-1} versus 47.7 kJ mol^{-1} for AAT and PK, respectively, $P < 0.05$). In *L. obtusata*, E_a was similar in White Sea and North Sea animals for the PK reaction (37.5 kJ mol^{-1} versus 45.7 kJ mol^{-1}, $P > 0.05$), but significantly higher in White Sea *L. obtusata* as compared to their North Sea counterparts for the PEPCK reaction (49.5 kJ mol^{-1} versus 32.6 kJ mol^{-1}, $P < 0.05$).

Table 1 *Littorina* spp. Arrhenius breakpoint temperatures (*ABT*) and activation energies (E_a) of key metabolic enzymes in *L. saxatilis* and *L. obtusata* from different latitudes and shore levels. Activation energies below the respective ABTs were compared between animals from the same shore levels in different seas and, within each sea, in periwinkles from different shore levels (*n.s.* not significant; *df* degrees of freedom for the respective regression lines; *n.d.* not detected; –, no data)

Sea	Shore level	Enzyme	ABT (°C)	Temperature range				Significance of differences in E_a	
				Above ABT		Below ABT		Below ABT	
				df_1	E_a (kJ mol^{-1})	df_2	E_a (kJ mol^{-1})	Between seas	Between shores
L. saxatilis									
White	High	CS	20	34	28.15 ± 2.20	24	50.27 ± 3.72	n.s.	$P < 0.05$
	Low	CS	30	23	26.71 ± 3.14	36	41.94 ± 1.79	n.s.	
North	High	CS	30	15	26.72 ± 5.17	23	42.53 ± 1.78		n.s.
	Low	CS	35	9	11.27 ± 9.58	29	41.04 ± 1.63		
White	High	NADP-IDH	30	20	29.07 ± 3.49	21	78.65 ± 1.93	n.s.	n.s.
	Low	NADP-IDH	24.9	21	30.60 ± 2.55	23	80.41 ± 2.28	n.s.	
North	High	NADP-IDH	30	22	25.25 ± 11.62	37	83.45 ± 2.95		n.s.
	Low	NADP-IDH	24.9	25	15.49 ± 3.58	34	82.39 ± 3.94		
White	High	AAT	30	26	41.94 ± 1.97	24	52.50 ± 1.24	n.s.	$P < 0.05$
	Low	AAT	30	27	38.24 ± 1.57	23	55.73 ± 1.43	$P < 0.05$	
North	High	AAT	30	27	36.62 ± 1.81	23	55.44 ± 1.21		$P < 0.05$
	Low	AAT	35	19	35.85 ± 3.14	31	51.34 ± 0.76		
White	High	PK	n.d.	–	–	25	40.49 ± 2.98	n.s.	n.s.
	Low	PK	n.d.	–	–	25	35.20 ± 2.50	$P < 0.05$	
North	High	PK	n.d.	–	–	25	38.76 ± 5.96		n.s.
	Low	PK	n.d.	–	–	25	47.70 ± 3.04		
White	High	PEPCK	n.d.	–	–	25	53.32 ± 2.39	n.s.	n.s.
	Low	PEPCK	n.d.	–	–	25	48.36 ± 3.16	n.s.	
North	High	PEPCK	n.d.	–	–	25	46.41 ± 4.22		n.s.
	Low	PEPCK	n.d.	–	–	25	45.00 ± 3.93		
L. obtusata									
White	Low	PK	n.d.	–	–	16	37.54 ± 1.96	n.s.	–
North	Low	PK	n.d.	–	–	19	45.66 ± 4.02		–
White	Low	PEPCK	n.d.	–	–	16	49.54 ± 4.56	$P < 0.05$	–
North	Low	PEPCK	n.d.	–	–	19	32.55 ± 4.47		–

Within each sea, *L. saxatilis* from different shore levels mostly showed similar E_a values for the respective enzymatic reactions (Table 1). Thus, E_a for NADP-IDH, PK and PEPCK reactions did not differ between high and low shore animals from the White and North Seas. Activation energy of the CS reaction was similar in high and low shore periwinkles from the White Sea, but significantly higher in high shore snails than in low shore ones from the North Sea. Although the activation energy of the AAT-catalysed reaction differed significantly in snails from different shore levels, the pattern was inconsistent between the two seas. In the North Sea, it was higher, and in the White Sea – lower in high shore animals as compared to their low shore counterparts.

Anaerobic potential of *L. saxatilis* and *L. obtusata*

The ratio of PK and PEPCK activities is considered as an index of the anaerobic capacity of succinate production, such that a lower ratio is associated with a higher anaerobic potential (de Vooys 1980; Bowen 1984; Simpendörfer et al. 1995). In *L. saxatilis*, this ratio was significantly affected by temperature ($F_{2,96} = 5.6$, $P = 0.005$), shore level ($F_{1,96} = 6.5$, $P = 0.012$) and differed in snails from the White and the North Seas ($F_{1,96} = 33.8$, $P < 0.001$). North Sea periwinkles had generally higher PK/PEPCK activity ratios as compared to White Sea *L. saxatilis* (Fig. 7). The ratio of PK and PEPCK activities declined with temperature in *L. saxatilis*, except for North Sea snails from low shore level where it remained essentially the same over the studied temperature range. High shore periwinkles had lower PK/PEPCK activity ratios as compared to their low shore counterparts, suggesting a higher capacity for anaerobic succinate production in high shore snails.

Low shore *L. obtusata* had PK/PEPCK activity ratios similar to (at the White Sea, $P > 0.81$) or even higher (at the North Sea, $P < 0.02$) than those of low shore *L. saxatilis* (Fig. 8). Similar to *L. saxatilis*, North Sea *L. obtusata* had higher PK/PEPCK activity ratios than the White Sea conspecifics. In *L. obtusata* from the White Sea, the PK/PEPCK activity ratio decreased with temperature ($F_{2,15} = 4.1$, $P = 0.038$) similar to what was found in *L. saxatilis*. In contrast, in North Sea *L. obtusata* a temperature rise led to a significant increase in the ratio of PK and PEPCK activities ($F_{2,18} = 4.1$, $P = 0.035$).

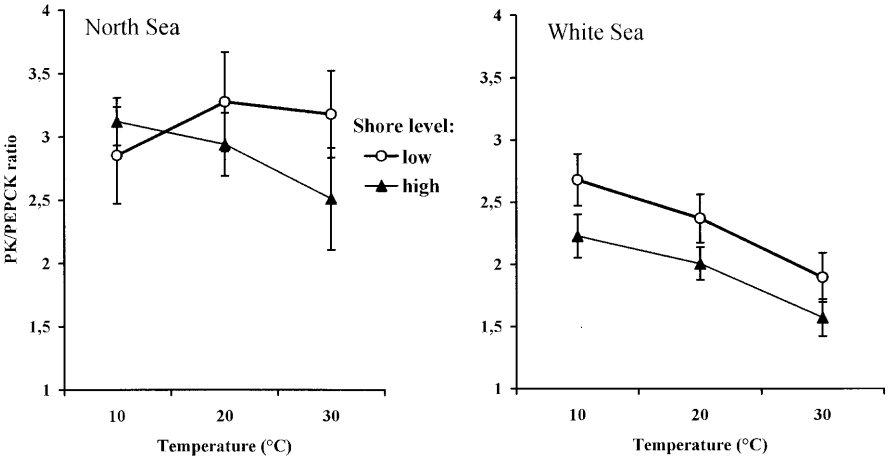


Fig. 7 *Littorina saxatilis*. Temperature effect on ratio of PK and PEPCK activities in *L. saxatilis* from different latitudes and shore levels. *Vertical bars* represent standard errors. For each temperature, $n = 9$

Discussion

Maximum activities of the studied key metabolic enzymes were found to vary both within and between populations of *Littorina saxatilis* and *L. obtusata*. Generally, the difference in enzyme activities may be genetically fixed or due to acclimatisation to contrasting environmental conditions. In the present study, V_{max} was compared in animals acclimated to standard laboratory conditions for a long time (3–6 weeks). This should minimise the effects of previous environmental history and allow to estimate the genetically based variation in enzyme characteristics. It should be mentioned that this variation may also partially involve irreversible non-genetic adaptations induced by "environmental imprinting" during early ontogenesis, as has been shown for salinity tolerance and temperature-dependence of metabolism in some marine fish and invertebrates (Tay and Garside 1975; Zamer and Mangum 1979). However, it was argued that the phenomenon of irreversible non-genetic adaptations is generally uncommon in animals (Kinne 1962). Thus, observed differences in V_{max} and temperature dependence of the studied enzymes in animals from different latitudes and shore levels are most likely genetically determined. This does not rule out a potential role of physiological plasticity and acclimatory changes of enzyme activities in adjustments to short-term (e.g. seasonal or diurnal) variations of ambient temperature in *Littorina* spp. Comparison of enzyme activities in autumn and spring samples of *L. saxatilis* from the same population maintained at different temperatures in the laboratory suggests that seasonal cold acclimation may indeed affect V_{max} of at least some enzymes. Cold acclimation in "spring" snails led to an increase in the activity of NADP-IDH as compared to warm-acclimated "autumn" animals, although the activity of another key mitochondrial enzyme, CS, remained unaffected. However, detailed analysis of effects of temperature acclimation on enzyme properties in *L. saxatilis* is beyond the scope of the present study and requires further investigation.

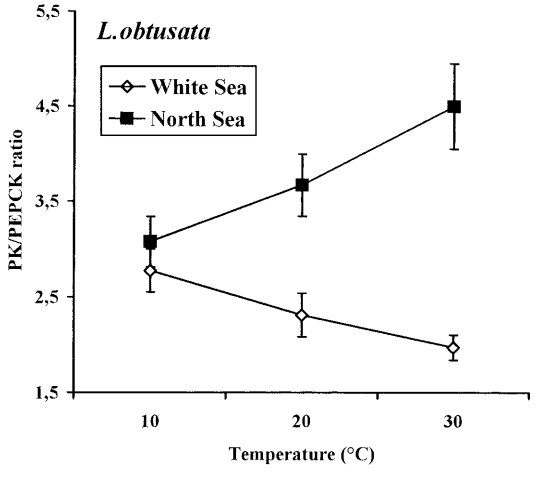


Fig. 8 *Littorina obtusata*. Temperature effect on ratio of PK and PEPCK activities in *L. obtusata* from different latitudes. *Vertical bars* represent standard errors. For each temperature, $n = 6$ (White Sea animals) or $n = 7$ (North Sea ones)

Comparisons of enzyme activities between *L. saxatilis* from high and low shores showed that in all cases when the differences were significant, low shore snails demonstrated higher enzyme activities than their high shore counterparts. This holds true for PEPCK, CS and NADP-IDH in North Sea animals (for the latter enzyme– only in a spring sample) and for AAT and PK in White and North Sea ones. Similarly, in cases when V_{max} values were significantly different in animals from different latitudes, White Sea periwinkles had higher enzyme activities than their conspecifics from the North Sea. This could be seen for NADP-IDH and PK in *L. saxatilis* and for PEPCK in *L. saxatilis* and *L. obtusata*. In general, this finding is in agreement with the concept of metabolic temperature compensation (Krogh 1916; Bullock 1955; Wohlschlag 1964), which suggests that warm-adapted animals (in this case, high shore animals or those from the temperate North Sea) will have lower basal metabolic rates and enzyme activities at any given temperature than their cold-adapted counterparts (e.g.

low shore snails or those from the sub-arctic White Sea, respectively). It should be noted, however, that the concept of metabolic temperature compensation sensu Krogh (1916) and Bullock (1955) is the focus of a heated controversy (for review see Clarke 1998). One reason for this may be the above-mentioned difficulty in finding comparable (i.e. closely related and similar in ecology and life style) model species at different latitudes (Eastman 1993). Only few studies have overcome this problem. For example, in an elegant study on 11 glycolytic enzymes, Pierce and Crawford (1997a,b) demonstrated that most of the latitudinal variation in enzyme activities in the genus *Fundulus* was attributed to the phylogenetic component and not to the latitudinal change in thermal regime. However, in three enzymes – glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH) and PK – significant negative correlations between enzyme activities and mean annual temperature were detected after the effects of phylogenetic history were accounted for (Pierce and Crawford 1997b). This indicates that activities of LDH, GAPDH and PK increase with decreasing ambient temperature and should compensate for the reduced activity of each enzyme at colder temperatures. In our study, possible phylogenetic effects on enzyme activities were avoided by using populations of the same species from different latitudes and shore levels. Results of the present comparison agree with the findings of Pierce and Crawford (1997a,b) in that several (but not all) enzymes respond to cold adaptation with an increase in V_{max}. These include both flux-limiting and equilibrium enzymes (Pierce and Crawford 1997b; present study), which suggests that many different enzymes (and not only so-called regulatory ones) are involved in modulating flux during temperature adaptation. Another point which should be considered when comparing metabolic cold adaptation in different species is the degree of eurythermality of the studied species, because the mechanisms of cold adaptation may differ in eurythermal and stenothermal species (Pörtner et al., in press). In general, metabolic cold compensation is characteristic of cold-acclimated or cold-adapted populations of eurythermal species (Pierce and Crawford 1997a,b; Guderley 1998; Sommer and Pörtner 1999; present study) but is not observed in cold-adapted stenotherms (e.g. in Antarctic species) (Guderley 1998; Johnston et al. 1994, 1998; Pörtner et al., in press). The results of the present study performed on an extremely eurythermal and thermotolerant intertidal gastropod, *L. saxatilis*, fit this general pattern.

Activation energies for most enzyme-catalysed reactions were similar in *L. saxatilis* from different shore levels. The only exceptions were the CS-catalysed reaction (E_a was significantly higher in high shore animals than in low shore ones in the White Sea) and the AAT-catalysed reaction (E_a was higher in the North Sea and lower in the White Sea animals from high shore as compared to the respective low shore counterparts). Similarly, comparisons of E_a between White and North Sea *Littorina* spp. did not reveal a uniform pattern. Activation energies of the respective enzymatic reactions were mostly equal in snails from different latitudes. However, in few cases enzymes from White Sea animals demonstrated lower (e.g. PK in low shore *L. saxatilis* and PEPCK in *L. obtusata*) or higher E_a values (e.g. AAT in low shore *L. saxatilis*) than in their conspecifics from the North Sea. This supports a conclusion that a cold-induced drop in E_a is not as obligatory as was suggested earlier (Hochachka and Somero 1973) and that there may be exceptions with unchanged or even higher values of E_a (Alexandrov 1977; Pörtner et al., *in press*).

It is worth noting that maximum activity of an enzyme, V_{max}, depends on concentration [*E*] and turnover number k_{cat} of the respective enzyme. Enzyme concentration is a parameter that readily and quickly responds to temperature acclimation and acclimatisation both on short-term and evolutionary time scales (Hochachka et al. 1988). At the same time, turnover number is thought to be a more conservative characteristic determined by the structure of the enzyme (Suarez et al. 1996; Feller et al. 1997). Usually the adjustments of k_{cat} follow changes in [*E*] and require a longer time for their accomplishment – e.g. as it is necessary to switch between gene expression of different isozymes during acclimation or to produce new allo- or isoenzymes during evolutionary adaptation to temperature. It also seems plausible that enzymatic variants with different E_a are represented by different iso- or allozymes (Gonzalez-Villaseñor and Powers 1986). In our study, although V_{max} of most studied enzymes showed significant differences between animals from different latitudes (e.g. NADP-IDH, PK, PEPCK in *L. saxatilis* and PEPCK in *L. obtusata*) or shore levels (e.g. CS, AAT, PK, PEPCK in autumn animals and CS and NADP-IDH in spring animals), activation energy of the respective enzymatic reactions remained more or less unchanged (see above). This, in turn, suggests that the observed variations in V_{max} of the studied enzymes of *L. saxatilis* and *L. obtusata* are primarily brought about by variations of enzyme concentrations. Even so, it should again be emphasised that differences in V_{max} between snails from different latitudes and shore levels are probably constitutive and did not disappear during prolonged laboratory acclimation under the same temperature conditions.

Thermal stabilities of enzymes in *L. saxatilis* from different latitudes and shore levels were compared using Arrhenius breakpoint temperatures and temperatures of heat inactivation of the respective enzymes. ABT reflects a change in the thermal properties of the protein itself or of the metabolic unit to which it belongs and may be considered as a first sign indicating loss of protein function or change in metabolic regulation (Pörtner et al., in press). In *L. saxatilis*, E_a of enzymatic reactions dropped by 30–70% beyond the respective ABT values, indicating a decrease in temperature dependence of enzymatic activities at high temperatures. ABT values for the three enzymes of *L. saxatilis* studied in this respect

ranged between 20°C and 35°C and were close to 30°C in most groups; these temperatures are well within the average highs experienced by *L. saxatilis* in its habitat during low tide. This suggests that considerable changes in metabolic regulation may occur within the "normal" temperature range of this species.

In contrast, thermal denaturation of the studied enzymes occurred only at temperatures >40°C, which is a value higher than the average upper temperatures in White and North Sea intertidal zones and close to the environmental extremes experienced by animals during summer low tides. Our data suggest that the resistance of *L. saxatilis* to acute heating simulating environmental conditions during summer low tide may be limited by the heat stability of key mitochondrial enzymes. A temperature rise to 45°C in vivo led to a considerable decrease in the activity of a key regulatory enzyme of the TCA cycle, CS. Under the same conditions, the activity of NADP-IDH decreased only slightly. As a result, the ratio of the two enzymes' activities shifted in favour of NADP-IDH. A similar decrease in the ratio of CS and NADP-IDH activities (from 3–4 at 0°C to <1 above 30°C) was observed during a temperature rise in vitro, although in this case the reason was not thermal inactivation but rather the difference in E_a of these two enzymes (Fig. 9). It is worth noting that NADP-IDH is probably involved in a mitochondrial substrate cycle leading to proton leakage without concomitant ATP production (Sazanov and Jackson 1994; Pörtner et al., in press). Thus, an increase of NADP-IDH over CS activity ratio may lead to a progressive metabolic imbalance at high temperatures, which, in turn, may limit whole-organism survival.

Our study did not reveal any significant differences in the heat resistance of most studied enzymes between *L. saxatilis* from different latitudes and shore levels, the only exception being AAT, which was more heat stable in high shore animals as compared to their low shore counterparts. It should be noted that considerable thermal inactivation of AAT in White and North Sea *L. saxatilis* occurred only above 50°C. However, it has been shown that at lower temperatures (45°C) thermal denaturation of AAT of *L. saxatilis* also occurs, although at a lower rate (Hull et al. 1999). Moreover, thermal stabilities of AAT in different littorinids measured at 45°C and 56°C were closely correlated (Hull et al. 1999). Taking this into account and considering that high shore periwinkles may spend a few hours at extreme temperatures (up to 45–47°C) during summer low tide, the observed variation in heat stability of AAT between animals from different shore levels may be of selective importance for high shore snails, ensuring the function of an important enzyme involved in early anaerobiosis and post-anaerobic recovery (de Zwaan 1991; Mommsen and Hochachka 1988). Similar findings were reported by Hull et al. (1999), who studied thermal inactivation of AAT in eight species of North Atlantic Littorinidae. They found that AAT was more heat stable in high shore *L. saxatilis* than in mid-shore ones, and that this enzyme had generally greater heat stability in high shore species of littorinids as compared to mid- and low shore ones. In the common mussel *Mytilus edulis*, intertidal specimens displayed higher thermal stability of AAT than subtidal ones (Read 1963). It is interesting that an analysis of allozyme polymorphism of *L. saxatilis* demonstrated a consistent cline of allele frequencies at the *Aat* locus associated with the vertical shore gradient of the intertidal in the North and White Seas, with the slow allele *Aat100* allele dominating in mid- and low shore and a faster one (*Aat120*) predominantly found at high shore levels (Johannesson and Johannesson 1989; M. Panova, unpublished results). This vertical cline is probably maintained by differential selection acting either directly upon this locus or upon other, closely linked loci (Johannesson et al. 1995b). Recent studies showed that *L. saxatilis* with different *Aat* genotypes significantly differ with respect to AAT activity (M. Panova, unpublished results). This suggests that the *Aat* locus may itself be a target of selection at different shore levels. Notably, homozygotes of the *Aat100* allele dominating in mid-shore had a higher V_{max} of AAT as compared to *Aat120* homozygotes, which are predominantly found at high shore levels (M. Panova, unpublished data). This agrees with our findings of relatively higher AAT activities in low shore *L. saxatilis* from the White and the North Seas (see above). It is worth mentioning that a similar vertical cline in allele frequencies associated with the tidal height was found for *IdhI* locus coding the NADP-IDH in White Sea *Macoma balthica* (Hummel et al. 1998). However, similarity in enzyme activities and E_a of NADP-IDH in *L. saxatilis* from different latitudes and shore levels suggests that this may be not the case in this species.

Besides comparison of V_{max} of different enzymes, analysis of activity ratios of enzymes belonging to

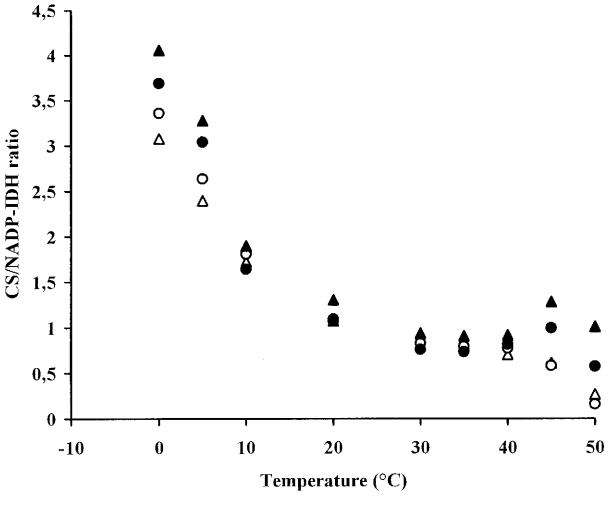


Fig. 9 *Littorina saxatilis*. Temperature dependence of ratio of CS and NADP-IDH activities in *L. saxatilis* from different latitudes and shore levels (*filled symbols* North Sea animals; *open symbols* White Sea animals; *circles* low shore snails; *triangles* high shore snails)

functionally related pathways may yield additional insight into mechanisms of metabolic regulation (Pette and Hofer 1980). Thus, two functionally linked enzymes PK and PEPCK involved in this study were used to estimate the potential anaerobic capacity of *Littorina* spp. These two enzymes are likely to compete for a common substrate, phosphoenolpyruvate, channelling it to aerobic (PK) or anaerobic (PEPCK) pathways (Saz 1971; Bryant 1975), so that a low PK/PEPCK activity ratio is indicative of a relatively higher anaerobic capacity. It was found that the ratio of PK and PEPCK activities differed consistently between animals from different latitudes and shore levels, as well as between the two studied species. Generally, the PK/PEPCK activity ratio correlated with the vertical distribution of the studied species/intraspecific groups and decreased in the order: *L. obtusata* > low shore *L. saxatilis* > high shore *L. saxatilis*. This suggests a higher potential of anaerobic succinate production in high shore *L. saxatilis* as compared to low shore ones and, especially, to *L. obtusata*, which is a typical mid- to low shore dweller unable to invade high intertidal horizons. These results agree with our previous findings that the capability to survive prolonged anaerobiosis during air exposure at elevated temperatures or during fresh water exposure is better in high shore *L. saxatilis* than in their low shore counterparts and better in *L. saxatilis* than in *L. obtusata* (Sokolova et al. 2000a,b). Previously, a correlation of the PK/PEPCK activity ratio with vertical distribution was reported for other species of molluscs. For example, in mid-shore *Perumytilus purpuratus* the PK/PEPCK activity ratio was nearly two times lower than in low shore *Mytilus chilensis* (Simpendörfer et al. 1995), and in subtidal *M. galloprovincialis* it was two to four times higher than in intertidal *M. edulis* (de Vooys 1980). In *Haliotis* spp. from habitats between the tidal zone and depths of 20 m, the ratio of PK and PEPCK activities ranged from 5.9–15.8 in high intertidal *H. rufescens* and *H. cracherodii* to 24.3 in subtidal *H. corrugata* (Bowen 1984). To the best of our knowledge, the present study is the first one to demonstrate that adaptation to life at different shore levels may involve considerable changes in anaerobic enzymatic potential within a single species. Moreover, intraspecific differences in the PK/PEPCK activity ratio between high and low shore *L. saxatilis* are quite comparable in magnitude to those between closely related species (de Vooys 1980; Bowen 1984; Simpendörfer et al. 1995; present study). Between-sea comparisons of the PK/PEPCK activity ratio showed that North Sea *L. saxatilis* and *L. obtusata* have higher values of this parameter as compared to their conspecifics from the White Sea, suggesting enhanced capacity for anaerobic energy production via the mitochondrial succinate pathway in White Sea animals. This suggestion is supported by the very high degree of succinate accumulation found during environmental anaerobiosis in White Sea *Littorina* spp. (Sokolova et al. 2000a), which is likely to be related to the higher mitochondrial density in cold-adapted subpolar invertebrates (Sommer et al. 1997).

The ratio of PK and PEPCK activities may be further modified by environmental conditions. In molluscs, aestivation, anoxia, temperature stress and air exposure lead to a decrease in PK activity, most likely due to phosphorylation of the active non-phosphorylated form of the enzyme (Churchill and Storey 1989; Rees and Hand 1991; Fields and Ellington 1992; Russel and Storey 1995; Simpendörfer et al. 1997) and result in decreased PK/PEPCK activity ratios (Greenway and Storey 1999). Additionally, PK is effectively inhibited by alanine, which is a common anaerobic end product during early anaerobiosis in marine invertebrates (McManus and James 1975; Zammit and Newsholme 1978; Holwerda et al. 1983). Presumably, these mechanisms serve to channel glycolytic pyruvate towards anaerobic succinate production when conditions inducing anaerobiosis persist. Our data suggest that a switch of metabolism towards anaerobic succinate production may be further enhanced by increased temperature in White Sea *L. saxatilis* and *L. obtusata* and in North Sea *L. saxatilis* from high shore levels, as shown by a decline of the PK/PEPCK activity ratio with rising temperature. This may be advantageous in the intertidal, especially at high shore levels, where periods of oxygen deprivation during low tide frequently coincide with elevated temperatures. In North Sea *L. obtusata* and low shore *L. saxatilis*, the PK/PEPCK activity ratio tended to increase with rising temperature, suggesting that anaerobic capacity is rather impaired by increasing temperatures in these animals. Presently, it is difficult to say whether this reflects an increasing dependence on aerobic pathways or, more likely, increasing metabolic disbalance at high temperatures in these animals, which rarely encounter extreme temperature fluctuations in low shore habitats of the North Sea with comparatively mild macro- and microclimatic conditions.

In conclusion, our data show that adaptation of *L. saxatilis* and *L. obtusata* to life at different latitudes and/or shore levels involves constitutive (possibly, genetic) changes in activity levels of key metabolic enzymes. The periwinkles, naturally adapted to (micro-) environments with lower mean temperatures (i.e. in the White Sea or at low shore levels), tend to have higher enzyme activities as compared to their counterparts from warmer habitats (e.g. the North Sea or high shore levels, respectively); this tendency may reflect metabolic temperature compensation of enzyme activities in these animals. Activation energies of the respective enzymes vary within and between populations to a much lesser extent than overall enzyme activities, suggesting that the characteristic E_a for a given enzyme may be a conservative feature in *Littorina* spp. Besides overly decreased V_{max} of key metabolic enzymes, adaptation to high shore life involves an increased cellular capacity of anaerobic energy production in *Littorina* spp., which is reflected in increased activity of PEPCK relative to PK, and improved heat resistance of another enzyme involved in anaerobic pathways, AAT. These features may be advantageous under conditions of periodical oxygen

deficiency, frequently combined with the extreme heating that snails encounter at high shore levels. A potential advantage of improved heat stability of enzymes for adaptation to high shore life is further emphasised by the finding that survival under conditions of acute heat stress in vivo seems to be limited by the thermal stability of a key mitochondrial enzyme, CS. However, it should be noted that unlike AAT, in vitro heat stability of most enzymes was similar in animals from thermally different environments, suggesting that other mechanisms (e.g. molecular chaperones and/or increased protein production to replace damaged enzymes) may play a foremost role in the protection of enzymatic activity from the deleterious effects of heating (Sanders 1988, 1993). This question is, however, beyond the scope of the present study and requires further investigation.

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