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Energetic aspects of cold adaptation: critical temperatures in metabolic, ionic and acid–base regulation?

Temperature is considered to be one of the most important abiotic factors shaping marine ecosystems due to its major impact on all biological processes. Therefore, low or high temperature extremes characterise the limits of geographical distribution of many species, and global change has already caused a change in the distribution of species (Southward, Hawkins & Burrows, 1995). An investigation of marine ectotherms surviving in seasonally or permanently cold ocean environments and their comparison with ectotherms from temperate and warm waters should help to reveal those biochemical or physiological mechanisms which determine geographical distribution limits. These studies should also reveal which molecular, cellular and systemic functions have been shifted to levels compatible with the steady-state maintenance of all life-sustaining processes in the cold. In permanent cold, the latter must also include growth and reproduction, whereas during seasonal cold exposure these processes may be suspended.

Adaptation to cold started from life forms that evolved in warm waters (e.g. Arntz, Brey & Gallardo, 1994; Thiel, Pörtner & Arntz, 1996). Therefore, the characteristics of the adaptational process must be seen in the light of this evolutionary trend. Cold adaptation then becomes a special physiological feature rather than a basic ability of all life forms. If life conquered the cold after having evolved in warm waters, the question arises about what were the limiting factors in this adaptational process and how would these limiting factors affect the whole organism, thereby preventing an easy access to cold ocean environments. Animals from latitudes outside the polar regions are therefore included in our analysis, in order to elaborate the general validity of those adaptational strategies. As a first step, critical thresholds need to be defined beyond which steady-state function is no longer possible. Furthermore, physiological or biochemical characteristics or processes have to be identified which are responsible for limiting survival. In a logical second step, key processes of physiological and biochemical

adjustment should be identified which support seasonal and permanent life in the cold, and which allow for a shift in tolerance and distributional limits under different and changing temperature regimes. Certainly, research has not yet provided final answers to these questions and the present study is intended to summarise current knowledge and stimulate further research in this direction.

Critical temperatures

As a first step towards a deeper understanding of the integrated regulatory cascades leading to adaptation in cold ocean environments, the mechanisms and limits of adaptation to cold were investigated in temperate zone animals (Zielinski & Pörtner, 1996; Sommer, Klein & Pörtner, 1997). The marine sipunculid worm, *Sipunculus nudus*, is a suitable invertebrate model for an animal without a circulatory system and, generally, with a simple organisational level at the low end of the animal kingdom. In its natural environment in sandy sediments of the intertidal zone around Brittany, France, this worm is subjected to regular fluctuations of environmental parameters like oxygen and CO_2 levels as well as temperature, and modifications of cellular set points are required to adjust to these fluctuations. Long-term exposure to unfavourable conditions (low oxygen and high CO_2 levels) is tolerated, based on an adaptive drop in metabolic rate (Hardewig *et al.*, 1991; Pörtner, Reipschläger & Heisler, 1997). However, fluctuations of abiotic factors will only be tolerated within certain limits. Limiting temperature thresholds may be reached during seasonal fluctuations. Extremely low temperatures including winter frost are very rare in the worm's natural environment, and if low temperature is a limiting factor these animals should show stress effects during cold exposure.

In a study with cannulated animals dwelling in their natural burrows the correlated changes in ventilatory activity, gas exchange and the mode of energy production were investigated, using anaerobic metabolites as stress indicators since they will indicate insufficiency of aerobic ATP production and transition to a time-limited situation (Zielinski & Pörtner, 1996). Ventilation decreased sharply below 4 °C, and blood gas values as well as tissue metabolite levels indicate that hypoxia developed owing to insufficient oxygen supply. Succinate and volatile fatty acids like acetate and propionate accumulated in the body wall musculature and in the coelomic fluid. These metabolites are formed in the mitochondria, emphasising that insufficient oxygen supply elicits anaerobic metabolism. Obviously, a low critical temperature exists in *S. nudus* (between 4 and 0 °C), that is characterised by a failure of ventilation and the transition to anaerobic metabolism which finally causes death of the animals.

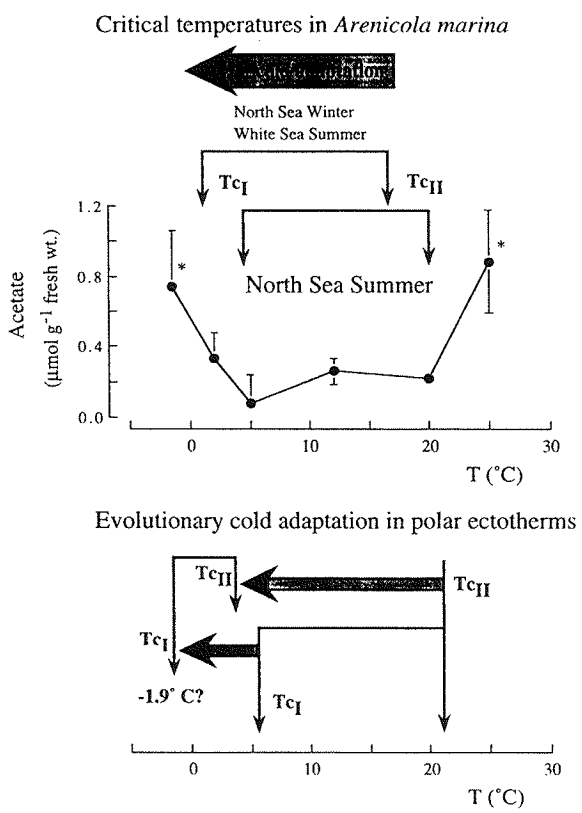


Fig. 1. Low (Tc_I) and high (Tc_{II}) critical temperatures in the lugworm, *Arenicola marina*, are characterized by the accumulation of anaerobic end products, especially acetate. Both Tc_I and Tc_{II} shift to lower values during adaptation to cold. They are found at lower values in animals collected at higher latitudes, e.g. the Russian White Sea, indicating cold adaptation (based on data by Sommer *et al.*, 1997, *=significantly different from controls, $P<0.05$). In polar ectotherms, Tc_I falls to below freezing associated with a large reduction in the distance between Tc_I and Tc_{II} (see text, supported by findings of H.O. Pörtner, L. Peck, S. Zielinski & L.Z. Conway, unpublished data, in the Antarctic bivalve *Limopsis marionensis*).

Work with *Arenicola marina*, another animal model well studied in hypoxia research, provided a comparison of low and high critical temperatures in populations of the same species in a latitudinal gradient (Sommer *et al.*, 1997, Fig. 1). *A. marina* is found from the Mediterranean to the North Sea, and its distribution ranges via Norway to the Russian White Sea. Although, in contrast to *S. nudus*, this species possesses a well-developed circulatory system, temperature stress also leads to insufficient oxygen supply and a transition to anaerobic metabolism. The comparison of populations from different latitudinal areas revealed that critical thresholds as defined above are set to low and high values depending on the latitudinal and seasonal temperature regime. When the animals experienced temperatures beyond low and high critical thresholds while dwelling in their natural burrows, they accumulated largely acetate, but also propionate in their body fluids. It appears that both low and high critical temperatures are characterised by the transition to anaerobic mitochondrial metabolism. Both temperature thresholds are set to lower values in White Sea than in North Sea animals (Fig. 1). During winter adaptation of North Sea animals, the low critical temperature was also found to decrease, but a value 1–2 °C below ambient was not eliminated when animals collected at 2 °C in January were kept at 2 °C in the laboratory. Consequently, animals leave the intertidal zone and migrate to areas below the low water line to avoid lethal cold exposure (Werner, 1956). Even animals exposed to seawater without sediment showed a transition to anaerobic metabolism in the cold, a phenomenon not evident in *S. nudus*. The comparison suggests that not only ventilatory mechanisms but also the performance of the circulatory system is prone to being disturbed by cold exposure, emphasising the threat of functional hypoxia. Further studies suggest that cold-induced anaerobiosis also develops in crustaceans such as the shore crab *Carcinus maenas* (DeWachter & Pörtner, 1997).

The complete set of mechanisms involved in cold adaptation is still unclear, but these data emphasise that mechanisms are required to eliminate the threat of functional hypoxia. North Sea animals appear unable to adjust to polar conditions and are less able, in general, than the White Sea animals to shift critical temperatures (Sommer, Hummel & Pörtner, 1996). White Sea animals adjust to Arctic water temperatures during winter and it is now known that these animals are genetically distinct from the North Sea population (Sommer *et al.*, 1996). The pattern of metabolite accumulation during temperature-induced anaerobiosis suggests that anaerobic mitochondrial metabolism is more pronounced in White Sea than in North Sea specimens (Sommer *et al.*, 1996, 1997).

Cold adaptation is therefore linked to the necessity to overcome the threat of cold-induced functional hypoxia. This process may contribute to the development of a high sensitivity to high temperatures (cf. Somero, 1991), i.e. a low upper Tc, Tc_{II}. Obviously, the distance between critical temperatures does not remain constant during evolutionary cold adaptation but rather falls when cellular functions in the cold are optimised (Fig. 1). It may very well be that the drop in the distance between the Tcs is obligatory for maintaining all life-sustaining functions in the extreme cold. The molecular

mechanisms responsible for setting the Tcs are currently under investigation, especially those shifting the low Tc to below polar ambient temperatures. Adaptational changes would include, among others, a rise in aerobic capacity (combined with mitochondrial proliferation, as found by Egginton & Sidell, 1989 in fish muscle), improvement of muscle function and nervous conductivity by adjustments of ionic exchange mechanisms (see below), and adjustments of the metabolic machinery (for example, enzyme quantities and kinetic properties, see Vetter & Buchholz, this volume; Guderley, this volume). These changes overall can be summarised as 'metabolic cold adaptation' (Thiel *et al.*, 1996). This definition should be preferred over the historical definition, which is restricted to the view that the adaptation to low temperature may be associated with energy expenditures elevated above the decrease expected from the Q_{10} effect. This is a continuing area of discussion (see Clarke, this volume; Somero, this volume).

In this context, we hypothesise that mitochondrial proliferation as required during cold adaptation would inevitably lead to elevated metabolic rates, not just owing to the transient cost of mitochondrial synthesis, but also to the cost of mitochondrial maintenance which comprises the baseline 'idling' of mitochondrial oxygen consumption associated with the maintenance of ionic gradients and the compensation of H^+ leakage across mitochondrial membranes. In consequence, it will be more costly to maintain mitochondria in the same volume of tissue or animal than other cellular elements. In support of this hypothesis, we found consistently higher rates of oxygen consumption in populations of the same species (!), *Arenicola marina* from the sub-Arctic White Sea than from the more temperate North Sea, when the two populations were compared at identical temperatures over a wide temperature range (A. Sommer & H.O. Pörtner, unpublished observations). A comparison of fiddler crab populations (animals reared in the laboratory under the same conditions) from along the North American Atlantic coast yielded similar results (Vernberg & Costlow, cited in Cossins & Bowler, 1987), even suggesting genetic differences to develop in a latitudinal gradient. It would be difficult to draw this conclusion from traditional interspecies comparisons.

These considerations also fit the widely held principle that animals with a higher level of activity or cost of locomotion must exhibit higher standard or resting metabolic rates than more sluggish species in order to attain high rates of metabolism during exercise. Extreme differences in this respect are seen between fish and squid where, owing to the costly mode of swimming in squid, rates of resting metabolism are about ten times higher in squid than in equally active fish in order to allow for the extreme rates of oxygen consumption at high swimming speeds (cf. O'Dor, Pörtner & Shadwick, 1990). Further support of this concept arises from the finding that a seven times

higher rate of oxygen consumption in an endotherm (the rat) compared to an ectotherm (a lizard) can be explained by the observation that overall mitochondrial density and mitochondrial leakiness for H^+ is higher in the rat than in the lizard (Brand *et al.*, 1991). More precisely, the level of standard metabolic rate is correlated with the total area of inner mitochondrial membrane and its degree of leakiness for H^+ according to body size, the level of endothermy (Brand, 1990; Brand *et al.*, 1992) and, most likely, the level and scope for activity (see above) of an animal. According to Brand (1990) a doubling of the number of mitochondria (of inner membrane surface area) without a concomitant rise in ATP demand will cause oxygen consumption to rise by 50 to 75%. The cost of maintaining the mitochondria (i.e. of compensating for the H^+ leak) is estimated to comprise up to 45% of the respiration rate of the individual cell or up to 70 % of the increment in respiration rate associated with an elevation of mitochondrial density! These numbers are vague estimates but qualify the price for maintaining a high aerobic capacity as needed for a maximisation of aerobic scope of activity (e.g. in squid) and immediately explain why an organism should strive to minimise the number of mitochondria and maximise 'fuel economy' in accordance with its mode of life and level of activity.

This discussion already suggests that with an obligatory mitochondrial proliferation in the cold, only a modification of inner mitochondrial membrane or a reduction of its surface area (both processes leading to a reduced leakiness for H^+) may offset some (but probably not all) of the metabolic rate increment following mitochondrial proliferation. This may be associated with a drop in mitochondrial oxidative capacity. Accordingly, mitochondria from fish living in permanent cold were modified to exhibit lower capacities of substrate oxidation and rates of oxygen consumption than mitochondria of temperate zone fish acclimated to cold water (Guderley & Johnston, 1996; Guderley, pers. comm.). To compensate for the Q_{10} effect the latter showed marked increases in oxidative capacity at low temperature (Guderley & Johnston, 1996), an adaptational change which was obviously reversed during evolutionary cold adaptation (Johnston *et al.*, 1994, Fig. 2), possibly associated with the adoption of a low activity mode of lifestyle (cf. Clarke, this volume; Thiel *et al.*, 1996).

In conclusion, mitochondrial proliferation leads to a higher cost and may contribute to an increment in whole animal standard metabolic rate in the cold which compensates for (some of) the Q_{10} effect (metabolic cold compensation). This metabolic rate increment is required to shift Tc_I to lower temperatures but will inevitably cause Tc_{II} to fall as well (Fig. 1). It may be balanced by a reduction in other processes like motor activity thus reducing the rise in SMR (Fig. 2). However, there may be further processes associated with a higher cost of maintenance than expected from the Q_{10} effect. One of

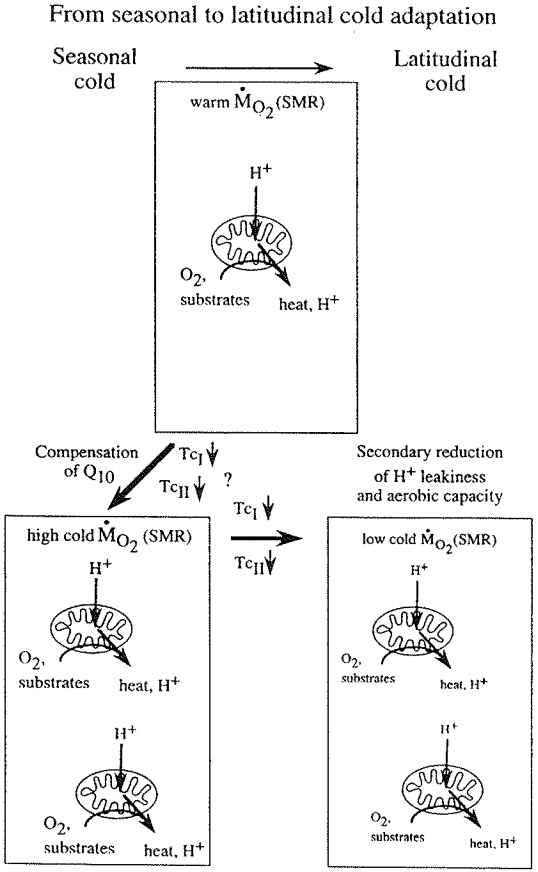


Fig. 2. Modelled depiction of the adjustment of mitochondrial density and of the H^+ leakiness of inner mitochondrial membranes during seasonal or latitudinal cold adaptation. The model assumes that an increase in mitochondrial density and aerobic capacity (possibly also via an increase in the surface area of cristae, Archer & Johnston, 1991) offsets the Q_{10} effect on standard metabolic rate (SMR, measured as the molar rate of oxygen consumption, $\dot{M}o_2$) and aerobic capacity, for example, during seasonal cold (if activity levels are to be maintained and seasonal dormancy does not occur). This process which very likely contributes to a shift of Tc_I and Tc_{II} to lower values (cf. Fig. 1), may partly be compensated during progressive evolutionary (latitudinal) cold adaptation at elevated levels of mitochondrial density when a reduction in aerobic capacity may occur to minimise futile proton cycling and associated energy dissipation. This is postulated to be achieved by a reduced H^+ leakiness of inner mitochondrial membranes (supported by a modification of the membrane or a reduction in surface area) and is interpreted to allow for even further reduction of the Tcs. Note that cold acclimated mitochondria ('seasonal cold') exhibit a higher oxidative capacity if compared to warm acclimated mitochondria at the same temperature (for further discussion and references, see text).

them may be especially important in marine fish, which are hypoosmotic relative to the ambient medium. In contrast to marine invertebrates, fishes maintain ion concentrations lower than ambient in the blood. Since the inward diffusion of ions along the osmotic gradient is only slightly altered by temperature, the metabolic costs for the elimination of surplus ions are maintained at low temperatures, whereas other metabolic processes such as respiration are more temperature-dependent. In the following section, the adaptive flexibility and cost of ion regulation will be dealt with in more detail.

Membrane transport mechanisms

Temperature effects on the cost of ion regulation

The preservation of ion balance despite changes in body temperature is crucial for ectothermal animals in order to maintain vital cellular functions. Ion homeostasis is influenced by active ion pumping, mostly via Na^+/K^+-ATPase, and opposing dissipative ion fluxes. Those dissipative fluxes, referred to as 'leaks', are caused by the diffusion of ions along the electrochemical gradients either non-specifically or mediated by specific pathways, such as cotransport, antiport, or propagation of electrical signals through voltage-gated channels.

Pump and leak processes are differentially affected by temperature changes. While Na^+/K^+-ATPase displays a Q_{10} of 2–4 (Ellory & Hall, 1987; Raynard & Cossins, 1991; Gibbs, 1995), leak processes are relatively temperature insensitive with Q_{10} values close to unity (Ellory & Hall, 1987; Raynard & Cossins, 1991). The disparity in Q_{10} values of the two opposing processes seems to widen at low temperature: generally, Q_{10} values for most physiological processes, including Na^+/K^+-ATPase activity, increase with decreasing temperature, whereas the Q_{10} for passive K^+ flux in red blood cells is smaller at low than at high temperatures (Hall & Willis, 1986). Accordingly, Raynard & Cossins (1991) found almost identical Q_{10} values for pumps and leaks in rainbow trout red cells between 20 and 10 °C. A disparity occurred only between 10 and 0 °C, where the Q_{10} for Na^+/K^+-ATPase increased to 3.3, while the Q_{10} for passive K^+ flux remained low at 1.4. This differential effect of temperature on ion movements may lead to an excess of dissipative fluxes over active ion pumping during cold exposure unless the organism is able to compensate for the difference in temperature sensitivity of these pathways. The necessity of compensatory mechanisms may be especially important during exposure to temperatures close to freezing, where the mismatch of Q_{10} values is particularly distinct.

Much literature is available on the compensatory mechanisms utilised to maintain ion homeostasis during seasonal acclimation to low temperatures

(Stuenkel & Hillegard, 1980; Raynard & Cossins, 1991; Rady, 1993; Ventrella *et al.*, 1993). However, data on the evolutionary adaptation to subzero temperatures in Antarctic species or polar species in general are scarce. According to the foregoing explanations Antarctic species are confronted with large dissipative ion fluxes, comparable to those in temperate species. This is particularly important in Antarctic teleosts, which are hypoosmotic to the ambient sea water, facing large inward fluxes of inorganic ions that have to be counterbalanced by appropriate rates of active ion pumping. Marine invertebrates, on the other hand, are isoosmotic to seawater and ion balance may therefore be less affected by temperature changes. However, gradients between haemolymph and ambient water are maintained for individual ions such as K^+ and Mg^{2+}. In addition, ion gradients (for Na^+, K^+, etc.) over cell membranes have to be maintained in all organisms despite temperature changes.

Generally, two strategies are possible to compensate for the differential effects of temperature on pumps and leaks: either the activity of ion pumps is upregulated during acclimation or adaptation to cold to match the relatively temperature insensitive dissipative fluxes, or mechanisms are employed to reduce those fluxes (Hochachka, 1988). A unifying trend is not yet visible, research has predominantly been carried out in fish, but much less is known about invertebrates.

Upregulation of pump activity in the cold

A compensatory increase in Na^+/K^+-ATPase activity during (seasonal) cold acclimation has been observed in different tissues of several eurythermal teleosts such as Atlantic cod *Gadus morhua*, trout *Oncorhynchus mykiss*, carp *Cyprinus carpio* or roach *Rutilus rutilus* (Raynard & Cossins, 1991; Schwarzbaum, Wieser & Niederstätter, 1991; Schwarzbaum, Niederstätter & Wieser, 1992*a*; Rady, 1993; Staurnes *et al.*, 1994, cf. Fig. 3). The increase in Na^+/K^+-ATPase activity can either be due to an enhanced number of carrier molecules or to an increased catalytic activity of individual transporters. In the fresh water roach *R. rutilus*, the increase of Na^+/K^+-ATPase activity in kidney and hepatocytes was at least partly due to an increase of the number of pumps as determined by ouabain binding studies (Schwarzbaum *et al.*, 1991, 1992*a*). While the binding sites in hepatocytes increased by a factor of 1.9 between fish acclimated to 20 and 5 °C, a 4.4-fold increase was observed in the kidney. Surprisingly, pump density decreased in gills at low acclimation temperatures despite a positive compensation of Na^+/K^+-ATPase activity (Schwarzbaum *et al.*, 1991). In trout erythrocytes, acclimation temperature had no influence on the number of ouabain binding sites, while total Na^+/K^+-ATPase activity was elevated in the cold (Raynard & Cossins,

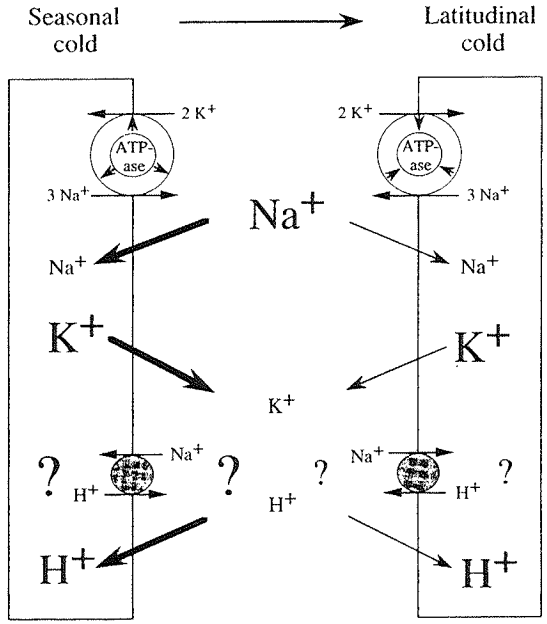


Fig. 3. Modelled depiction of the adjustment of cellular Na^+/K^+-ATPase levels during seasonal or latitudinal cold adaptation. Acclimatisation to seasonal cold is interpreted to reflect the short-term response to cold, whereas latitudinal cold adaptation reflects evolutionary adjustment to cold linked to a secondary reduction of the initial increment of Na^+/K^+-ATPase capacity. Downward arrows represent passive fluxes of the respective ions, the magnitude of dissipative flux being represented by line thickness. Question marks indicate that nothing is known about the potential adjustment of acid–base exchangers (represented by the Na^+/H^+-exchanger) during cold exposure. During seasonal cold the larger question marks indicate that an upregulation of the Na^+/H^+-exchanger is expected owing to large dissipative proton fluxes (see text).

1991). The difference in response between different cell types may correlate with the cellular protein turnover and biosynthetic capacity of the respective tissue. While highly active tissues such as kidney and liver produce more enzymes, tissues with a lower capacity of biosynthesis like red blood cells and gills may increase Na^+/K^+-ATPase activity by increasing the substrate turnover number of existing molecules. Changes in Na^+/K^+-ATPase turnover number may be induced by post-translational modification, such as enzyme phosphorylation, or by non-covalent interactions with allosteric modulators

like cardiotenic steroids or membrane lipids (Blaustein & Hamlyn, 1985; Beguin *et al.*, 1994; Gibbs, 1995). The influence of membrane composition on the activity of membrane-bound enzymes has received considerable attention in the past. It has been proposed that desaturation of membrane lipids during cold adaptation, a phenomenon termed homeoviscous response, may lead to enhanced Na^+/K^+-ATPase activity (Cossins, Bowler & Prosser, 1981; Gibbs, 1995). In several cases, reductions of the level of membrane order following cold acclimation have been correlated with higher rates of Na^+/K^+-ATPase activity (Gibbs & Somero, 1989; Raynard & Cossins, 1991; Schwarzbaum, Wieser & Cossins, 1992*b*; Rady, 1993; see Storelli *et al.*, this volume). However, in the Arctic charr *Salvelinus alpinus* no compensation of Na^+/K^+-ATPase activity occurs during cold acclimation despite a large homeoviscous response (Schwarzbaum *et al.*, 1992*b*). Similar observations were made in trout erythrocytes where changes in membrane composition did not correlate with an increase in Na^+/K^+-ATPase activity in animals caught during the winter (Raynard & Cossins, 1991).

Whether or not cold adaptation also leads to the modulation of other ion transport activities is virtually unknown. In the context of the present study consideration of changes in transporters relevant in acid–base regulation is interesting. During seasonal cold an upregulation of the Na^+/H^+-exchanger is expected owing to a rise in dissipative proton fluxes (see below and Fig. 3).

Hochachka (1988) proposed that, in latitudinal cold adaptation, ion pump capacities are increased in polar compared to temperate zone fish to maintain the balance between pumps and leaks in the cold. A partial compensation may occur for Ca^{2+}-ATPase, since the sarcoplasmatic reticulum (SR) prepared from the fast muscle of the Antarctic *Notothenia rossii* accumulated calcium six times faster than SR prepared from a tropical fish at 0 °C. However, assayed at their respective environmental temperatures, calcium pumping rates were about five times faster in the warm acclimated species (McArdle & Johnston, 1980). Somero, Giese and Wohlschlag (1968) showed that gill filaments from an Antarctic species at 0 °C consume O_2 at the same rate as the goldfish gill at 10–15 °C, which may indicate cold adaptation of Na^+/K^+-ATPase activity. However, comparison of specific activities of Na^+/K^+-ATPase between polar, temperate and tropical species leads to a different picture (Fig. 4). The accumulated data suggest that evolutionary cold adaptation causes a reduction of Na^+/K^+-ATPase activity. In their study on pressure adaptation of Na^+/K^+-ATPase, Gibbs & Somero (1989) investigated Na^+/K^+-ATPase activity in the gills of 19 fish species of different geographical origin including several Antarctic teleosts. Unfortunately, the authors did not present the specific activities of Na^+/K^+-ATPase, but they did mention that Na^+/K^+-ATPase activity was barely measurable in Antarctic fish while activities in other investigated species

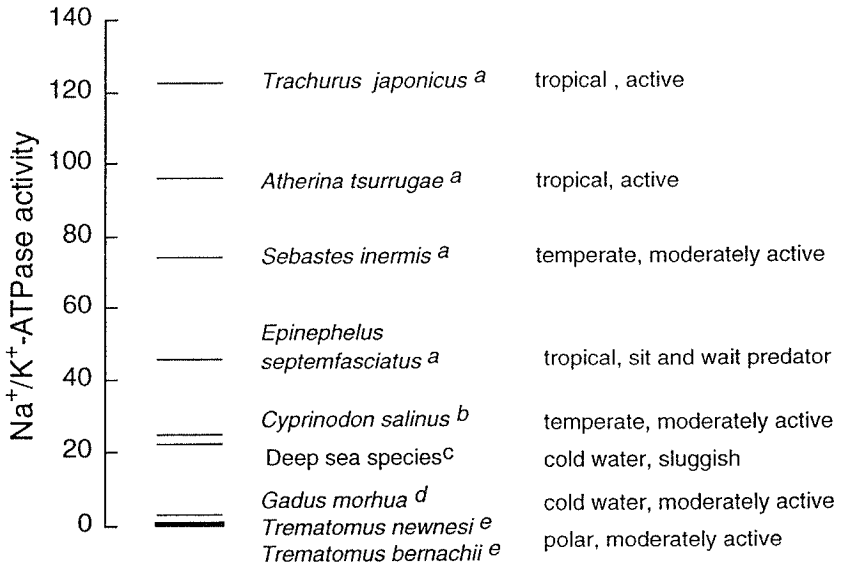


Fig. 4. Semi-quantitative depiction of branchial Na^+/K^+-ATPase activity (μmol P_i mg protein^{-1} h^{-1}) in fish species of different climatic zones and with different lifestyles. The data suggest that evolutionary adaptation to cold goes along with reduced Na^+/K^+-ATPase activities. Enzyme activities were determined at 37 °C or are extrapolated from lower assay temperatures using a Q_{10} of 2. Note that the method of analysis differs between studies and influences the absolute values of Na^+/K^+-ATPase activity. A trend is visible for the activity of Na^+/K^+-ATPase to decline in latitudinal cold. [a] Kamiya & Utida, 1969 (25 °C); [b] Stuenkel & Hillyard, 1980 (37 °C); [c] Gibbs & Somero, 1989 (10 °C); [d] Staurnes *et al.*, 1994 (37 °C); [e] Gonzales-Carbrera *et al.*, 1995 (37 °C).

ranged from 2–10 μmol P_i mg prot^{-1} h^{-1} at 10 °C. These data suggest no positive and possibly even a negative compensation of Na^+/K^+-ATPase activity in the gills of Antarctic teleosts. Accordingly, these animals appear to use a strategy of reduced ion leakage to maintain ion balance in the cold.

Reduction of ion leakage at low temperature

A reduction of ion leakage during cold acclimation has been demonstrated in the Arctic charr *Salvelinus alpinus*. Rb^{2+} efflux in kidney preparations was reduced by a factor of 2.3 at low temperatures (Schwarzbaum *et al.*, 1991). However, an adaptional decrease of membrane conductivity has not been observed in carp or trout erythrocytes (Bourne & Cossins, 1981; Raynard & Cossins, 1991). It is widely accepted that ions pass through membranes via

water-filled pores and channels rather than through the lipid bilayer (Hochachka, 1986). A reduction of ion loss at low temperature may therefore be caused by decreasing channel density or by regulating the activity of existing channels. This phenomenon is termed channel arrest and has been suggested as an adaptive strategy during hypoxic or hypothermal exposure (Hochachka, 1986). Channel inhibition through Ca^{2+}, and modulation of channel activity by phosphorylation of the channel protein, are the most common features of channel regulation (Latorre *et al.*, 1989; Levitan, 1994). However, none of these mechanisms has yet been shown to become involved during cold acclimation. The role of homeoviscous adaptation in the down-regulation of dissipative fluxes during cold acclimation has been discussed (Cossins, Schwarzbaum & Wieser, 1995). In the Arctic charr *S.alpinus*, the reduction of passive K^+ fluxes is accompanied by a nearly perfect homeoviscous compensation (Schwarzbaum *et al.*, 1991). It seems unlikely, however, that membranes with a higher fluidity are less permeable to ions. Proton permeability of the inner mitochondrial membrane in rats is enhanced by the desaturation of membrane lipids (Brand *et al.*, 1992). Therefore, homeoviscous adaptation of the membranes may even lead to enhanced ion leakage in the cold, so the animal must compensate for ion leakage in a different manner.

A very interesting observation with respect to channel arrest during cold exposure was made by Rubinsky, Arav and Fletcher (1991). Using patch-clamp techniques they revealed that antifreeze proteins block ion channels in mammalian tissues and, therefore, prevent ion leakage. This indicates that antifreeze proteins in polar teleosts may serve not only as agents to avoid freezing, but may also have a function in ion regulatory processes.

Another possible mechanism to reduce passive ion fluxes is the reduction of ion gradients over cell membranes, which may be used during exposure to extreme cold. Burton (1986) reviewed the effects of temperature on the ion composition of the blood of several teleosts. The available data suggest that acclimation temperature has only minor effects on ion composition in most cases. Only acclimation to temperatures around 0 °C may lead to increased plasma osmolarities (Burton, 1986). Prosser, Mackay and Kato (1970) determined elevated concentrations of monovalent ions in the plasma of some cold stenothermal fish. Antarctic teleosts show plasma osmolarities about twice that of temperate fish (O'Grady & DeVries, 1982; Gonzalez-Cabrera *et al.*, 1995). This phenomenon agrees well with the lack of a positive compensation of Na^+/K^+-ATPase activity in the gills of Antarctic teleosts, and has been interpreted as an adaptive strategy to reduce the energetic costs of ion regulation (Prosser *et al.*, 1970). However, higher plasma osmolarity imposes higher Na^+ and K^+ gradients over the cell membranes in all tissues, since the ratios of intra- and extracellular concentrations of these ions

remain constant (Prosser *et al.*, 1970). Thus, the energy conserved in the gills by reducing the gradient between plasma and ambient water may be used to counteract enhanced dissipative fluxes over the cell membranes in muscle and nerve tissues. This conclusion is supported by a decrease in muscle Na^+/K^+-ATPase activity in warm acclimated *Trematomus bernacchii* and *T. newnesi* where plasma osmolarity is reduced to values comparable to those in temperate species (Gonzalez-Carbrera *et al.*, 1995). Therefore, the role of hyperosmolarity of Antarctic teleosts in energy conservation remains questionable. Investigations by O'Grady & DeVries (1982) indicate that Antarctic fish are able to actively regulate plasma osmolarity despite changes in ambient salinity. Therefore, plasma osmolarity is not determined by the insufficient capacity of ion regulation but is actively adjusted by a shift of the set point of the ion pumps involved. Accordingly, elevated ion concentrations in the plasma may serve to decrease the freezing point of the plasma rather than represent a strategy to reduce the cost of ion regulation in the cold (O'Grady & DeVries, 1982).

The above survey suggests that two different strategies may be used to maintain ionic equilibrium during temperature changes. Eurythermal species like cod *Gadus morhua*, trout *Oncorhynchus mykiss* and roach *Rutilus rutilus* display a positive compensation of ATPase activity either by enhancing the number of enzyme molecules or by modulating the turnover number of existing proteins. The energetic costs of ion regulation in fish have been estimated to be 25–30% of the total metabolic rate, based on oxygen consumption measurements in euryhaline teleosts at different salinities (Rao, 1968; Febry & Lutz, 1987). A lower estimate of 10% is given by Gibbs & Somero (1989) based on maximum Na^+/K^+-ATPase activities in the gills of marine teleosts. The compensatory strategy used by eurythermal species implies that the energy requirement for ion regulation remains almost unchanged and comprises a larger fraction of energy turnover during cold acclimation when the overall metabolic rate is depressed (Fig. 3). Indeed, Rao (1968) determined that a larger percentage of the standard metabolic rate is attributed to ion regulatory processes in rainbow trout at 5 °C than at 15 °C.

As an alternative strategy, dissipative ion fluxes are reduced in the cold-stenothermal teleost *Salvelinus alpinus*. This energy saving strategy may also be used by Antarctic species through a reduction of osmotic gradients and through possible inhibition of ion leakage by antifreeze proteins. This strategy may be even more important when the mode of life allows for maximum reduction of ion exchange mechanisms, as has been discussed extensively in a comparison of lifestyles and energy savings in Antarctic vs. deep sea fish (for recent reviews, see Thiel *et al.*, 1996; Somero, this volume).

The previous discussion suggests that the change in the cost of ion regulation in polar fish may not be substantially different from the respective

change expected for marine invertebrates. Actually, similar mechanisms may be involved in the reduction of dissipative ion fluxes. In general, the reduced cost of ion regulation at low temperature may help to shift the low critical temperature to below polar ambient temperature by reducing the energy requirements of the animals and the extent to which mitochondrial proliferation would otherwise be required. These cost reductions may also free enough energy for the purpose of growth and reproduction. Further research is needed to substantiate the different strategies discussed in this section.

Integrative signals of cold adaptation

Alphastat-regulation of pH

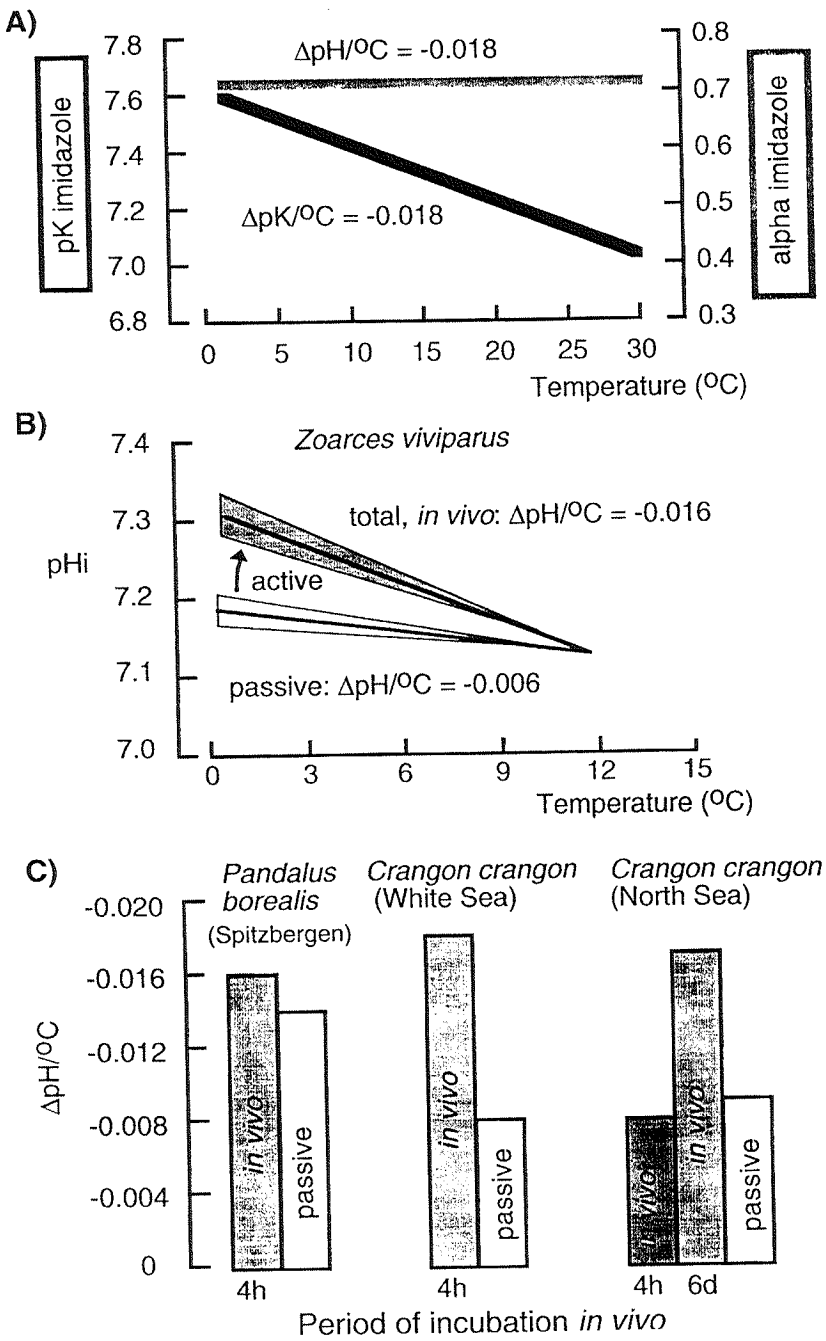
One important aspect of ionic regulation is the regulation of pH, which is known to be a parameter that integrates cellular functions (Busa & Nuccitelli, 1984). Passive proton distribution over the cell membrane is determined by the membrane potential and would lead to a ΔpH of about one pH unit between intra- and extracellular compartments (Thomas, 1984). A typical pH gradient of 0.4–0.6 pH units is maintained in most vertebrates and invertebrates, which is achieved by active proton extrusion from the cell through H^+-ATPases or by secondary active ion exchange, mostly via the Na^+/H^+-exchanger, which depends upon the Na-gradient established by Na^+/K^+-ATPase (see above, Fig. 3). Accordingly, an ATP cost of acid-base regulation has been quantified (A. Reipschläger & H.O. Pörtner, unpublished observations). The Na^+/H^+-exchanger displays an unusually high Q_{10} of 7.9 in trout erythrocytes (Cossins & Kilbey, 1990). In the cold, even a reduction of pH gradients between intra- and extracellular spaces has been observed (Heisler, 1986*b*; Sommer *et al.*, 1997), which would require enhanced rates of proton extrusion. Possibly, an up-regulation of Na^+/H^+-exchanger activity is required, associated with the larger deviation from thermodynamic equilibrium (see Fig. 3). However, these conclusions are valid only if the membrane potential remains unchanged during cold exposure.

A study of temperature effects on the regulation of pH may also give further insight into the regulatory signals causing shifts in critical temperatures. This question is not restricted to how the limiting temperatures are set but also to how the animals adjust to those between the critical temperatures. Each temperature change will not just mean a change in overall energy turnover according to Q_{10}, but also requires a readjustment of energy production and consumption, as discussed before. The temperature-dependent regulation of pH may relate to the fine tuning of these adjustments and support maintenance of steady-state function at different temperatures.

Reeves (1972) introduced the imidazole α-stat (=alphastat) hypothesis

postulating that poikilotherms regulate the pH of their body fluids such that the degree of protonation (α) of imidazole groups is maintained despite changes in body temperature. α-stat pH regulation has been under reinvestigation during recent years, both concerning its existence or not in various tissues and animal species, and its potential importance for enzymatic regulation. Briefly, the alphastat process is proposed to play an important role in the maintenance of the structural integrity of proteins, which appears to be a prerequisite for the maintenance of cellular, especially enzyme functions (Hochachka & Somero, 1984). Since, on average, the pK of imidazole groups changes at -0.018 units $°C^{-1}$ a shift in intra- and extracellular pH with body temperature by $\Delta pH/\Delta T \sim -0.018\ °C^{-1}$, as observed in many poikilotherms, is sufficient to keep histidine protonation (α) constant (Fig. 5 A). However, the picture is not uniform and deviation from intracellular α-stat control has been observed in many species and tissues (Heisler, 1986*b*; Butler & Day, 1993; Whiteley & Taylor, 1993; Whiteley *et al.*, 1995*a*,*b*). $\Delta pH/\Delta T$ values range from $-0.003\ °C^{-1}$ in crayfish claw muscle (Whiteley *et al.*, 1995*a*) to $-0.031\ °C^{-1}$ in the red muscle of the dogfish *Scyliorhinus stellaris* (Heisler & Neumann, 1980). Some of these studies may not have considered the existence of critical temperatures beyond which the onset of anaerobic metabolism and a shift of the setpoints of pH regulation (Pörtner, 1993) may lead to the observed deviation from a linear pH/temperature relationship (Sommer *et al.*, 1997). Therefore, we are interested in the temperature range of α-stat control and in the mechanisms involved as well as in how these mechanisms are influenced by environmental change.

Animals acclimated to low temperatures during the winter season frequently exhibit relatively low pH values deviating from the alphastat pattern. Low intracellular pH has been proposed as a key mechanism eliciting metabolic depression, for example, in hibernating mammals (Malan, 1985; for further examples and recent review, see Hand & Hardewig, 1996). Accordingly, the shrimp *Palaemon elegans* tends to be inactive at temperatures below 10 °C in winter, metabolic depression being reflected by a drop in intracellular pH and an increase in the concentration of sugar phosphates (Thebault & Raffin, 1991, Fig. 6). Low pHi values were also reported by Whiteley *et al.* (1995*a*) for the crayfish *Austrapotamobius pallipes* in winter. Only tissues like abdominal muscle, which remain operative in winter, followed α-stat while less active tissues like claw muscle and hepatopancreas did not. The authors explained the relative acidosis in these tissues by low rates of protein synthesis and a lowered overall metabolic rate at low temperatures when crayfish are inactive. Based on these results they speculated that the relative acidosis observed in the haemolymph of *Glyptonotus antarcticus* and *A. pallipes* may be characteristic of crustaceans living at low temperatures when rates of protein synthesis and possibly catabolism are low (Whiteley *et al.*,



1995*b*). In contrast, a reduction in metabolic activity could not be observed in *C. crangon* originating from the North Sea in winter (Sartoris & Pörtner, 1997*a*,*b*) and, possibly as a consequence, both winter and summer animals followed the α-stat pattern.

The question remains open whether lower pH for lower metabolism is true in all cases and whether a drop in pHi causes or is only a correlate of metabolic depression. To save energy, animals may tolerate passive changes of pHi during periods of relative inactivity, since the active transport of proton equivalents depends on energy supply. A down-regulation of ion exchange could lead to a reduction of pHi since pH would then approach thermodynamic equilibrium. In *S. nudus* extracellular pH is the key acid–base parameter eliciting metabolic depression during acidosis. Metabolic depression is not induced by moderate (0.3 pH units) decreases in pHi (Reipschläger & Pörtner, 1996). Since extracellular pH appears to play a predominant role in metabolic regulation, future work should investigate whether the observed changes in the relationship between intra- and extracellular pH with temperature (see above) affect metabolic rate.

The assumption that a deviation from α-stat at low temperatures is a correlate of metabolic depression deserves further consideration. In this context, it is interesting to determine the pH/temperature relationship in

Fig. 5. Changes in the pH of body fluids with temperature, the pattern of which is considered to be important for the maintenance of physiological functions. A) Effects of temperature on the dissociation constant for protons (pK) of the imidazole group in histidine, an important amino acid component of proteins. A parallel change in pK and cellular pH at -0.018 $°C^{-1}$ is postulated to occur in 'cold-blooded' animals and maintains the degree of dissociation, α-imidazole, which is thought to be essential for protein structure and function (alphastat hypothesis). B) Contributions of passive and active processes to adjustments in intracellular pH after temperature change in white muscle of *Zoarces viviparus*. pH_i decreases with rising temperature at a slope of -0.016 $°C^{-1}$. The framed areas represent the standard error of the slopes. **shaded frame**: pH/temperature relationship determined *in vivo* as the sum of active and passive mechanisms. **open frame**: pH/temperature relationship *in vitro*, representing the passive response of intracellular buffers to temperature change. C) Contributions of passive processes to the adjustment of intracellular pH *in vivo* (shaded) after temperature change (ΔpH $°C^{-1}$) in boreal (North Sea) and subarctic (White Sea) populations of *Crangon crangon* and *Pandalus borealis*. Intracellular pH was analysed after 4 hours (h) (*P. borealis* and *C. crangon* from the White Sea) and after 4 h and 6 days (d) (North Sea *C. crangon*) of incubation at various temperatures. Note that pH regulation was complete after 4 h in White Sea animals, whereas it was delayed and only found complete after 6 days in North Sea specimens (after Sartoris & Pörtner, 1997*a*; van Dijk *et al.*, 1997).

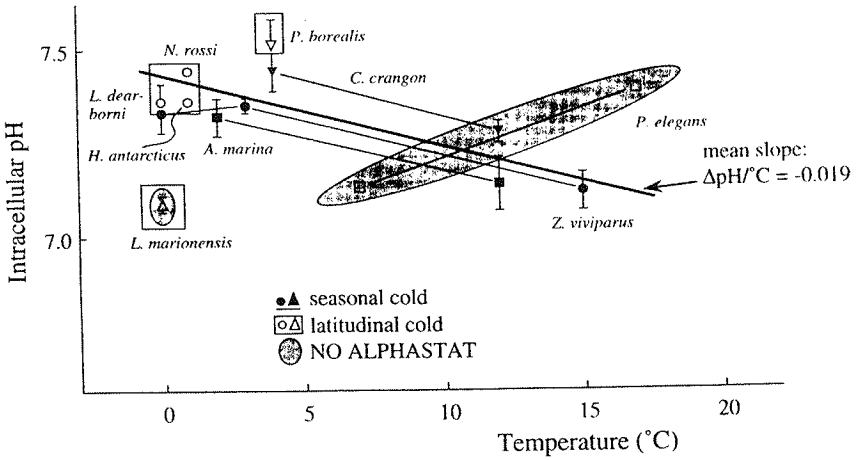


Fig. 6. Realisation of intracellular α-stat control during seasonal or latitudinal cold in marine ectotherms: Fish (Antarctica): *Harpagifer antarcticus* (white muscle, Egginton & Moerland, 1993, SD not shown), *Notothenia rossi* (red blood cells, Egginton *et al.*, 1991, SD not shown), *Lycodichthys dearborni* (white muscle, I. Hardewig, P. van Dijk & H.O. Pörtner, unpublished data), *Zoarces viviparus* (white muscle, North Sea; van Dijk *et al.*, 1997). Invertebrates: *Pandalus borealis, Crangon crangon* (tail muscle, Sartoris & Pörtner, 1997*a*), *Arenicola marina* (body wall muscle, Sommer *et al.*, 1996, 1997) *Palaemon elegans* (tail muscle, Thebault & Raffin, 1991), *Limopsis marionensis* (adductor muscle, H.O. Pörtner, S. Zielinski & L.S. Peck, unpublished data). Methods used by the various authors include the homogenate technique (Pörtner *et al.*, 1990) and ^{31}P-NMR.

species from a latitudinal temperature gradient as a test whether the activity level or a temperature-induced reduction of metabolism towards polar areas influences α-stat control. Actually, measurements in the deep water shrimp *Pandalus borealis* (Spitzbergen) and the Antarctic eelpout *Lycodichthys dearborni* indicate that cold-stenothermal animals follow α-stat regulation of intracellular pH (Fig. 6). The finding of α-stat-pHi by ^{31}P NMR in the Antarctic teleost *Harpagifer antarcticus* (Egginton & Moerland, 1993) supports this conclusion. However, a uniform picture is, again, not evident. For example, pH values in the Antarctic bivalve, *Limopsis marionensis,* do not reflect α-stat regulation (Fig. 6). It remains to be established whether this is a consequence of hypometabolism. Reduced activity at low temperatures could not explain the deviations from α-stat in the brown trout *Salmo trutta*, where a reduction in standard metabolic rate or swimming ability could not be observed in winter acclimated fish (Butler & Day, 1993). In this species pH

was independent of temperature in the musculature, whereas α-stat regulation was observed in the blood plasma. In contrast, a reduction of swimming performance was found in rainbow trout at low temperature which was supposedly associated with relatively acidotic intra- and extracellular pH values deviating from the α-stat pattern only at low temperatures (Taylor, Taylor & Egginton, 1993, 1996).

α-stat pH control was observed in the body wall musculature of *A. marina*, whereas coelomic fluid pH remained independent of changing temperature (Sommer *et al.*, 1997). An important point to be considered in such analyses is that α-stat pH regulation only occurs in the range between low and high critical temperatures. pH becomes independent of temperature beyond these thresholds (Sommer *et al.*, 1997). Accordingly, the temperature window for α-stat regulation is expected to be much smaller in polar species than in temperate zone species as verified in the Antarctic bivalve *Limopsis marionensis* (H.O. Pörtner, S. Zielinski & L.S. Peck, unpublished data).

The mechanisms of α-stat control have not yet been sufficiently characterised. According to Reeves (1985), α-stat regulation consists of both passive and active components. The passive component depends upon the physicochemical composition of intra- and extracellular buffers and results from proton binding or release owing to the change in pK values and associated dissociation equilibria of the buffer components with temperature. The active component comprises adjustments in either ventilation or ion exchange or both. In air breathers, ventilation changes with temperature and causes a shift in P_{CO_2} associated with a pH shift. In water breathers, ion exchange predominantly determines the active component of the temperature-induced pH change. Distinguishing between respiratory and ion exchange mechanisms is very important in order to understand the velocity of α-stat regulation. Ventilatory adjustment is faster than the adjustment of a new steady state of acid–base homeostasis by means of ion transport across gills and cell membranes.

Previous model calculations of the relative contributions of various processes to pHi adjustment exclusively relied on the $\Delta pK/\Delta T$ value of the imidazole group. However, $\Delta pK/\Delta T$ depends upon local charge configurations in the environment of the imidazole group as well as on ionic strength and, therefore, varies between -0.016 and $-0.024\ ^{\circ}C^{-1}$ for histidine and free imidazole compounds (Heisler, 1986*a*,*b*), and ranges from -0.0010 to $-0.051\ ^{\circ}C^{-1}$ for histidine residues in proteins, leading to a large uncertainty about the accuracy of these model calculations.

These theoretical problems could be solved by experimental analyses of active and passive processes. The homogenate technique (Pörtner *et al.*, 1990) allows the rapid measurement of pHi in tissue samples and to distinguish active and passive elements in α-stat pH regulation. To quantify

passive mechanisms, animals were exposed to control temperature and their tissues were analysed *in vitro* at different temperatures, thereby excluding the influence of biochemical reactions or ion exchange mechanisms. Therefore, measured pH changes result from passive, physicochemical buffering. In contrast, *in vivo* values determined in animals exposed to various temperatures prior to the collection of tissue samples can be interpreted to reflect the summed effects of active and passive processes (Fig. 5 B). The passive component comprises fast and temperature dependent proton binding or release by intracellular buffers, and the active component in water breathers represents ATP-dependent ion exchange which is considerably slower.

The results of these analyses suggest that the contribution and velocity of active processes to the pH shift differs between species and populations from different latitudes (Sartoris & Pörtner, 1997*a*; van Dijk, Hardewig & Pörtner, 1997; Fig. 5B and C). In the North Sea eelpout, *Zoarces viviparus*, 65% of the pH changes were elicited by active, energy requiring ion transport mechanisms, whereas only 35% were contributed by passive buffer processes. In the eurythermal shrimp *Crangon crangon*, from both the Russian White Sea and the North Sea, 50% of the pH change occurred by means of active mechanisms. Also, the passive $\Delta pHi/\Delta T$ relationship was identical in summer and winter animals from the North Sea ($\Delta pHi/\Delta T = -0.009$ units °C^{-1}), suggesting that passive pHi adjustment does not depend upon the season and that the concentrations of relevant intracellular buffer substances remains unchanged (Sartoris & Pörtner, 1997*a*). In contrast, the active component of pH regulation amounted to only 10% in the Arctic deep sea shrimp *Pandalus borealis*, which is more sensitive to temperature fluctuations (stenothermal). In conclusion, the active component may be more prominent in eurythermal species. However, since these are the first results on the relative contribution of active and passive processes to α-stat regulation in polar animals, it may only be speculated that pH adjustment mostly occurs by passive mechanisms in cold stenothermal ectotherms, with a small contribution of active components. Future work is required to show whether the relative contribution of active and passive mechanisms to α-stat regulation is involved in determining the limits of temperature tolerance and is related to the geographical distribution of species. It may be important in this context that stenothermal animals are often stenohaline and, therefore, the capacity of ion regulatory mechanisms may be less developed than in euryhaline species which frequently are also eurythermal. These findings are also relevant with respect to the allocation of energy to acid-base regulation. Cold-stenothermal (and stenohaline) species may reduce the energy requirements of α-stat regulation by using predominantly passive mechanisms for pH adjustment, whereas eurythermal (and euryhaline) species emphasise ion regulatory mechanisms to allow for a flexible response to environmental change. A larger active than

Table 1. *Temperature-dependent elements influencing the Gibbs' free energy of ATP hydrolysis and thus cellular energy status* in vivo

1. pK values of H^+ and Mg^{2+} binding to
   ATP^{4-}, $HATP^{3-}$, ADP^{3-}, $HADP^{2-}$, AMP^{2-}, Pi^{2-}, PLA^-

2. Reaction equilibra (simplified) of

   ATPase
   $MgATP^{2-} + H_2O \rightarrow MgADP^- + HPO_4^{2-} + H^+$

   Arginine kinase
   $PLA^- + MgADP^- + H^+ \rightarrow MgATP^{2-} + L\text{-}Arg^+$

   Adenylate kinase
   $MgADP^- + MgADP^- \rightarrow MgATP^{2-} + MgAMP$

   ⇒ ***ATP free energy change***

   $dG/d\xi_{ATP} = \Delta G^{\circ}obs + RT \cdot \ln ([ADP]_{free,tot} \cdot [Pi]_{free,tot} / [ATP]_{free,tot})$

   $dG/d\xi_{ATP} = \Delta G^{\circ}obs + RT \cdot \ln ([L\text{-}Arg]_{free,tot} \cdot [Pi]_{free,tot} / [PLA]_{free,tot} \cdot [H^+] \cdot Kapp_{AK})$

Note:
Apparent equilibria determined experimentally will include all species complexed with H^+ and/or Mg^{2+}, but unbound to cellular protein $[X]_{free,tot}$.
Source: cf. Figure 7; for the calculation procedure see Pörtner *et al.* (1996).

passive component of α-stat regulation may therefore be a prerequisite to colonise shallow coastal waters.

An additional result of these comparisons was that, in White Sea *Crangon*, active pH regulation was faster and, therefore, reached the final pH earlier than in North Sea animals (Fig. 5 C). Obviously, the velocity of these active ion exchange processes was increased as a consequence of metabolic cold adaptation. Animals living at lower temperatures may, in general, be able to compensate acid–base disturbances faster. In the cold stenothermal *Pandalus borealis* the faster response can be attributed to the large passive fraction, while in the more eurythermal White Sea *Crangon crangon* cold adaptation is likely to increase the capacity of pH regulatory mechanisms.

Factors limiting tolerance to cold exposure: cellular energetics?

As outlined above, critical temperatures characterise the onset of functional hypoxia and an anaerobic metabolism and, thereby, a time-limited situation.

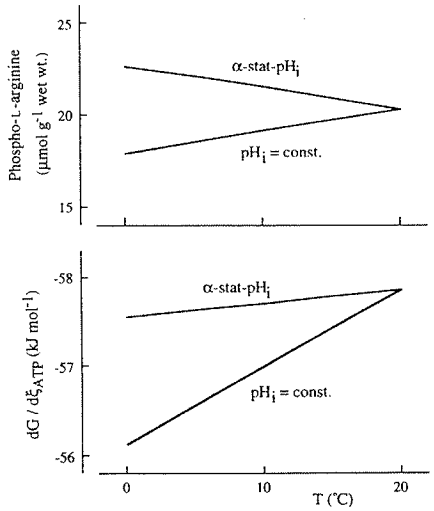


Fig. 7. Model calculations emphasise the importance of α-stat regulation for the maintenance of energy homeostasis during cooling. The calculation of the Gibbs' free energy change of ATP hydrolysis at different temperatures followed the rationale outlined by Pörtner *et al.* (1996). Numbers used are valid for the mantle muscle of the squid, *Lolliguncula brevis*. Levels of phospho-L-arginine were calculated from the relevant equilibrium of arginine kinase. Enzyme equilibria were calculated for each temperature by use of the van't Hoff equation. Fractional levels of reaction partners as they varied with fluctuating Mg^{2+} levels and pH were calculated from the respective dissociation equilibria (for details see Pörtner *et al.*, 1996). If it is assumed that the cells maintain constant levels of phospho-L-arginine plus L-arginine, as well as free ATP, ADP, Pi and Mg^{2+}, the concentrations of phospho-L-arginine and the level of Gibbs' free energy change of ATP hydrolysis will be maintained by α-stat pH regulation compared to constant pH. With α-stat control ATP free energy change will only fall by 0.3 kJ mol^{-1}, whereas a drop by about 1.7 kJ mol^{-1} is expected when pH remains constant.

Obviously, α-stat regulation of pH is also restricted to the specific window between critical temperatures of a species. The question arises which mechanisms limit tolerance to cold below the low Tc. As a precondition of low temperature tolerance, the maintenance of energy status as it depends upon temperature and the regulation of intracellular pH will be discussed. The onset of anaerobic metabolism is usually associated with a net decrease in cellular energy levels and may cause disturbances of ionic distribution and α-stat control below the Tc.

The actual energy level of a cell is quantified by the *in vivo* Gibbs' free energy change of ATP hydrolysis, a concept which has only recently been

applied to studies of marine ectotherms (Pörtner, 1993; Pörtner *et al.*, 1993; Combs & Ellington, 1996; Pörtner, Finke & Lee, 1996, Pörtner *et al.*, 1997; Zielinski & Pörtner, 1996). The assessment of cellular energy levels depends upon the quantification of a series of factors compiled in Table 1 and the consideration of their temperature dependence in relation to the regulation of intracellular pH during temperature change (Fig. 7). The results of this analysis emphasise the importance of intracellular pH in energy homeostasis since α-stat pH regulation supports maintenance of the levels of the phosphagen, phospho-L-arginine and of the Gibbs' free energy change of ATP hydrolysis. This becomes evident from the depiction in Fig. 7 which shows that the maintenance of a constant pH with falling temperature will cause a decrease in phospho-L-arginine and ATP free energy change values.

The question arises of how the results of these model calculations compare with the *in vivo* situation. Figure 8 depicts the levels of ATP free energy change as evaluated for the body wall musculature of a marine sipunculid worm during cooling. Intracellular pH follows α-stat predictions until temperature falls below the critical threshold found between 4 and 0 °C for this species (see above). The ability of the animals to recover ceased after longer than 2 days of exposure to 0 °C. Intracellular pH returned to control values when recovery was still possible, but intracellular pH continued to fall at temperatures below the critical temperature, reaching even lower values in those animals which were brought back to 12 °C but were no longer able to recover. The correlated fall in ATP free energy change levels to a low, possibly critical value (Fig. 8 B) may characterise the limitation of survival (point of no return) and contribute to the development of lethal cold injuries (Zielinski & Pörtner, 1996). It remains to be investigated which cellular functions would be affected by the decrease in cellular energy levels. Similarly, low energy levels may indicate irreversible temperature stress in the Antarctic bivalve *Limopsis marionensis* where an upper critical threshold is surpassed when temperature rises from 0 to values between 2 and 4 °C (Pörtner, H.O., S. Zielinski & L.S. Peck, unpublished data).

Summary and conclusions

Critical temperature thresholds (Tc) can be defined for various invertebrate species which are characterised by the transition to an anaerobic mode of metabolism, once temperature reaches low or high extremes. Beyond critical temperatures, it is not the availability of ambient oxygen that is limiting. Both low and high Tcs are either set by the failure of oxygen uptake and transport in the blood, or by the insufficiency of ventilatory mechanisms in the cold. Metabolic cold adaptation can be understood as a downward shift of both low and high critical temperatures. Critical temperatures shift

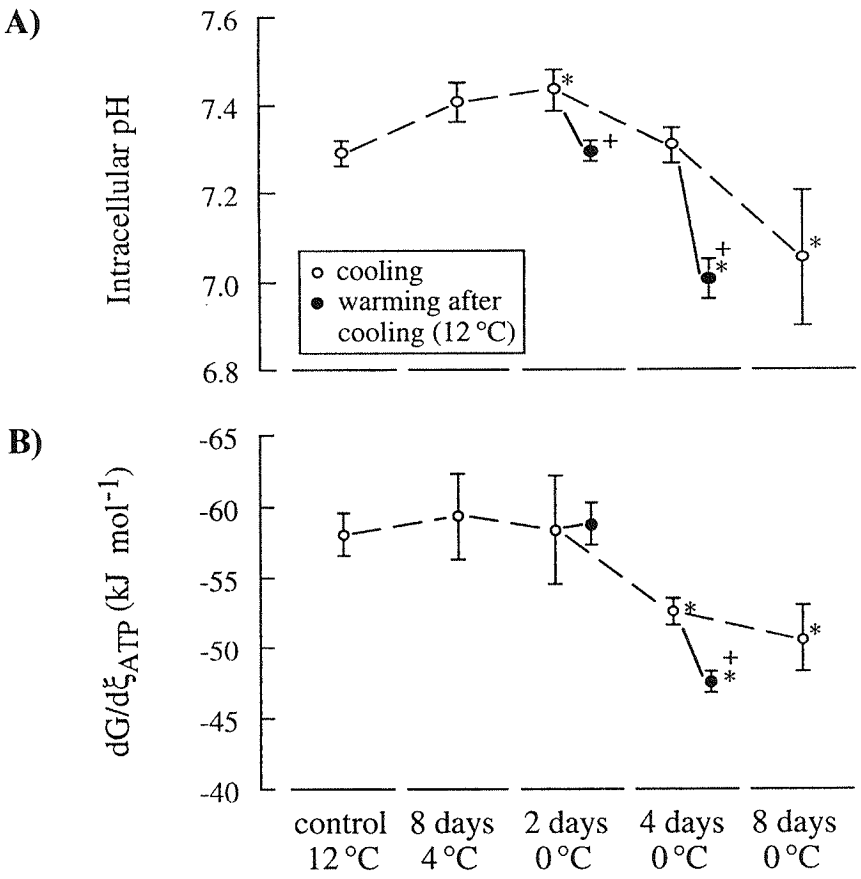


Fig. 8. A) Intracellular pH determined in the body wall musculature of the sipunculid worm, *Sipunculus nudus,* after different periods of exposure to control temperature (12 °C) and to decreasing temperatures (4 and 0 °C). The ability to recover from cold exposure was tested after 2 and 4 days at 0 °C, respectively. Animals were no longer able to recover after 4 days, when a severe metabolic acidosis developed during warming, which was partly caused by the irreversible accumulation of anaerobic end products (not shown). B) Energy content of ATP, quantified as the Gibbs' free energy change of ATP hydrolysis $dG/d\xi_{ATP}$, in the body wall musculature of *S. nudus* during long term exposure to cold temperatures and subsequent rewarming to 12 °C. Critical exposure is indicated by the fall in energy levels associated with the failure to recover from exposure to 0 °C for longer than 2 days (after Zielinski & Pörtner, 1996).

within, and differ between, populations depending on seasonal temperature adaptation and latitude. These differences may be related to genetic distances between populations. Low thresholds significantly above freezing observed in temperate zone species have been eliminated during evolutionary low temperature adaptation in polar ectotherms. In these organisms this process has led to a high sensitivity to elevated temperatures. The shift of the low Tc to below polar temperature is thought to require mitochondrial proliferation, but also a reduction in the basic cost of some ATP-dependent cellular functions. Both critical thresholds will be affected if the Tcs are set by mitochondrial density, which increases during cold adaptation but will then have to decrease during warm adaptation. The disadvantage of mitochondrial proliferation, in that a higher density of mitochondria causes a rise in energy turnover by itself, may be compensated to some extent. With a high mitochondrial density in polar species, the metabolic 'idling' of individual mitochondria may be reduced by membrane modifications which lead to a reduction in proton leakage and thus energy expenditure. Dissipative ion fluxes across cell membranes and epithelia are reduced at low temperatures allowing the energy turnover of Na^+/K^+-ATPase to decrease. Such a decrease appears to be the strategy found in animals exposed to permanent (polar) as opposed to seasonal cold and even more so in deep sea fish, where a mode of life more sluggish than found in Antarctic fish supports the development of such a strategy even further. The question of a potential reduction in the cost of acid–base regulation remains open.

Critical temperatures may also set the limits for an adjustment of pH regulation to changing temperatures. A rise in pH with falling temperature as required to maintain the degree of protonation (α) of imidazole moieties in proteins and, thus, protein function, is no longer observed beyond low or high critical temperatures. Alpha-stat regulation of pH supports the maintenance of cellular energy levels quantified as the Gibbs' free energy change of ATP hydrolysis. A decrease in these energy levels seen during lethal cold exposure below the Tc is interpreted to limit cold tolerance and impair ATP dependent cellular functions. Low levels of available energy may, for example, compromise acid–base and ionic regulation as well as muscular function relevant in the maintenance of ventilatory and circulatory function.

Future efforts must address the regulatory integration of the different biochemical and physiological processes depending on the temperature regime focusing on pH, both as a regulated parameter and a parameter effective in metabolic regulation. Those molecular mechanisms which are responsible for setting the critical temperatures need to be identified as well as those which are modified for a change in critical temperature values. Furthermore, it needs to be evaluated further to what extent the temperatures critical for physiological processes are related to distributional limits of a species in the

natural environment. These efforts should also address to what extent the temperature-induced transition to anaerobic metabolism causes behavioural changes and migratory movements under environmental stress, as has recently been demonstrated for terrestrial ectotherms (Pörtner *et al.*, 1994).

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B.D. SIDELL and M.E. VAYDA

Physiological and evolutionary aspects of myoglobin expression in the haemoglobinless Antarctic icefishes

Fish fauna of the Southern Ocean present an unique combination of biological characteristics and evolutionary history compared with those from other marine systems. Two features figure prominently in setting these organisms apart from fishes of temperate zone and even polar boreal seas.

First, the level of endemism of Antarctic fishes is unparalleled in other ocean systems. Of the 250+ species of fish known to inhabit the Southern Ocean, the dominant group, in terms of both species numbers (>100) and abundance (50–90% of captures) are members of the perciform suborder Notothenioidei (Dewitt, 1971; Anderson, 1990; Eastman, 1993). With few exceptions, fishes of the six notothenioid families are indigenous to waters surrounding Antarctica where they have evolved during the last 25–40 My in isolation under conditions that are both thermally stable and severely cold. Within this monophyletic group are species displaying a wide diversity of ecologies and life histories, from sluggish demersal to active pelagic habits.

The second major feature that sets Antarctic notothenioid species apart from the ichthyofauna of other marine systems is their long geographical isolation in waters that are the most severely cold, thermally stable aquatic habitat on the planet. The best estimates are that thermal isolation of Antarctica began with the development of circumpolar currents in the late Oligocene and was followed shortly thereafter with the establishment of the Antarctic Convergence (about 20 million years ago) (Kennett, 1977, 1980). The demise of most non-notothenioid fishes and radiative expansion of this suborder in coastal Antarctica apparently began with the significant ocean cooling that predated these events (Anderson, 1990). At present, mean annual temperature in McMurdo Sound is −1.86 °C and varies only by about 0.1 °C seasonally (Littlepage, 1965). The Antarctic Peninsula shows only slightly greater variance with average summer and winter temperatures running between −1.1° and +0.3 °C (summer) and −1.1 °C in winter (Dewitt, 1971).

The very cold oxygen-rich waters of the Southern Ocean coincidentally provide both challenges and benefits with respect to respiratory requirements for oxygen. On the benefit side, exceptionally cold body temperature