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Energy metabolism and ATP free-energy change of the intertidal worm *Sipunculus nudus* below a critical temperature

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Abstract The intertidal worm *Sipunculus nudus* was exposed to various temperatures for an analysis of the integrated changes in energy and acid-base status. Animals were incubated in sea water or maintained for up to 8 days at 4 and 0 °C while dwelling in the sediment. Cannulation of the animals prior to experimentation allowed the analysis of blood gas parameters (P_{O_2} , P_{CO_2} and pH). P_{O_2} fell to 0 torr within 8 days at 0 °C. A simultaneous reduction of ventilatory activity was derived from measurements of the pattern of coelomic fluid pressure changes associated with ventilatory movements. The increase in P_{CO_2} and an onset of anaerobic metabolism, indicated by the accumulation of end products like acetate and propionate both in the coelomic fluid and the body wall musculature, led to the development of a progressive acidosis and a deviation from the alaphastat regulation of intracellular pH seen in unburied animals. The drop in intracellular pH together with the depletion of the adenylates and the phosphagen, phospho-L-arginine, reflect a significant decrease in the Gibb's free-energy change of ATP hydrolysis. These changes are interpreted to indicate lethal cold injuries, because recovery was not possible when the animals were returned to 12 °C after more than 2 days of exposure to 0 °C. A low critical temperature indicating the onset of cold-induced anaerobiosis is concluded to exist below 4 °C owing to the insufficient response of the ventilatory system to the developing hypoxia.

Key words Cold-induced anaerobiosis · Acid-base status · Blood gas parameters ·

Gibb's free-energy change of ATP hydrolysis · Intracellular pH · Critical temperature

Abbreviations ΣAde summed concentrations of ATP, ADP, AMP · ΣArg summed concentrations of L-arginine containing metabolites · *L-Arg* L-arginine · $dG/d\xi$ Gibb's free-energy change of ATP hydrolysis · *PCA* perchloric acid · pH_e extracellular pH · pH_i intracellular pH · P_i inorganic phosphate · *PLA* phospho-L-arginine · T_c critical temperature

Introduction

Intertidal animals like *Sipunculus nudus* are frequently subjected to temperature changes between tidal cycles, as well as to seasonal temperature fluctuations. For many invertebrates it has been shown that an increase in temperature leads to an increase in O_2 consumption until a temperature threshold is surpassed, above which O_2 consumption decreases (Newell and Pye 1970a, b; Kristensen 1983). Furthermore, temperature influences the ventilation rate. Baumfalk (1979) demonstrated for *Arenicola marina* that the pumping rate increases linearly with temperature between 5 and 22 °C (for further examples see Discussion). However, some species inhabiting the intertidal zone may exhibit rates of physiological activity which are temperature independent (Newell and Pye 1970a, b). Some investigators looked at temperature-induced changes in biochemical tissue composition depending on the season. Zandee et al. (1980) for instance showed that the concentrations of proteins, glycogen and lipids in the muscles of *Mytilus edulis* rose in autumn. In winter at low temperatures these stores were broken down.

Temperature not only affects metabolism but also the acid-base status, usually in such a way that pH rises as temperature decreases. Reeves (1972) postulated that pH is not kept constant and independent of temperature but typically changes at -0.017 pH units per °C

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($\Delta\text{pH}/\Delta T$) such that the dissociation of the histidine imidazole residues of proteins is kept constant (alphastat hypothesis). The constant degree of protonation will maintain the functional properties of a protein. This is supported by the finding that activities of several enzymes were almost unchanged when pH was increased according to alphastat during a temperature decrease, while a temperature drop at constant pH led to significant changes in enzyme activities (Hazel et al. 1978; Michaelidis and Storey 1990). As a corollary, pH regulation and metabolic regulation are interdependent processes.

The studies mentioned so far have not addressed the question of whether energy metabolism remains completely aerobic over the whole temperature range investigated. Murphy (1977) suggested for *Modiolus demissus* that anaerobic metabolism may become involved in energy production at low temperatures. His conclusion was based on the finding of elevated alanine and proline concentrations. Since proline is not an end product and alanine no major product of anaerobic metabolism the data are not sufficient for the conclusion that an onset of anaerobic metabolism occurs. Therefore, the present study was designed to investigate the influence of long-term exposure to low temperatures on metabolism and acid-base status of *Sipunculus nudus*. This worm irrigates its burrow by peristaltic waves of the body wall moving from head to tail, which induces a water current along the body surface (Pörtner 1982). Since the body of worms like *Sipunculus nudus* works as a hydraulic system, all movements are related to pressure changes in the coelomic fluid produced by the musculature (Zuckerandl 1950; Trueman and Foster-Smith 1976). When ventilation of *Sipunculus nudus* is reduced at low temperatures this might induce internal hypoxia. Particularly interesting was the question whether anaerobic metabolism truly becomes involved in energy production and how the temperature threshold for a potential transition to anaerobiosis is defined.

In many marine invertebrates opines are formed as end products of anaerobic glycolysis. During prolonged anaerobiosis glycolytic phosphoenolpyruvate is converted into malate, which undergoes disproportionation in mitochondria and finally leads to the formation and accumulation of succinate, propionate and acetate (Grieshaber et al. 1994). The production of anaerobic metabolites would be advantageous for the animals at freezing temperatures, since Loomis et al. (1989) demonstrated that alanopine, strombine, propionate, acetate and lactate prevented the inactivation of lactate dehydrogenase and phosphofructokinase in a freeze-thaw cycle. This study also investigates whether the energy status, quantified as the Gibb's free-energy change of ATP hydrolysis ($dG/d\xi$), can be maintained under these conditions or whether this parameter can be used as an indicator of low temperature stress.

Materials and methods

Animals

Specimens of *Sipunculus nudus* from the intertidal flats near Morgat (small animals of 4.1–11.4 g) and Locquemeau (large animals of 28.5–55.1 g) were provided in February 1994 by the Biological station of Roscoff, Brittany, France. In Bremerhaven the animals were kept in tanks with a bottom layer of sand (10–20 cm) and recirculated natural sea water at $12 \pm 1^\circ\text{C}$.

Experimental procedures

All incubations were carried out in darkened aquaria containing 15 l of aerated natural sea water. The aquaria were placed in a water bath thermostatted to the experimental temperature.

Large specimens of *Sipunculus nudus* were incubated for 24 h at 0, 5, 10, 15, 20 and 25°C . The animals were directly transferred to water of 5, 10 and 15°C . For the incubation at 0°C they were transferred to water of 5°C which was cooled to 0°C within 14 h. Incubations at 20 and 25°C started at a water temperature of 15°C and the experimental temperatures were reached after 7 and 7.5 h, respectively.

In a second set of experiments a bottom layer of sand (10 cm) was added to the aquaria. Large specimens were catheterized using the technique described by Pörtner et al. (1986). The animals were then allowed to dig into the sand at a water temperature of 12°C . After 24 h the temperature was decreased and the animals were incubated for another 8 days. One group of animals was exposed to 0°C , another to 4°C . These temperatures were reached after 9 and 7 h, respectively. The third group was incubated for 4 days at 0°C ; after this time the temperature was increased within 10 h and maintained at 12°C for another 4 days. Owing to repeated sampling of coelomic fluid these animals were sacrificed only at the end of the experimental period.

One animal of each group was connected to a pressure transducer (UFI Type 1050, Morro Bay, Calif. USA) via the indwelling catheter. Ventilatory body movements associated with pressure fluctuations in the coelomic fluid were monitored using a MacLab system (ADI Instruments, Hastings, UK).

Small specimens were allowed to dig into the sand at a water temperature of 12°C . After 24 h the temperature was decreased to 0°C . A group of ten animals was incubated at 0°C for 2 days, when five animals were removed and the temperature was returned to 12°C . The remaining five animals were kept at this temperature for another 3 days. A second group was incubated for 4 days at 0°C . After removing five animals, the temperature was increased to 12°C and the remaining animals removed after 3 days. Another five animals were directly taken from the aquarium tank as control animals. All temperatures $\pm 0.5^\circ\text{C}$.

Analyses

Coelomic fluid samples were withdrawn once every day via the indwelling catheter and analyzed for extracellular pH (pH_e), P_{O_2} and P_{CO_2} using Eschweiler (Kiel, Germany) equipment (MT3R-monitor and electrodes, thermostatted to the incubation temperature of the animals). The electrodes were calibrated with precision buffers (Radiometer, Copenhagen, Denmark) or humidified calibration gases prepared from pure N_2 , CO_2 and CO_2 -free air by gas-mixing pumps (type 2M303/a-F, Wösthoff, Bochum, Germany).

At the end of each experiment the animals were dissected quickly in the dorsoventral plane. Body wall tissue was freeze clamped, wrapped in aluminium foil and stored under liquid N_2 until analyzed. Intracellular pH was determined using the homogenate

technique described by Pörtner et al. (1990). After grinding the tissue under liquid N₂ and transfer of the tissue powder into closed Eppendorf caps containing 250 µl of 160 mmol l⁻¹ KF and 0.5 mmol l⁻¹ nitrilotriacetic acid, the Eppendorf caps were filled with medium, closed and treated with ultrasound for 2 min. During this period samples were kept cold at a temperature below -6 °C in a mixture of ice and salt.

The remaining tissue powder was extracted in PCA following the procedure of Beis and Newsholme (1975), modified according to Pette and Reichmann (1982). Coelomic plasma, which was obtained after centrifugation of coelomic fluid (for 4 min at 14 000 g), was extracted according to Pörtner et al. (1984).

The following metabolites were determined enzymatically in the body wall musculature: ATP, ADP, AMP and succinate (Bergmeyer et al. 1989), P_i (Pörtner 1990), PLA, L-Arg and octopine (Grieshaber et al. 1978). The concentrations of acetate, propionate, strombine and alanopine were analysed with a Dionex BioLC ion chromatograph (Idstein, Germany). The measurement of acetate and propionate followed a procedure modified after Hardewig et al. (1991), using 0.125 mmol l⁻¹ octane sulphonic acid as an eluent. The column was thermostatted to 40 °C. Separation of strombine and alanopine was performed with a Dionex RT 100-6,5 Polyspher ARAC column, using 5 · 10⁻⁵ mol l⁻¹ HCl as an eluent with a flow rate of 0.6 ml min⁻¹. The column was thermostatted to 45 °C. Peaks were monitored with a conductivity detector. Standard opine solution for calibration was prepared by means of in vitro synthesis (Siegmond and Grieshaber 1983). IMP levels were measured in PCA extracts with a Pharmacia HPLC (Uppsala, Sweden) after Tekkanat and Fox (1988) using 0.55 mol l⁻¹ acetonitrile, 0.06 mol l⁻¹ KH₂PO₄ and 0.45 mmol l⁻¹ tetrabutylammoniumdihydrogenphosphate as an eluent (pH 4.85) to which 8% methanol was added.

The levels of free ADP and AMP and dG/dζ were calculated based on the equilibrium of arginine kinase and myokinase. Equilibrium constants for both enzymes were corrected for experimental temperature and pH dependence as related to changing proton and magnesium binding of the adenylates and the proton turnover of the arginine kinase reaction (Pörtner 1993; Pörtner et al. 1996).

The significance of changes in extracellular pH, P_{O₂} and P_{CO₂}, intracellular pH and in the concentrations of metabolites was tested at the 5% level by one-way analysis of variance (ANOVA) and Student-Newman-Keuls posthoc tests as well as Student's *t*-test for unpaired samples.

Results

Incubation of *Sipunculus nudus* at low temperatures (4 and 0 °C) in sand led to the sedimentation of coelomic cells, indicated by a change of the coelomic fluid samples to a clear solution on the second day. No significant changes in coelomic gas parameters and volatile fatty acid concentrations were seen at 4 °C. A trend for P_{O₂} and pH_e to decrease was observed between day 5 and day 7, at the same time the highest acetate concentrations were found. P_{CO₂} values fluctuated between 2.6 ± 0.8 mm Hg on day 1 and 3.5 ± 0.6 mm Hg after 7 days. pH_e, P_{CO₂} and propionate levels were not significantly different owing to high inter-individual variability.

Lowering temperature to 0 °C (Fig. 1) resulted in a significant decrease in P_{O₂} from day 7 until on the last day when O₂ was no longer detectable. P_{CO₂} and propionate levels rose at the same time. The concentration of acetate increased to a maximum of 0.64 ± 0.34 mmol l⁻¹ coelomic fluid on the last day.

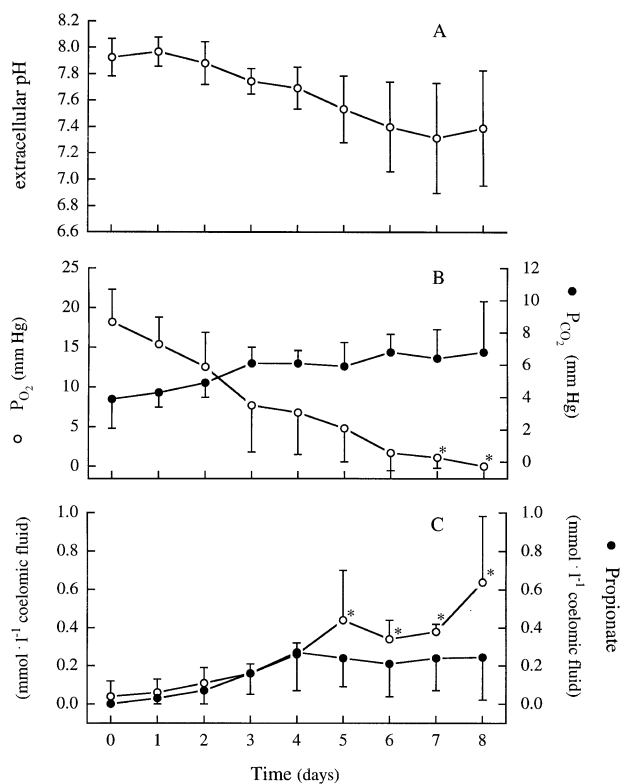


Fig. 1A–C Extracellular pH, oxygen and CO₂ partial pressures, and the concentrations of acetate and propionate in the coelomic fluid of *Sipunculus nudus* (large animals) dwelling in sediment. After 24 h (= day 1) the incubation temperature was lowered from 12 to 0 °C within 9 h (* significantly different from controls; day 0–7: *n* = 5; day 8: *n* = 3)

The values from days 5–8 were significantly different from the concentrations found at 12 °C.

Similar results were obtained in a second experiment when the temperature was first lowered to 0 °C and after day 4 raised to 12 °C again (Fig. 2). Within 8 days coelomic P_{O₂} decreased significantly to 0 mm Hg and pH_e fell to pH 6.68 ± 0.09. P_{CO₂} increased significantly from 3.0 ± 0.4 to 15.8 ± 2.0 mm Hg. Concentrations of acetate and propionate also rose to maximum values of 6.66 ± 3.59 mmol l⁻¹ coelomic fluid and 0.32 ± 0.24 mmol l⁻¹ coelomic fluid, respectively.

All animals incubated at 0 °C were in a very poor condition at the end of the experiments. One of those large animals, which was rewarmed to 12 °C after 4 days at 0 °C and two of those large animals, which were exposed to 0 °C for 8 days, died (no longer included in data set).

The recording of pressure changes in the coelomic fluid revealed ventilatory movements typical for animals which had constructed a burrow in the sediment (Fig. 3A). At 12 °C individual, larger peaks associated with digging movements were recorded at irregular intervals. These digging movements ceased when the temperature was lowered to 4 and 0 °C. At 4 °C ventilatory movements were observed at mean values of 0.03 Hz

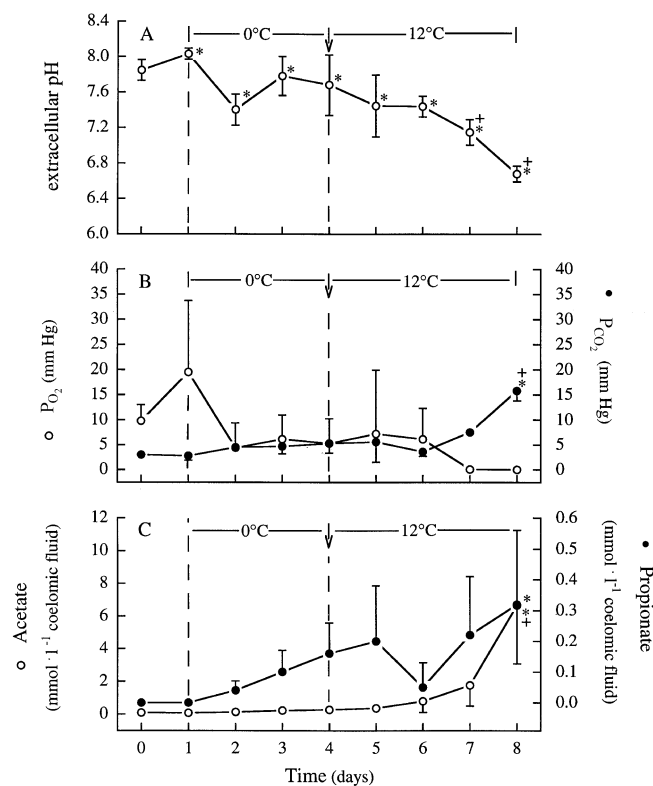


Fig. 2A–C Extracellular pH, oxygen and CO₂ partial pressures, and the concentrations of acetate and propionate in the coelomic fluid of *Sipunculus nudus* (large animals) during incubation in sediment. Initially, the temperature was lowered from 12 to 0 °C within 9 h. After 4 days the animals were warmed to 12 °C within 12 h (* significantly different from controls, + significantly different from the values after 4 days at 0 °C; day 0–5: $n = 4$; day 6–8: $n = 3$)

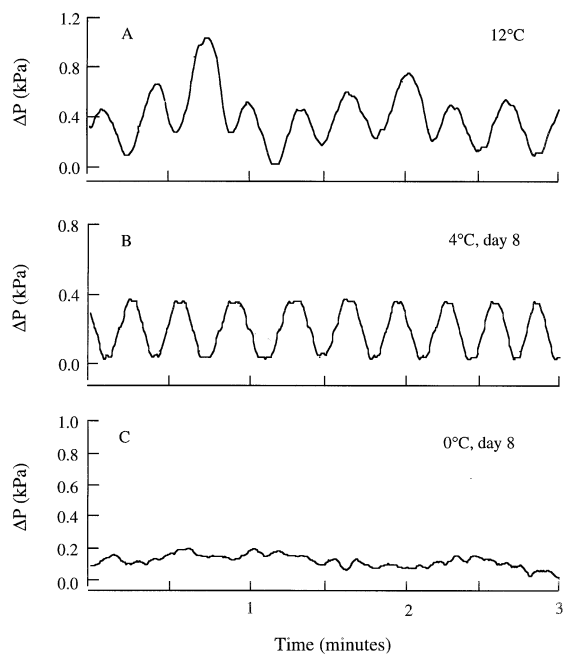


Fig. 3A–C Ventilatory activities of *Sipunculus nudus* (large animal) burrowed in the sediment at 12 °C (A), 4 °C (B) and at 0 °C (C). ($n = 1$)

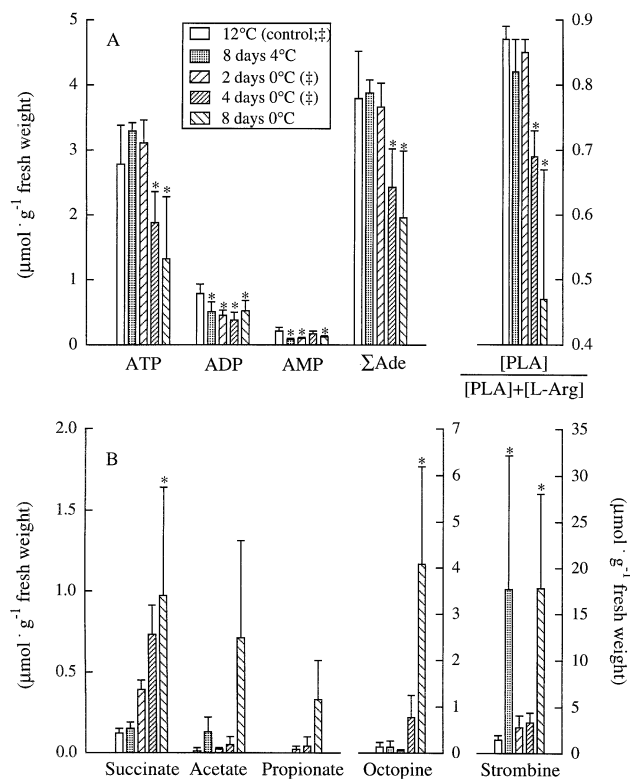


Fig. 4A, B Concentrations of ATP, ADP, AMP and ΣAde , the ratio of the levels of phospho-L-arginine over the sum of the concentrations of PLA and L-arginine (A) and the levels of succinate, acetate, propionate, octopine and strombine (B) in the body wall musculature of *Sipunculus nudus* (large and small animals) during exposure to 0 °C in the sediment (* significantly different from controls; $n = 5$, except after 8 days at 0 °C: $n = 3$; † indicates data collected from small animals)

and 0.6 kPa during the whole experimental period. At 0 °C, the ventilation frequency decreased from 0.03 Hz at 12 °C to 0.01 Hz within 8 days and the amplitude fell from 0.7 kPa at 12 °C to 0.2 kPa during this period. Towards the end of the experiments ventilatory activity was no longer visible (Fig. 3).

In the body wall musculature the concentrations of the adenylates ATP and ADP and the summed contents of ATP, ADP and AMP (ΣAde) decreased during exposure to 0 °C (Fig. 4a), while the concentration of P_i rose (Table 1). The depletion of ATP did not lead to elevated AMP levels, but rather to a significant decrease after exposure to 4 and 0 °C (for 2 and 8 days). At the same time the concentrations of IMP rose reaching significantly higher levels during exposure to 0 °C for 8 days and during rewarming to 12 °C after 4 days at 0 °C (Table 1). Furthermore, the concentrations of the phosphagen PLA fell, while arginine levels rose leading to a significantly reduced ΣArg and a drop of the ratio of PLA over L-Arg plus PLA concentrations after 8 days at 0 °C (Table 1, Fig. 4A). Data from small and large animals were combined in Figs. 4, 7 and Table 1 since a comparison of metabolite levels under

Table 1 Concentrations ($\mu\text{mol g}^{-1}$ fresh weight) of inorganic phosphate, IMP, phospho-L-arginine, L-arginine and the summed levels of L-arginine containing metabolites ($\Sigma\text{Arg} = [\text{PLA}] + [\text{L-Arg}] + [\text{Octopine}]$) in the body wall musculature of *Sipunculus*

Temperature ($^{\circ}\text{C}$)	Time (days)	PLA	L-Arg	ΣArg	P_i	IMP
12 (control)		45.03 ± 4.42	6.66 ± 0.85	52.12 ± 4.77	2.92 ± 1.34	0.03 ± 0.02
4	8	33.69 ± 4.11^a	7.22 ± 1.99	41.02 ± 3.56	2.55 ± 1.80	0.04 ± 0.01
0	2 ^b	46.66 ± 3.48	8.58 ± 1.04	55.29 ± 3.96	4.02 ± 1.32	0.04 ± 0.02
0	4 ^b	29.15 ± 5.62^a	12.95 ± 1.69^a	42.87 ± 6.85	8.86 ± 3.69	0.03 ± 0.02
0	8	15.40 ± 10.84^a	13.91 ± 1.73^a	33.39 ± 9.61^a	9.74 ± 2.48	0.15 ± 0.02^a
12 (after 2 days at 0°C)	3 ^b	45.01 ± 7.04	7.90 ± 2.23	53.06 ± 5.58	3.02 ± 1.03	0.04 ± 0.01
12 (after 4 days at 0°C)	3 ^b	7.84 ± 3.10^a	26.34 ± 3.98^a	29.97 ± 11.39^a	13.31 ± 6.85^a	0.29 ± 0.13^a

^aSignificantly different from controls

^bData collected from small animals

Table 2 Intracellular pH, the ratio of the levels of phospho-L-arginine over the sum of the concentrations of PLA and L-arginine and the concentrations ($\mu\text{mol g}^{-1}$ fresh weight) of octopine, ATP, ADP, AMP, the summed concentrations of the adenylates and the levels of

nudus (large and small animals) after exposure to 4°C and 0°C for up to 8 days in sediment, and after rewarming to 12°C after different periods at 0°C . Values are means \pm SD

acetate, propionate and strombine in the body wall musculature of *Sipunculus nudus* (large animals) after 24 h of incubation in cold sea water without sediment (^asignificantly different from control values; 0, 5 and 10°C : $n = 6$; 15, 20 and 25°C : $n = 5$)

	0°C	5°C	10°C	15°C	20°C	25°C
pHi	7.45 ± 0.01	7.42 ± 0.01	7.30 ± 0.02	7.25 ± 0.01	7.20 ± 0.02	7.07 ± 0.03
[PLA]/ [PLA] + [L-Arg]	0.93 ± 0.01			0.92 ± 0.01		0.93 ± 0.01
Octopine	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
ATP	3.23 ± 0.53			3.28 ± 0.45		3.71 ± 0.33
ADP	0.42 ± 0.06	0.47 ± 0.08	0.38 ± 0.04	0.36 ± 0.11	0.39 ± 0.13	0.40 ± 0.08
AMP	0.08 ± 0.03	0.08 ± 0.03	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.04	0.08 ± 0.01
$\Sigma[\text{Adenylate}]$	3.77 ± 0.63			3.71 ± 0.52		4.19 ± 0.37
P_i	0.68 ± 0.48	0.67 ± 0.41	1.53 ± 0.66	0.73 ± 0.68	0.66 ± 0.68	1.17 ± 0.53
Acetate	0.00 ± 0.00			0.02 ± 0.04		0.01 ± 0.03
Propionate	0.04 ± 0.06			0.14 ± 0.05		0.02 ± 0.03^a
Strombine	0.84 ± 0.04			0.85 ± 0.42		0.51 ± 0.31

control conditions and after 4 days at 0°C and 3 days of subsequent warming at 12°C revealed no significant difference between small and large specimens and led to the conclusion that small and large animals are equally sensitive to temperature change.

Depending upon the length of the incubation period, keeping the animals at 0°C caused a rise in the concentrations of succinate, of the volatile fatty acids acetate and propionate and of the opine strombine in the body wall (Fig. 4B). None of these changes were observed in the unburied animals exposed to 0°C for 24 h (Table 2).

Recovery of small animals at 12°C was only possible when exposure to 0°C did not exceed 2 days. Under these conditions the concentrations of ADP and AMP decreased during low temperature exposure but the ADP level returned to control values after returning the animals to 12°C (Fig. 5A). Warming after 4 days at 0°C resulted in further depletion of these metabolites and of the phosphagen PLA (Fig. 5B, Table 1). Concentration changes of anaerobic end products confirmed the observation that the ability to recover depended upon the length of the incubation period. While exposure to 0°C caused the levels of succinate, acetate,

propionate, octopine and strombine to rise, this trend was reversed during rewarming after 2 days of cold exposure, but not during rewarming after exposure to 0°C for 4 days (Fig. 6).

Incubation of unburied animals between 0 and 25°C led to a decrease of pH_i with increasing temperature (Table 2), following a slope of -0.015 pH units per $^{\circ}\text{C}$. Similar slopes were seen for the pH_i of animals incubated in sand at 4°C and for 2 days at 0°C with -0.014 or -0.012 pH units per $^{\circ}\text{C}$, respectively, leading to a significant rise in pH_i above the control value of $\text{pH } 7.29 \pm 0.03$ (Fig. 7A). This pH was restored when the animals were brought back to 12°C after 2 days at 0°C . Eight days at 0°C and rewarming the animals after 4 days at 0°C resulted in the development of an uncompensated acidosis. This acidosis together with the accumulation of P_i and the depletion of the adenylates led to a significant drop in $dG/d\xi_{\text{ATP}}$ from -56.1 ± 1.5 kJ mol^{-1} (control animals) to a minimum value of -45.5 ± 0.8 kJ mol^{-1} (Fig. 7B, C) in those animals which had been rewarmed after 4 days at 0°C . The levels of free AMP and ADP also rose when animals were rewarmed after 4 days but not in those rewarmed after 2 days of exposure to 0°C .

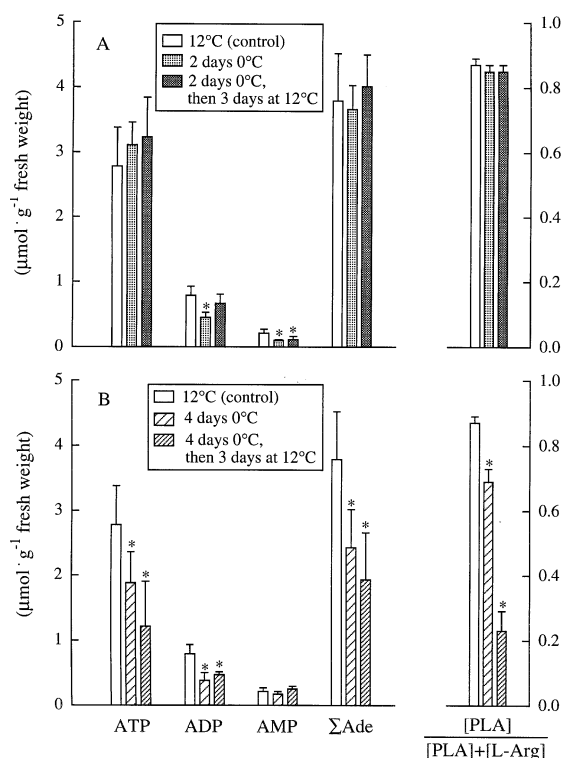


Fig. 5A, B Effect of rewarming of *Sipunculus nudus* (small animals) after 2 days (A) and 4 days (B) of exposure to 0 °C in sediment on the concentrations of ATP, ADP, ANP, Σ Ade and the ratio of the levels of PLA over the sum of the concentrations of PLA and L-Arg (* significantly different from controls; $n = 5$, except 2 days 0 °C, then 3 days at 12 °C: $n = 4$)

Discussion

Cold-induced anaerobiosis

The incubation of *Sipunculus nudus* in sand at low temperatures caused the production of anaerobic metabolites. The onset of anaerobic metabolism is correlated with the decrease in O_2 partial pressure in the coelomic fluid. Pörtner et al. (1985) demonstrated that 24 h of incubation at low O_2 tensions between 15 and 7.5 mm Hg in the ambient water, which corresponds to a P_{O_2} below 4 mm Hg in the coelomic fluid, led to an accumulation of anaerobic products like succinate, propionate and acetate. In the present experiments the concentrations of anaerobic metabolites actually increased significantly when O_2 partial pressures in the coelomic fluid fell below about 5 mm Hg. Insufficient provision of O_2 at low temperature is therefore concluded to elicit the onset of anaerobic metabolism.

The drop in coelomic fluid P_{O_2} was probably caused by the reduction and finally elimination of ventilatory activity during low temperature exposure. A strong influence of temperature on ventilatory activity of marine invertebrates has already been reported for other species. Theede (1972) showed that the movements of cilia in isolated gills of the mussel *Spisula solida* slowed

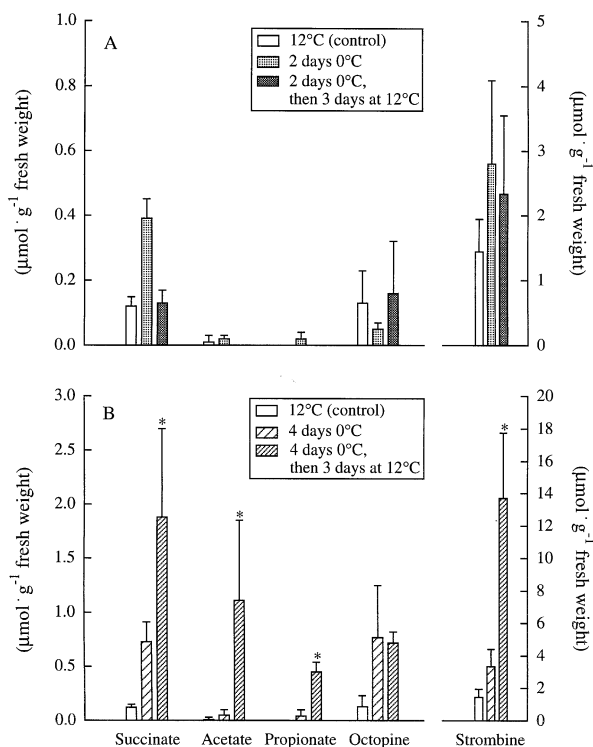


Fig. 6A, B Effect of rewarming of *Sipunculus nudus* (small animals) after 2 days (A) and 4 days (B) of exposure to 0 °C in sediment on the concentrations of succinate, acetate, propionate, octopine and strombine (* significantly different from controls; $n = 5$ except 2 days 0 °C, then 3 days at 12 °C: $n = 4$)

down drastically at temperatures below 5 °C. Measurements of the ventilation rate of three species of the polychaete *Nereis* revealed that the rate decreased below 25 °C and finally reached very low values (Kristensen 1983). We conclude that an insufficiency of ventilatory mechanisms develops at low temperature which causes inadequate O_2 supply and the transition to anaerobiosis. This conclusion is supported by the observation that coelomic cells sediment at low temperature reflecting insufficient suspension of the cells by peristaltic movements of the body wall. For comparison, *Sipunculus nudus* incubated in cold sea water without sediment did not switch to anaerobiosis within 24 h (Table 2), whereas *Arenicola marina* did (Sommer et al. 1996). *Arenicola* possesses a circulatory system, the pulsation frequency of which is reduced at low temperatures (Seymour 1972). *Sipunculus* possesses no circulatory system emphasizing that insufficient ventilation and mixture of body fluids go hand in hand below the critical temperature.

In the present study, the production rate of anaerobic metabolites was reduced compared to 24 h of experimental anaerobiosis at 15 °C (Pörtner et al. 1984). Further comparison reveals that more propionate than acetate is produced at high temperatures, whereas acetate reached higher levels than propionate at low temperature. It is completely unknown whether

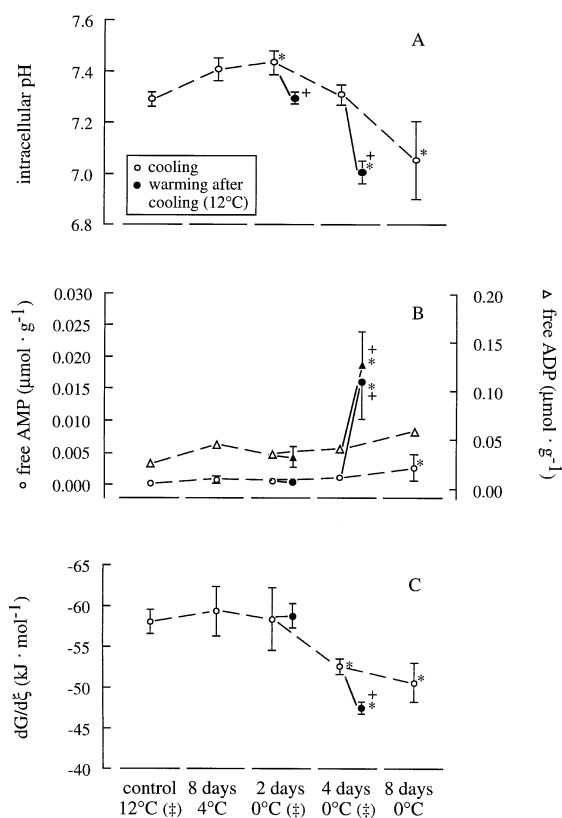


Fig. 7A–C Intracellular pH (A) in the body wall musculature of *Sipunculus nudus* [large and small (†) animals] after exposure to 4 °C and 0 °C and after rewarming to 12 °C, as well as the levels of free AMP and ADP (B) and the free-energy change of ATP hydrolysis (C)

propionate synthesis is more temperature sensitive than acetate. During anaerobic propionate formation, the rate-limiting step is the decarboxylation of methylmalonyl-CoA by the propionyl-CoA carboxylase which releases bicarbonate. Kinetic measurements in purified enzyme preparations of propionyl-CoA carboxylase from the body wall musculature of *Arenicola marina* showed that a non-respiratory acidosis (low bicarbonate levels) enhances the rate of decarboxylation. Measurements at a constant pH of 7.0 revealed that HCO_3^- has an inhibitory effect (Hardewig et al. 1994). Exposure of *Sipunculus nudus* in sand at low temperatures caused a rise in pH and most likely bicarbonate levels, thereby possibly impeding propionate production and favouring acetate formation. Activities of enzymes involved in acetate formation may be less affected by the temperature decrease than enzymes of the propionate pathway, but more data are needed before more definite conclusions can be drawn.

Adenylate catabolism

Energy turnover during anaerobiosis is reduced in comparison to aerobic metabolism (Zebe et al. 1980)

which is also the case in *Sipunculus nudus* (Hardewig et al. 1991). During 24 h of anaerobiosis at constant temperature in open sea water adenylate concentrations did not change significantly in the body wall, since ATP levels were buffered at the expense of PLA (Pörtner et al. 1984; Pörtner 1987b). However, long term incubation of *Sipunculus nudus* in the sediment caused a reduction in both PLA and ATP concentrations after 4 and 8 days at 0 °C (Fig. 4A). ATP depletion can occur due to an enhanced utilisation and/or a reduced production rate. The latter could be caused by a decrease in glycolytic rate due to the fact that polymeric enzymes dissociate into subunits at low temperatures as has been shown for the tetrameric glucose-6-phosphate dehydrogenase and phosphofructokinase (Prosser 1991). However, ATP depletion was not associated with an accumulation of AMP, but AMP levels fell instead, together with a decrease in the summed levels of the adenylates. This implies that a degradation beyond AMP occurred, possibly caused by a temperature-dependent shift in the reaction equilibrium of the deamination of AMP to IMP by AMP deaminase. This is suggested by the decrease in AMP and rise in IMP levels at 4 °C when [ATP] is still constant. The temperature decrease possibly favoured the breakdown of adenylates, starting with AMP and subsequently causing the decrease of the other adenylates through the equilibrium of the adenylate kinase, the temperature dependence of which is known to be very small (Tewari et al. 1991).

Acid-base regulation and ATP free-energy change

During long-term incubation at 4 °C and during 2 days at 0 °C the pH_i of *Sipunculus nudus* changed according to the alaphastat hypothesis. In this respect, *Sipunculus nudus* behaves like many other animals. Howell et al. (1970) determined a slope of -0.016 pH units per °C in the arterial blood of the frog *Rana catesbeiana* and the toad *Bufo marinus* for the temperature range 5–37 °C. For *Rana catesbeiana* musculature $\Delta\text{pH}/\Delta T$ was -0.015 between 5 °C and 30 °C (Malan et al. 1976). Other authors demonstrated that there can be differences between intra- and extracellular compartments. The pH in the blood of *Salmo trutta* followed alaphastat regulation, whereas pH_i in different muscle tissues was kept constant and independent of the temperature (Butler and Day 1993). The opposite was true for *Arenicola marina*, where pH_i changed according to alaphastat predictions, while pH_e was kept constant (Sommer et al. 1996).

Long-term exposure of *Sipunculus nudus* to 0 °C caused a decrease in pH_i . The drop in ventilatory activity led to an accumulation of CO_2 , most likely linked to the development of an intracellular respiratory acidosis. The production of opines, propionate and acetate from glycogen is accompanied by the

production of protons (Pörtner 1987a). In contrast, the depletion of PLA utilises protons (Pörtner et al. 1984) and the depletion of PLA, ATP and ADP leads to an accumulation of P_i which increases the non-bicarbonate buffer value (Pörtner 1989). Hydrolysis of MgATP releases protons, but the amount is reduced by the dissociation of Mg^{2+} from MgADP as well as by the conversion of AMP to IMP (Pörtner 1987b). The net effect of these mechanisms, however, did not prevent proton accumulation. The depletion of the adenylates, the accumulation of phosphate and the acidosis led to a significant drop in the free-energy change of ATP hydrolysis, which represents the driving force for any ATPase reaction. A drop in $dG/d\xi$ below a critical level may cause severe disturbance of cellular functions and finally death. This might be one reason why animals were not able to recover after 4 days at 0°C , while after 2 days at 0°C the change was not significant and animals recovered. If the change in $dG/d\xi$ is limiting survival then a level of -52 kJ mol^{-1} (see Fig. 7) appears as the limiting level at 0°C . However, little is known about the limiting levels of ATP free-energy change in marine invertebrates or their temperature dependence [for a more detailed discussion see Pörtner (1993)].

Conclusions

These results indicate that a low critical temperature exists between 4°C and 0°C for *Sipunculus nudus* dwelling in sediment. In its natural environment of the intertidal zone of Brittany, *Sipunculus nudus* will hardly ever encounter temperatures this low, since this species lives close to the low water line and mean water temperature is 9°C in January and February (Isemer and Hasse 1985). The low critical temperature is characterized by the onset of anaerobic metabolism as indicated by the accumulation of acetate, propionate and succinate and by the development of acidosis which is not only related to metabolism, but also to respiratory acidification. In support of our present findings, a low and a high critical temperature were correlated with the onset of anaerobic metabolism in *Arenicola marina* (Sommer et al. 1996). In *Sipunculus nudus* the low critical temperature is explained by the drop in ventilatory activity seen below 4°C . Recovery from cold-induced anaerobiosis is only possible when cold exposure occurs during a limited time period. The "point of no return" is characterized by a decrease in the free-energy change of ATP hydrolysis which may fall below a critical level thus impairing the maintenance of cellular functions at low temperatures. Temperature is also interpreted to influence the steady-state levels of the adenylates by favouring ATP catabolism at low temperatures still above the T_C when the mode of energy metabolism is still completely aerobic.

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