

METABOLIC RATES AT DIFFERENT OXYGEN LEVELS DETERMINED BY DIRECT AND INDIRECT CALORIMETRY IN THE OXYCONFORMER *SIPUNCULUS NUDUS*

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Summary

Oxygen uptake and the mode of energy production in *Sipunculus nudus* L. were determined at different oxygen levels by means of direct and indirect calorimetry. Oxygen consumption declined linearly with decreasing ambient P_{O_2} . A similar decrease in heat production was observed down to a P_{O_2} of 8.66 kPa. At lower oxygen tensions, a discrepancy between aerobic and total heat production indicated the onset of anaerobic metabolism. The occurrence of the critical P_{O_2} between 8.66 and 2.66 kPa was confirmed by estimation of anaerobic end products in the body wall musculature. The contributions of aerobic and anaerobic metabolism to total ATP production were determined at a P_{O_2} of 2.66 kPa and were found to be 48 and 52%, respectively. Measured heat dissipation under extreme hypoxia (P_{O_2} nominally zero) ($21.5 \pm 3.5 \text{ mJ h}^{-1} \text{ g}^{-1}$) agreed with the enthalpy changes calculated from the rates of formation of anaerobic end products ($17.9 \pm 4.7 \text{ mJ h}^{-1} \text{ g}^{-1}$). For the sake of redox balance maintenance, saturation of fatty acids was assumed; this would be accompanied by an additional heat production of $3.3 \text{ mJ h}^{-1} \text{ g}^{-1}$, so that the total calculated enthalpy change amounted to $21.2 \text{ mJ h}^{-1} \text{ g}^{-1}$.

Introduction

Most intertidal invertebrates, in particular those of the infauna, are temporarily exposed to hypoxic conditions when the supply of oxygenated water is interrupted during low tide. Prosser (1973) described two different patterns of reaction to situations of reduced oxygen availability: oxyregulators keep their oxygen consumption independent of the ambient oxygen tension down to a certain P_{O_2} ,

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below which regulation ceases. Oxyconformers, however, steadily reduce their oxygen uptake with decreasing P_{O_2} .

The occurrence of various patterns of oxygen consumption in relation to the ambient P_{O_2} , found even in different individuals of the same species (Mangum and van Winkle, 1973; Bayne, 1971), makes it necessary to define clearly the transition phases in oxygen consumption: the traditional approach is to select the critical P_{O_2} (P_{cI}), below which oxygen consumption declines. In addition, a second critical P_{O_2} (P_{cII}) may be defined, below which anaerobic metabolism starts to compensate for the decline in aerobic energy production (Pörtner *et al.* 1985).

For the lugworm *Arenicola marina*, the available data suggest that P_{cI} and P_{cII} differ. P_{cI} , indicating the transition to oxyconformity, lies near air saturation at 16 kPa (Toulmond and Tchernigovzeff, 1984), whereas the onset of anaerobic processes (P_{cII}) was observed only at oxygen tensions below 6 kPa (Schöttler *et al.* 1983). In oxyconforming *Mytilus edulis*, however, calorimetric experiments revealed that anaerobic metabolism is switched on as soon as oxygen uptake declines (Famme *et al.* 1981). Consequently, P_{cI} and the P_{cII} seem to be identical for this species. Anaerobic ATP generation could presumably compensate for the decrease in aerobic energy provision, thus keeping the total ATP supply constant down to a certain P_{O_2} level, below which both aerobic and anaerobic metabolic rates decline rapidly.

For the marine worm *Sipunculus nudus* L., Pörtner *et al.* (1985) suggested that aerobic oxyconformity prevails over the entire range of ambient partial pressures from normoxia to 6.7 kPa. At lower oxygen tensions oxygen consumption decreases more rapidly. The accelerated decline of O_2 uptake coincides with the onset of anaerobic metabolism, as indicated by the formation of anaerobic end products.

Low anaerobic metabolic rates, however, may not be detectable by biochemical analyses. Small amounts of accumulated end products may be masked by large individual variations in metabolite concentrations, thus requiring additional evidence in the characterization of metabolic transition phases. In addition, it is still uncertain whether all the anaerobic pathways for energy production and their end products are known. Indeed, calorimetric measurements and biochemical analyses for *Lumbriculus variegatus* and *Mytilus edulis* suggest that a significant fraction of anoxic heat flux cannot be explained on the basis known anaerobic pathways (Gnaiger, 1980a; Shick *et al.* 1983). In contrast, Famme and Knudsen (1984) postulated an agreement between biochemical and calorimetric analyses in *Tubifex tubifex*. Re-analysing Famme and Knudsen's (1984) results, Gnaiger and Staudigl (1987) claimed that the caloric equivalent employed for excreted volatile fatty acids was too high. Recalculation of the data resulted in an unexplained heat fraction of 34%.

In the present study the non-invasive techniques of simultaneous direct calorimetry and oxygen consumption measurements were applied to determine the oxygen uptake and the prevailing energy metabolism in the marine worm *Sipunculus nudus*. Heat dissipation measured during extreme hypoxia was

compared with enthalpy changes calculated from changes in metabolite levels. Using this approach we investigated whether the known linear decline in oxygen consumption was reflected by a similar reduction in heat production. In addition, we estimated the relative contributions of aerobic and anaerobic energy production to the total energy expenditure below the critical P_{O_2} (P_{cII}). We tried to re-evaluate the existence of additional, hitherto unknown, hydrogen acceptors possibly necessary to maintain redox balance during extreme hypoxia.

Materials and methods

Experimental animals

Specimens of *Sipunculus nudus* with a mean live mass of 40 ± 8 g were collected in March 1989 along the coast of Brittany, France. The animals were kept for several weeks in aquaria containing sand from the site of collection and artificial sea water (34‰ salinity) at a temperature of 15 ± 1 °C.

Direct and indirect calorimetry

The calorimetric experiments were carried out at the Gorlaeus Laboratoria, University of Leiden, The Netherlands. Heat production and oxygen consumption of groups of 4–5 animals were measured simultaneously. Direct calorimetry was performed with a twin-flow microcalorimeter Setaram GF 108, equipped with a 1 dm³ animal chamber, as described by van Waversveld *et al.* (1988). At a flow rate of 16 cm³ min⁻¹ a sensitivity of approximately 94 ± 0.5 μ V mW⁻¹ was achieved. The system was calibrated with a 10 mW pulse for 6 h before and after each experiment with a reproducibility of ± 0.05 mW. Baseline drift was less than 0.1 mW during calibration.

For measurements of oxygen consumption, samples of the water flowing out of both measurement and reference vessels were alternately led through an electrode cell with a flow rate of 2 cm³ min⁻¹. The oxygen sensor (E 5046 Radiometer, Copenhagen) was calibrated with air-saturated water and a solution of 10% Na₂SO₃ (0% O₂). Oxygen consumption rates were determined by integrating the differential P_{O_2} readings between measurement and reference vessel in order to correct for blank rates of oxygen consumption. The solubility of oxygen in sea water (α) was calculated from the values of Carpenter (1966): $\alpha = 12.4$ μ mol l⁻¹ kPa⁻¹ at 15 °C and 34‰ salinity.

Measurements started after the equilibration of the animal chamber at each oxygen tension. Corrections for instrumental lag were not considered necessary, since heat dissipation and oxygen consumption were integrated over extended periods (2.8 h) compared to the time constants for calorimetric and respirometric measurements ($\tau = 54$ min and 62 min, respectively).

Experimental procedure

The experiments were carried out at 15 °C. Prior to the measurements, weighed animals were kept for 24 h under experimental conditions, i.e. in a calorimeter

vessel with a flow-through system without sediment, in order to reduce stress. After acclimation, animals were put into the calorimeter. Stability of the calorimeter signal was achieved within 24 h.

After 48 h of normoxia, the P_{O_2} was successively decreased to 8.66, 2.66 and 0 kPa and kept at each level for 24 h. Different P_{O_2} levels were obtained by equilibrating the inflowing water with a mixture of air, N_2 and CO_2 , delivered by gas-mixing pumps (M30F and M303a-F; Wösthoff, Bochum, FRG). Actual oxygen concentrations to which the animals were exposed were calculated as the mean levels in inflowing and outflowing water. P_{CO_2} was kept constant between 0.03 and 0.04 kPa. Oxygen diffusion into the flow-through system was prevented by the use of stainless-steel tubes. During extreme hypoxia (P_{O_2} nominally zero) the P_{O_2} was kept below the detection limit of the oxygen electrode ($=66$ Pa). After 24 h of extreme hypoxia animals were allowed to recover for 48 h under normoxic conditions.

Samples of the outflowing water were collected at each oxygen level for the determination of the mean rate of acetate and propionate release. Evaporation of the volatile fatty acids was prevented by adding 0.4% (v/v) 1 mol dm^{-3} NaOH to each sample.

For biochemical determinations the same experiments were performed outside the calorimeter under identical conditions, using an identical calorimeter cell. After the incubation, animals were dissected rapidly and the coelomic fluid was collected. Body wall musculature was freeze-clamped in liquid nitrogen (Wollenberger *et al.* 1960) and both coelomic fluid and muscle samples were kept frozen at -80°C . For the body wall musculature the mass fraction coefficient, which is the mass of the body wall musculature divided by the total wet mass, was found to be 0.34 ± 0.03 (mean \pm s.d., $N=20$).

Biochemical analyses

After perchloric acid (PCA) extraction of muscle tissue (Beis and Newsholme, 1975) and of coelomic fluid (Pörtner *et al.* 1984b) the following metabolites were determined enzymatically: succinate (Michal *et al.* 1976), phospho-L-arginine, L-arginine and octopine (Grieshaber *et al.* 1978) and aspartate, ATP, D- and L-alanine (Bergmeyer, 1974). Alanopine and strombine were estimated by high pressure liquid chromatography (Siegmond and Grieshaber, 1983).

Acetate and propionate were determined in diluted PCA extracts with a Dionex Bio LC ionchromatograph (Idstein, FRG). Separation was performed with an ion exclusion column (Dionex HPIC E-AS 1), using $0.25\text{--}0.5 \text{ mmol dm}^{-3}$ octane sulphonic acid as an eluent with a flow rate of $1 \text{ cm}^3 \text{ min}^{-1}$. The column was thermostatted at 32°C . Peaks were monitored with a conductivity detector with a sensitivity of $3 \mu\text{V}$ for the full scale. Background conductivity was decreased using a micro membrane suppressor (Dionex AMMS-ICE) regenerated with 5 mmol dm^{-3} tetrabutylammonium hydroxide. Calibration curves were linear between 10 and $100 \mu\text{mol dm}^{-3}$ acetate and propionate with a reproducibility of

$\pm 4\%$. Water samples were extracted and concentrated by water vapour distillation prior to analysis. The recovery efficiency for internal standards was $86 \pm 5\%$.

The significance of differences was tested using Student's *t*-test at a significance level of 5%.

Results

Direct calorimetry

A typical time course of heat flux of *Sipunculus nudus* incubated at different oxygen levels is shown in Fig. 1. During the first 24 h after inserting the animals into the calorimeter, peaks of heat dissipation appeared periodically, probably reflecting enhanced activity by the animals. After 24 h these activity peaks occurred less frequently. With decreasing oxygen tensions, heat dissipation dropped steadily from $110.1 \pm 10.8 \text{ kJ h}^{-1} \text{ g}^{-1}$ fresh mass (mean \pm s.d., $N=4$) under normoxic conditions to 66.7 ± 11.8 and $36.5 \pm 6.1 \text{ kJ h}^{-1} \text{ g}^{-1}$ fresh mass at 8.66 and 2.66 kPa, respectively. During extreme hypoxia a value of $21.5 \pm 3.5 \text{ kJ h}^{-1} \text{ g}^{-1}$ fresh mass was observed, which amounts to only 20% of the normoxic value. Each change in ambient P_{O_2} was accompanied by a short and sudden increase in heat flux, the size of which decreased with decreasing P_{O_2} . Except for these peaks, the activity of the animals was reduced under moderate ($P_{\text{O}_2}=8.66$ and 2.66 kPa) and under extreme hypoxia as shown by the general decrease in the number of peaks. After 112 h, reoxygenation coincided with an immediate rise in heat production, which reached a slightly higher level than the control value and decreased steadily thereafter. However, control values were not reached even after 48 h of recovery. During recovery, the animals did not seem to be active, since peaks of heat dissipation were absent.

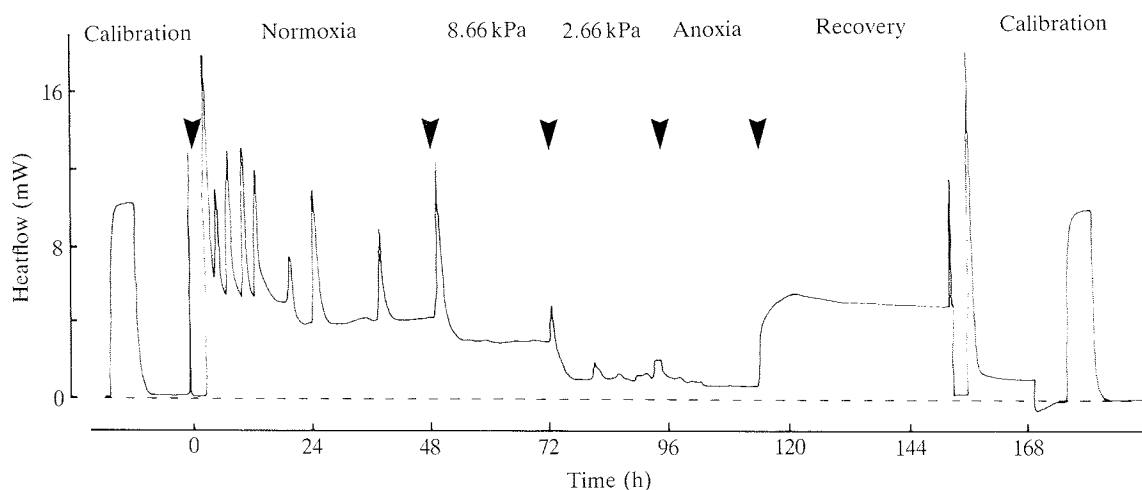


Fig. 1. Heat dissipation of four specimens of *Sipunculus nudus* (154.5 g fresh mass in total) subjected to a stepwise reduction in ambient P_{O_2} . Arrowheads indicate the changes in oxygen tension.

Aerobic and anaerobic metabolism

Rates of heat dissipation determined by calorimetry and calculated from oxygen consumption are compared in Fig. 2. The diagram shows gaps during transitions between oxygen tensions, because oxygen consumption could not be measured under non-steady-state conditions. To calculate the aerobic heat flux an oxycaloric equivalent for mixed substrates of $450 \text{ kJ mol}^{-1} \text{ O}_2$ (Gnaiger, 1983a) was used. An excess of measured heat flux over the values predicted from oxygen consumption must be due to anaerobic metabolism, whereas a rise in the caloric equivalent of oxygen uptake above measured heat production, the so-called aerobic overshoot, may be caused by endothermic processes or by oxygenation of haemerythrin and the physical solution of oxygen in the coelomic fluid.

Under normoxic conditions the energy demand was largely met by aerobic metabolism. Only during activity bursts, when heat dissipation reached values almost twice as high as the steady rate, did anaerobic metabolism occur in addition to enhanced aerobic metabolic rates. The resulting oxygen debt was discharged during the resting periods, as reflected by the aerobic overshoot. The overall calorimetric/respirometric ratio was determined in four experiments of 24 h of normoxia to be $423.4 \pm 27.1 \text{ kJ mol}^{-1} \text{ O}_2$.

At an oxygen tension of 8.66 kPa, aerobic metabolism, albeit decreased to 55 % of the normoxic value, was sufficient to maintain the animal's energy demand. At a

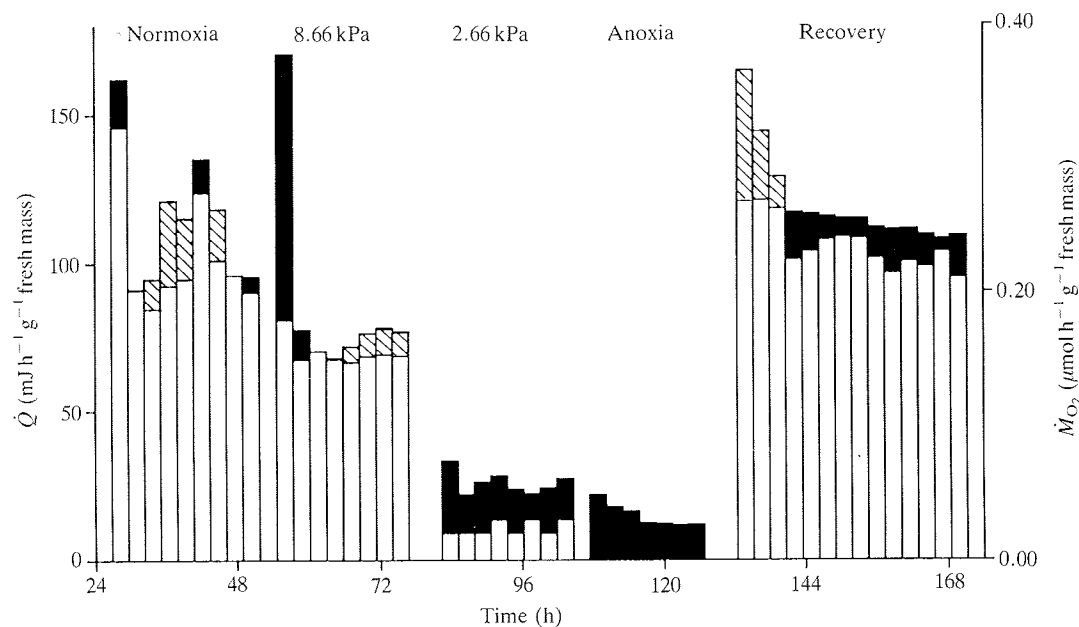


Fig. 2. Comparison of measured rates of heat production (\dot{Q}) and those calculated from oxygen consumption (\dot{M}_{O_2}) in four specimens of *Sipunculus nudus* at different oxygen tensions. An oxycaloric equivalent of $450 \text{ kJ mol}^{-1} \text{ O}_2$ was used. Open columns, aerobic heat production; filled columns, anaerobic heat production, hatched columns, aerobic overshoot.

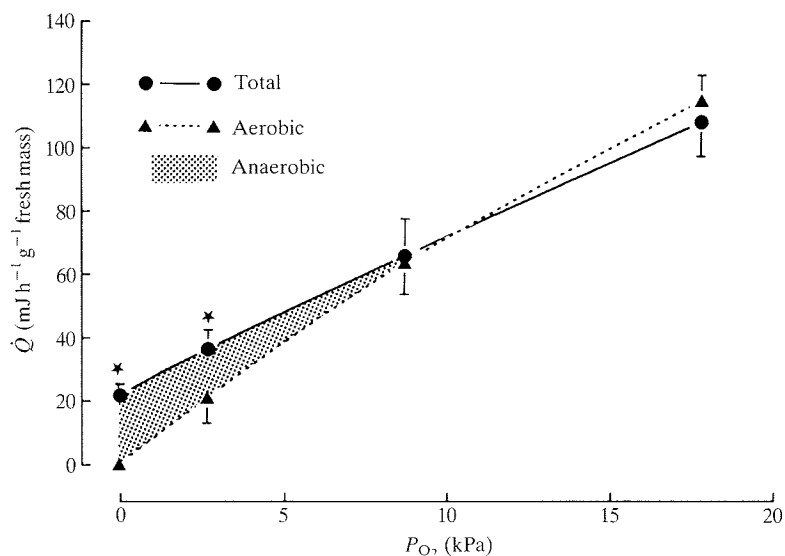


Fig. 3. Comparison of indirectly (oxygen consumption) and directly (calorimetry) determined mean rates of heat production (\dot{Q}) in *Sipunculus nudus* at different oxygen tensions using an oxycaloric equivalent of $450 \text{ kJ mol}^{-1} \text{ O}_2$ (mean \pm s.e.; $N=4$). Asterisks indicate a significant difference between directly and indirectly measured values ($P \leq 0.05$, unpaired t -test).

P_{O_2} of 2.66 kPa the aerobic metabolic rate was reduced to 18 % of control values. At this oxygen tension, however, the anaerobic component increased to 42 % of the total heat flux. During extreme hypoxia heat production was even further reduced and derived exclusively from anaerobic metabolism.

During the first hours of recovery the animals consumed more oxygen than under control conditions. This increase was not quantitatively reflected by a concomitant rise in heat dissipation. Later, anaerobic heat production began and continued for the next 30 h of recovery.

The relationship between total heat flux and aerobic (calculated) heat production at different oxygen levels is shown in Fig. 3. Aerobic heat production fell almost linearly with decreasing P_{O_2} . At a P_{O_2} of 8.66 kPa, oxygen consumption amounted to 55 % of the normoxic control value. Total heat production matched the aerobic (calculated) values above 8.66 kPa, but at lower oxygen levels a significant discrepancy between total and aerobic heat production was observed, indicating the onset of anaerobic metabolism. The anaerobic heat flux increased with decreasing P_{O_2} from $15.3 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass at 2.66 kPa to $21.5 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass under extreme hypoxia.

Changes in metabolite concentrations

To interpret the metabolite concentrations given in Tables 1 and 2 it is necessary to bear in mind that the animals were subjected to a stepwise reduction in P_{O_2} . This

means that animals were kept at 8.66 kPa, then at 2.66 kPa, before finally being exposed to extreme hypoxia.

Metabolite concentrations (Tables 1, 2) did not change significantly between normoxic and hypoxic (8.66 kPa) animals except for acetate, which decreased in the body wall musculature, propionate, which increased in the coelomic fluid, and succinate, which increased slightly in both compartments. Aspartate was degraded to some extent in the muscle tissue. A significant accumulation of anaerobic end products was found at a P_{O_2} of 2.66 kPa. Strombine concentrations rose from 0.78 ± 0.30 to $3.69 \pm 1.80 \mu\text{mol g}^{-1}$ body wall muscle. The increases in octopine, L-alanine, succinate and propionate contents were less pronounced. Because of high

Table 1. Concentrations of metabolites ($\mu\text{mol g}^{-1}$ body wall muscle) in *Sipunculus nudus* after incubation at different oxygen levels

	Control	8.66 kPa	2.66 kPa	0 kPa
ATP	2.17±0.44	2.51±0.33	2.20±0.52	1.71±0.19
Phosphoarginine	28.83±3.12	30.43±0.25	19.83±5.10*	13.50±1.43*
Arginine	6.59±1.22	7.87±1.96	15.40±6.62*	15.83±0.78
Octopine	0.12±0.05	0.12±0.08	0.86±0.57*	1.31±0.24
Strombine	0.78±0.29	0.78±0.30	3.69±1.80*	7.57±2.92*
L-Alanine	1.24±0.46	1.07±0.23	1.95±0.76*	2.63±0.61
D-Alanine	1.09±0.46	1.89±1.74	1.98±0.46	2.36±0.44
Aspartate	2.15±0.61	1.43±0.09*	0.86±0.21*	0.53±0.03*
Succinate	0.07±0.02	0.20±0.12*	0.80±0.26*	1.32±0.42*
Acetate	0.37±0.06	0.01±0.03*	0.03±0.03	0.17±0.07*
Propionate	<0.05	0.03±0.04	0.17±0.08*	0.59±0.22*

Values are mean±s.d. ($N=5$).

* Significant difference ($P \leq 0.05$, unpaired t -test) compared with values for the previous O_2 tension.

Table 2. Concentrations of succinate, acetate and propionate in the coelomic fluid (CF; $\mu\text{mol ml}^{-1}$ coelomic fluid) and their release into the incubation water (IW; $\text{nmol h}^{-1} \text{g}^{-1}$ fresh mass) after incubation at different oxygen partial pressures

	Control	8.66 kPa	2.66 kPa	0 kPa
Succinate				
CF	0.02±0.03	0.14±0.03*	0.19±0.11	0.35±0.21
Acetate				
CF	0.18±0.25	0.42±0.27	0.40±0.13	1.26±0.54*
IW	<1	<1	<1	21.3±4.5*
Propionate				
CF	<0.01	0.09±0.07*	0.43±0.19*	1.29±0.40*
IW	<1	<1	<1	5.7±5.3*

Values are mean±s.d. ($N=5$).

* Denotes a significant difference ($P \leq 0.05$, unpaired t -test) compared with values for the previous O_2 tension.

individual variations, the rise in D-alanine concentration was not significant. Acetate was not produced at a P_{O_2} of 2.66 kPa. A significant drop in phosphagen levels from 30.43 ± 0.25 to $19.83 \pm 5.10 \mu\text{mol g}^{-1}$ body wall muscle corresponded to an increase in arginine concentration from 7.87 ± 1.96 to $15.40 \pm 6.62 \mu\text{mol g}^{-1}$ body wall muscle. ATP levels remained constant.

After the transition to extreme hypoxia most anaerobic end products accumulated at similar rates to those under exposure to 2.66 kPa (Table 1). The formation of propionate was enhanced and, additionally, acetate production started. Tissue concentrations remained relatively low (acetate 0.17 ± 0.07 and propionate $0.59 \pm 0.22 \mu\text{mol g}^{-1}$ body wall muscle), whereas most of the fatty acids were excreted into the coelomic fluid (acetate 1.26 ± 0.54 and propionate $1.29 \pm 0.40 \mu\text{mol ml}^{-1}$) and the ambient water (acetate 21.3 ± 4.5 and propionate $5.7 \pm 5.3 \mu\text{mol g}^{-1}$ fresh mass). Phosphagen levels continued to decrease during extreme hypoxia but the decrease was less pronounced than under 2.66 kPa (from 19.83 ± 5.10 to $13.50 \pm 1.43 \mu\text{mol g}^{-1}$ body wall muscle). ATP levels fell from 2.20 ± 0.52 to $1.71 \pm 0.19 \mu\text{mol g}^{-1}$ body wall muscle during severe hypoxia. In all samples, concentrations of alanine were below the detection limit. Previous measurements have shown that after 24 h of anoxia no D- or L-lactate is detectable in the body wall musculature of *S. nudus*. The increase in malate level under these conditions can be neglected (Pörtner, 1982).

The heat balance

To calculate the expected metabolic heat dissipation from the changes in concentrations of anaerobic metabolites and to compare it with the measured heat production, anoxic rates of metabolite formation have to be multiplied by the molar enthalpy change for the respective end products (Gnaiger, 1983b; see Table 3):

$${}_k\dot{Q} = \sum \dot{N}_p \Delta_k H_p, \quad (1)$$

where ${}_k\dot{Q}$ is the catabolic heat dissipation ($\text{mJ h}^{-1} \text{g}^{-1}$ fresh mass), \dot{N}_p is the rate of end product formation ($\mu\text{mol h}^{-1} \text{g}^{-1}$ fresh mass) and $\Delta_k H_p$ is the molar enthalpy change of end product formation (kJ mol^{-1}) calculated from standard enthalpies of formation in dilute aqueous solution at pH 7, H_f° :

$$\Delta_k H_p = \sum \Delta H_f^\circ(\text{products}) - \sum \Delta H_f^\circ(\text{substrates}). \quad (2)$$

Since standard enthalpies of formation for octopine and strombine are not available, catabolic molar enthalpy changes for these two end products were assumed to be close to the value for lactate formation. Additional enthalpy changes, due to the condensation reaction between pyruvate and the respective amino acid, occurring in the pathway for opine formation, were considered to be negligible.

Table 3 shows the rates of metabolite formation during 24 h of severe hypoxia, based on the mean values given in Tables 1 and 2, and the associated heat and proton production.

Table 3. *Metabolic proton release and total heat production calculated from rates of end product formation (\dot{N}_p) under 24 h of anoxia*

	ν_{H^+}	\dot{N}_p^* (nmol h ⁻¹ g ⁻¹)	$\dot{N}_p \nu_{H^+}$ (nmol h ⁻¹ g ⁻¹)	$\Delta_k H_p$ (kJ mol ⁻¹)	$\dot{N}_p \Delta_k H_p$ (mJ h ⁻¹ g ⁻¹)
ATP	+0.61†	-6.9	-4.2	+27‡	-0.186
Phosphoarginine	-0.34†	-89.7	+30.5	+40§	-3.588
Octopine	-1.00	+6.4	-6.4	-55	-0.352
Strombine	-1.00	+55.0	-55.0	-55	-3.025
Alanine	-1.00	+15.0	-15.0	-77	-1.555
Succinate (from glycogen)	-2.00	+2.9	-5.8	-110	-0.319
Succinate (from aspartate)	+0.34	+4.6	+1.6	-54	-0.248
Acetate	-1.00	+20.6	-20.6	-65	-1.339
Propionate	-2.00	+24.5	-49.0	-113	-2.768
		Total	-123.9		
Released acids					
Acetate	-1.00	+21.3	-21.3	-65	-1.384
Propionate	-2.00	+5.7	-11.4	-113	-0.644
		Total	-32.7		-15.0

* Calculated using values from Table 1, with mass fraction coefficients of 0.34 for body wall musculature and 0.52 for coelomic fluid (Pörtner, 1987b).

† Pörtner (1987a) for pHi=7.1.

‡ Gnaiger (1980b).

§ Curtin and Woledge (1978).

Proton coefficients (ν_{H^+}) were derived from stoichiometric equations listed in Table 4. Molar enthalpy changes of end product formation ($\Delta_k H_p$) were calculated using values of ΔH_f° from Wilhoit (1969) and Gnaiger (1980b).

The released hydrogen ions undergo side reactions with physiological buffers, which are associated with additional enthalpy changes:

$${}_b\dot{Q} = \dot{N}_p \nu_{H^+} \Delta_b H_{H^+}, \quad (3)$$

where ${}_b\dot{Q}$ is the catabolic heat dissipation due to buffer reactions (mJ h⁻¹ g⁻¹), $\dot{N}_p \nu_{H^+}$ is the metabolic proton production, derived from the stoichiometric equations given in Table 4, ($\mu\text{mol h}^{-1} \text{g}^{-1}$) and $\Delta_b H_{H^+}$ is the enthalpy of neutralization (kJ mol⁻¹).

The main buffer substances occurring in the body fluids of *S. nudus* and their respective molar enthalpy changes are listed in Table 5. Metabolic proton production amounts to 123.9 nmol h⁻¹ g⁻¹ fresh mass (Table 3). The proton fractions neutralized by each buffer component were calculated as follows. (1) The change in phosphate protonation with a pH drop from 7.2 to 7.1, which was found after 24 h of anoxia in *S. nudus* (Pörtner *et al.* 1984a), was determined. Based on phosphagen depletion, the increase in phosphate concentration was calculated as 7.2 $\mu\text{mol g}^{-1}$ fresh mass. This results in a proton buffering of 14.7 nmol h⁻¹ g⁻¹

Table 4. *Stoichiometric equations of anaerobic end product formation*

Opines	$C_6H_{10}O_5 + 2 \text{ amino acid}^- \longrightarrow 2C_3H_3O_2 - \text{amino acid}^- + 2H^+ + H_2O$
Alanine	$C_6H_{10}O_5 + 2NH_4^+ \longrightarrow 2C_3H_7O_2N + H_2O + 2H^+$
Succinate from glycogen*	$C_6H_{10}O_5 + 0.86CO_2 + 0.14H_2O \longrightarrow 1.71C_4H_4O_4^{2-} + 3.42H^+$
Succinate from aspartate	$C_4H_6O_4N^- + 0.57H_2O + 0.29H^+ \longrightarrow 0.86C_4H_4O_4^{2-} + NH_4^+ + 0.57CO_2$
Acetate*	$C_6H_{10}O_5 + H_2O \longrightarrow 3C_2H_3O_2^- + 3H^+$
Propionate	$C_6H_{10}O_5 + 0.86CO_2 + 1.86H_2O \longrightarrow 1.71C_3H_5O_2^- + 1.71HCO_3^- + 3.42H^+$

* Gnaiger (1980b).

Redox balance is assumed between each substrate and the respective end product. The equations consider the actual participation of CO_2 or bicarbonate in the metabolic reactions. The proton stoichiometry for each end product is in accordance with the proton balance recently proposed by Pörtner (1987a).

Table 5. *Main buffer components in the body fluids of Sipunculus nudus and their molar enthalpy changes of proton neutralization ($=\Delta_b H_{H^+}$)*

	$\Delta_b H_{H^+}$ (kJ mol ⁻¹)	F_{H^+} (nmol h ⁻¹ g ⁻¹)	$F_H \Delta_b H_{H^+}$ (mJ h ⁻¹ g ⁻¹)
HPO ₄ ²⁻ /H ₂ PO ₄ ⁻	-6*	14.7	0.088
NH ₃ /NH ₄ ⁺	-52†	6.9	0.358
HCO ₃ ⁻ /CO ₂	-11*	54.8	0.603
Histidine residues	-32‡	47.5	1.520
Sea water (HCO ₃ ⁻ /CO ₂)	-11	32.7	0.360
		Total	2.929

Proton fractions, buffered by each component (F_{H^+}), and the resulting heat production were calculated for 24 h of anoxia (see text).

* Gnaiger (1980b).

† Wilhoit (1969).

‡ Curtin and Woledge (1978).

fresh mass by phosphate protonation. (2) Proton consumption due to NH_3 formation expected from ATP depletion was calculated as 6.9 nmol h⁻¹ g⁻¹ fresh mass. (3) Proton buffering by titration of bicarbonate was calculated as 24.6 nmol h⁻¹ g⁻¹ fresh mass by the drop in HCO₃⁻ content after 24 h of anoxia (based on Pörtner, 1987b), and as 30.2 nmol h⁻¹ g⁻¹ fresh mass by bicarbonate formation during propionate synthesis (see Tables 3, 4). Neutralization of the remaining 47.5 nmol H⁺ h⁻¹ g⁻¹ fresh mass was attributed to buffering by histidine residues. For volatile fatty acids, released into the ambient water, a $\Delta_b H_{H^+}$ of -11 kJ mol⁻¹ was employed for bicarbonate buffer.

The sum of the enthalpy changes of end product formation ($15.0 \pm 4.5 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass, $N=5$) and of proton buffering ($2.9 \pm 1.2 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass, $N=5$) amounted to $17.9 \pm 4.7 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass, which is not significantly different from the calorimetrically measured heat production of $21.5 \pm 3.5 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass.

Discussion

Heat dissipation under progressive hypoxia

As an inhabitant of the intertidal zone, the marine worm *Sipunculus nudus* is regularly exposed to reduced water, and therefore oxygen, availability. The animal responds to hypoxic conditions by reducing its oxygen consumption correspondingly (see Fig. 3). This oxyconformic behaviour of *S. nudus* had already been demonstrated by Henze (1910) and was recently confirmed by Pörtner *et al.* (1985).

The comparison of heat production and oxygen consumption demonstrates that energy production remains fully aerobic down to P_{O_2} values between 8.66 and 2.66 kPa. These results differ from the observations made by Famme *et al.* (1981) for *Mytilus edulis* and by Hammen (1983) for *Crassostrea virginica*. These authors found that the two bivalve molluscs compensated for the decline in aerobic energy production down to very low oxygen tensions by increasing anaerobic metabolism, such that the overall heat dissipation was kept constant. This pattern of energy expenditure is associated with a high rate of substrate consumption. *S. nudus*, however, adjusts its rates of energy expenditure and substrate depletion to the availability of oxygen and is thus able to use its body stores economically.

At oxygen tensions below 8.7 kPa aerobic metabolism alone cannot satisfy the energy demands of *S. nudus* and additional anaerobic energy production becomes necessary (Fig. 3). This is consistent with the accumulation of anaerobic end products observed at 2.66 kPa (Tables 1, 2). From these results we conclude that the critical P_{O_2} (the so-called P_{crit}) that indicates the onset of anaerobic metabolism must lie between 2.66 and 8.66 kPa. These data correspond well with those of Pörtner *et al.* (1985), who determined the critical P_{O_2} to be close to 6.7 kPa in large specimens of *S. nudus*.

The overall heat production rate is further reduced with decreasing P_{O_2} until it amounts to only 20% of the normoxic value under extreme hypoxia. This value may have a small aerobic component because very low rates of oxygen diffusion into the experimental system cannot be ruled out. In these experiments the P_{O_2} at extreme hypoxia was kept below the detection limit of the oxygen electrode (i.e. 66 Pa = 0.5 mmHg). At a flow rate of $0.1 \text{ ml min}^{-1} \text{ g}^{-1}$ fresh mass, complete extraction of the residual oxygen by the animals would yield an aerobic heat production of $2.2 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass, which is only about 10% of the measured total heat production of $21.5 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass. Thus, the anaerobic metabolic rate certainly amounts to 18–20% of the normoxic value, which agrees well with values measured in other worms, such as the oligochaetes *Lumbriculus variegatus* (23% ;

Table 6. ATP turnover ($\mu\text{mol ATP h}^{-1} \text{g}^{-1}$ fresh mass) and glycolytic rates ($\text{nmol glycosyl units h}^{-1} \text{g}^{-1}$ fresh mass) in *Sipunculus nudus* under different oxygen levels

P_{O_2} (kPa)	ATP turnover			Glycolytic rate (aerobic; anaerobic)
	Aerobic	Anaerobic	Total	
17.99	1.60	—	1.60	43.3 (43.3;0)
8.66	0.88	—	0.88	23.9 (23.9;0)
2.66	0.29	0.31	0.60	52.9 (7.9;45.0)
0	—	0.44	0.44	77.0 (0;77.0)

Gnaiger and Staudigl, 1987) and *Tubifex tubifex* (10–15 %; Famme and Knudsen, 1984) and the polychaete *Nereis diversicolor* (10 %; A. D. F. Addink, unpublished results).

ATP turnover and glycolytic rates at different oxygen levels

Unfortunately, the turnover of ATP cannot be deduced from the total heat dissipation, because there is no constant relationship between these variables in aerobic and anaerobic pathways. The dissipated heat per unit of ATP turnover is approximately -80 kJ mol^{-1} for fully coupled aerobic metabolism, but less than -40 kJ mol^{-1} for the acetate–propionate pathway (Gnaiger, 1983b). Therefore, ATP turnover rates must be calculated from biochemical data, using stoichiometric coefficients of ATP production for aerobic and anaerobic glycogen degradation, as published by Gnaiger (1980b, 1983b) and Pörtner (1987a). For the glycolytic end products D- and L-alanine, octopine and strombine, 1.5 mol ATP per unit of product are derived from the Embden–Meyerhof–Parnas pathway. For succinate and propionate, formation *via* fumarate reductase has to be assumed for the sake of redox balance maintenance, which yields 2.5 and 3.5 mol ATP, respectively, per unit of product. Finally, acetate production is accompanied by the formation of 2.5 mol ATP per unit of product.

The metabolite flux through the Embden–Meyerhof–Parnas pathway can be calculated from the oxygen consumption and from the accumulation of anaerobic end products, assuming that glycogen is the only substrate used by *S. nudus*.

During normoxia the turnover rate of ATP is $1.6 \mu\text{mol h}^{-1} \text{g}^{-1}$ fresh mass and the metabolite flux through the Embden–Meyerhof–Parnas pathway amounts to $43.3 \text{ nmol glycosyl units h}^{-1} \text{g}^{-1}$ fresh mass, as calculated from oxygen consumption (see Table 6). The contribution of anaerobic metabolism can be excluded, because a caloric/respirometric ratio of $423.4 \pm 27.1 \text{ kJ mol}^{-1} \text{O}_2$, found during normoxia, indicates fully aerobic metabolism.

When the animals are exposed to moderate hypoxia at a P_{O_2} of 8.66 kPa, the ATP demand and the rate of glycogen depletion are reduced to 55 % of the

normoxic values. From this result it is evident that under steady-state conditions of moderate hypoxia *S. nudus* reduces its energy expenditure. Except for the small amount of aspartate degradation (Table 1) and a small, but significant, accumulation of succinate, no marked anaerobic energy contribution can be seen. The slight anaerobiosis might be due to an anaerobic burst occurring at the beginning of moderate hypoxia (Figs 1, 2). It can therefore be concluded that, during moderate hypoxia, ATP is still provided by aerobic metabolism, albeit at a depressed rate.

At a lower steady state of oxygen provision (P_{O_2} 2.66 kPa) the ATP turnover is further decreased to 37.5 % of the normoxic rate, but now 52 % of the total ATP yield is supplied by anaerobic processes (Table 6). Despite the pronounced depression of energy expenditure, the metabolite flux through the Embden–Meyerhof–Parnas pathway is increased compared to normoxic values. During acute hypoxia (P_{O_2} nominally zero) a further depression of heat dissipation to 19.5 % of the normoxic rate occurs. Although the ATP turnover yields only 27.5 % of the normoxic value, the ATP requirement can only be met by an enhanced degradation of glycogen, which amounts to 178 % of the normoxic flux. Thus, at least in *S. nudus*, there is no suppression of the Embden–Meyerhof–Parnas pathway during extreme hypoxia; instead, it is activated to almost twice its normoxic rate.

From these results one must conclude that under moderate hypoxia metabolic depression leads to a lower basal metabolic rate which is sustained by an aerobic energy metabolism. As a consequence, the animal will save substrate. When the critical oxygen tension (P_c) is passed, the animal can lower its energy expenditure still further, but it cannot save stored substrate, because the low ATP yield of anaerobic energy provision requires an increased degradation of glycogen. As a corollary, *S. nudus* may save substrate when the tide is receding and it may thus compensate for the enhanced glycogen degradation during acute hypoxia at the end of low tide. Overall, the degradation of glycogen during ebb may not be very different from that during normoxic conditions.

Recovery from anaerobiosis

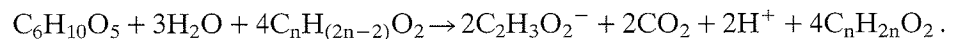
During the first hours of recovery a marked increase in oxygen uptake above control values was observed, whereas heat production was only slightly enhanced. This leads to a calorimetric/respirometric (CR) ratio of 335 kJ mol⁻¹ O₂, which is lower than the value for fully dissipative aerobic metabolism (450 kJ mol⁻¹ O₂, Gnaiger, 1983a). In *Mytilus edulis* the CR ratio was 360 kJ mol⁻¹ O₂ during the first hour of recovery from 72 h of anoxia (Shick *et al.* 1988). For recovering oyster larvae a value as low as 180 kJ mol⁻¹ O₂ has been observed (Widdows *et al.* 1989). CR ratios below 450 kJ mol⁻¹ O₂ may indicate non-metabolic oxygen uptake, i.e. oxygenation of the haemerythrin store and saturation of the coelomic fluid. For *S. nudus*, an additional O₂ uptake of 0.47 μmol O₂ g⁻¹ fresh mass is necessary to saturate the coelomic fluid and reoxygenate the haemerythrin after extreme hypoxia (calculated using data from Pörtner *et al.* 1985). The occurrence of

endothermic anabolic reactions, such as gluconeogenesis or phosphagen repletion, also results in significantly decreased CR ratios, down to $200 \text{ kJ mol}^{-1} \text{ O}_2$ for succinate clearance for instance (Shick *et al.* 1988). In *S. nudus*, accumulated succinate is degraded within 6 h and the phosphagen is restored within 3 h after the end of anaerobiosis (Pörtner *et al.* 1986).

During prolonged recovery, anaerobic processes become involved, a phenomenon that has also been observed in *M. edulis* by Shick *et al.* (1986). The authors found a correlation between the frequency of contractions of the adductor muscle and anaerobic metabolic rate during recovery, which led to the conclusion that additional anaerobic energy is used for shell movements. In case of *S. nudus* increased respiratory peristaltic movements could require anaerobic energy provision.

Correlation between direct and indirect calorimetry during anaerobiosis

In this study the anaerobic heat production calculated from biochemical analysis is close to the directly measured anaerobic heat production. Calculations of metabolic enthalpy changes are based on the maintenance of redox balance. During anaerobiosis this is achieved if a maximum ratio of acetate:(propionate + succinate) production of 0.5 is maintained (Gnaiger, 1980*b*, 1983*b*). In our experiments, we found a value of 1.11. Including the additional NADH derived from alanine production by transamination, we found an excess of $50.7 \text{ nmol NADH h}^{-1} \text{ g}^{-1}$ fresh mass. Assuming an oxygen consumption of $5.0 \text{ nmol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ fresh mass, due to O_2 diffusion into the experimental system under extreme hypoxia (see above), regeneration of $11 \text{ nmol NADH h}^{-1} \text{ g}^{-1}$ fresh mass can be explained by oxidative phosphorylation. For reoxidation of the remaining $39.7 \text{ nmol NADH h}^{-1} \text{ g}^{-1}$ fresh mass additional NAD-regenerating reactions must occur. Zs-Nagy (1977) suggested an endogenous anoxic oxidation of NADH by electron transfer to unsaturated fatty acids. In this case, the stoichiometric equation for acetate formation would be:



If oleic acid were to serve as the final electron acceptor, for instance, the $\Delta_k H$ of this reaction would yield $230.8 \text{ kJ mol}^{-1}$ acetate [calculated with H_f from Wilhoit (1969), Weast (1976) and Gnaiger (1980*b*)]. These enthalpy changes exceed the values for acetate formation in combination with succinate and propionate production by $165.8 \text{ kJ mol}^{-1}$. The calculated value of heat dissipation under extreme hypoxia ($17.9 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass) must therefore be corrected for the additional aerobic heat dissipation caused by residual oxygen contamination ($=2.2 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass) and for the formation of $19.8 \text{ nmol acetate h}^{-1} \text{ g}^{-1}$ fresh mass ($\triangleq 39.7 \text{ nmol NADH h}^{-1} \text{ g}^{-1}$ fresh mass) associated with fatty acid saturation ($=3.3 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass). The corrected value is $23.4 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass, which is in fair accordance with the calorimetrically measured heat production of $21.5 \pm 3.5 \text{ mJ h}^{-1} \text{ g}^{-1}$ fresh mass.

Famme and Knudsen (1984) assumed that the results obtained by direct and

indirect calorimetry were in agreement for *Tubifex tubifex*. Their calculations were criticized, however, by Gnaiger and Staudigl (1987), who stated that the caloric equivalent for excreted acids of -146 kJ mol^{-1} acid used by Famme and Knudsen (1984) was too high. Using Gnaiger's (1983b) values, the biochemically explained heat amounts to only 66% of the calorimetrically measured heat production. Gnaiger (1980a) found a similar discrepancy for the values in *Lumbriculus variegatus*. He explained this 'anoxic gap' by postulating unknown sources of anaerobic heat production. Gnaiger's studies should only be considered as rough estimations, since he compared the measured heat production of *L. variegatus* with the release of fatty acids by *Tubifex* spp. (actually a mixture of different species) determined by Schöttler and Schroff (1976). Even if the metabolism of the animals used is comparable, as assumed by Gnaiger (1980a), the differences in experimental conditions and seasonal variations might have caused the observed discrepancy. A more detailed study was carried out by Shick *et al.* (1983). They determined heat production and end product accumulation simultaneously in *Mytilus edulis* and found an unexplained heat fraction of 63% between 3 and 48 h of anoxia. The authors explained the observed discrepancy by postulating additional enthalpy changes caused by protonation of CO_3^{2-} from the shell, which is observed under prolonged anoxia. But since the caloric coefficients for anaerobic end products employed by Shick *et al.* (1983) incorporated the enthalpy changes due to physiological proton buffering ($\Delta_b H_{\text{H}^+} = -30 \text{ kJ mol}^{-1}$), this explanation has to be rejected (J. M. Shick, A. de Zwaan and A. M. Th. de Bont, personal communication).

A close agreement between the results obtained by direct and indirect heat production, as we found in the present study, implies that all essential processes in anaerobic metabolism have been considered. This is also confirmed by the recent finding that changes in the acid-base status in anaerobic *Sipunculus nudus* could be completely explained by the formation of anaerobic end products and the associated protons (Pörtner, 1987b).

In conclusion, we have demonstrated that changes in oxygen consumption above P_{cII} reflect changes in total metabolism of the animals. We have thus verified our earlier study (Pörtner *et al.* 1985), which, based on biochemical analyses, led us to define P_c as the critical P_{O_2} below which anaerobiosis starts. The use of non-invasive calorimetry also allowed quantification of the metabolic rate below P_c . Heat production under acute hypoxia could be attributed to the formation of known anaerobic end products. In accordance with calculations based on anaerobic metabolic changes, ATP expenditure decreased to 27% of normoxic values and reflected the energy-saving strategies of the animals during anaerobiosis.

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