J Comp Physiol B (1992) 162:297–304

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Influence of long-term hypoxia on the energy metabolism of the haemoglobin-containing bivalve *Scapharca inaequivalvis*: critical O_2 levels for metabolic depression

G. van den Thillart[1]*, G. van Lieshout[2], K. Storey[3], P. Cortesi[4], and A. de Zwaan[2]

Consorzio di studi, ricerche ed interventi sulle risorse marine, viale Vespucci 2, 47042 Cesenatico (FO), Italy

[1] University of Leiden, Department of Biology, Gorlaeus laboratoria, POB 9502, 2300-RA, Leiden, The Netherlands
[2] Delta Institute for Hydrobiological Research, vierstraat 28, 4401-EA, Yerseke, The Netherlands
[3] Carleton University, Department of Biology, Ottawa, Ontario, Canada K1S 5B6
[4] University of Bologna, Department of Biochemistry, via Belmeloro 8/2, 40126 Bologna, Italy

Accepted January 10, 1992

Summary. The oxygen consumption rate of *Scapharca inaequivalvis* measured under normoxic conditions over 48 h showed a significant daily cycle with lowest values occurring shortly after the dark period; all hypoxia exposure experiments were carried out during the declining part of the cycle. Animals were exposed to a constant level of hypoxia for a 12-h period in a series of 14 experiments, each at a different oxygen tension. The oxygen consumption was measured continuously, and the extent of accumulation of end-products (succinate and propionate), and the inhibitory effect of adenosine triphosphate on phosphofructokinase were determined at the end of exposures. All three parameters (oxygen consumption, end-product accumulation, phosphofructokinase inhibition) showed a remarkable correlation with major changes occurring between 2.5 and 1.5 ppm (7 and 4 kPa) O_2. The oxygen consumption rates showed a drop to 6% of the normoxic rate, but a consistent low consumption remained below 2 ppm (5.5 kPa) which partly recovered over the 12-h exposure period by about three-fold. Succinate and propionate accumulated progressively between 2.5 and 1.5 ppm (7 and 4 kPa); at $[O_2] < 1.5$ ppm (4 kPa) the concentration did not increase further, indicating that anaerobic metabolism had reached a maximum. Over the same range, phosphofructokinase showed an increased sensitivity for adenosine triphosphate, the lower inhibitor concentration at 50% V_{max} value pointing to depression of glycolytic rate. Despite the activation of anaerobic metabolism and the evident depression of aerobic metabolism, simple calculation demonstrates that *Scapharca inaequivalvis* relies mainly on aerobic metabolism even during severe hypoxia. It is assumed that the occurrence of haemoglobin in this species is essential for its capacity to survive long periods of hypoxia.

Key words: Hypoxia – Phosphofructokinase – Energy metabolism – Haemoglobin – Bivalve, *Scapharca inaequivalvis*

Introduction

Anaerobic metabolism in bivalves has been studied almost exclusively under anoxic conditions; an impressive anoxia resistance is found, ranging from days to weeks depending on species, weight, season and temperature (Theede et al. 1969). *Scapharca inaequivalvis* is able to survive anoxia at 18 °C for 17 days during which a significant production of propionate and succinate is observed (Brooks et al. 1991). However, the energy generated under environmental anoxia is only a fraction of the aerobic energy requirements, demonstrating that the animals survive environmental anoxia mainly by means of metabolic depression. Since anaerobic metabolism in bivalves is obviously not capable of compensating for the loss of aerobically generated energy, it follows that the contribution from anaerobic metabolism will be effective only under rather low O_2 levels when energy consumption is depressed. Therefore, it is likely that anaerobic metabolism is activated at low O_2 levels.

Scapharca inaequivalvis is a "blood-clam" recently introduced in the northern Adriatic Sea. This species is very successful in this area and outcompetes native species of economic value such as *Venus gallina*. One of the reasons for this success may be the presence of haemoglobin-containing erythrocytes in the blood-clam. According to Weber (1980), the presence of haemoglobin in invertebrates improves oxygen transport at low O_2 levels.

Abbreviations: ATP, adenosine triphosphate; I_{50}, inhibitor concentration at 50% V_{max}; PFK, phosphofructokinase; P_c, critical PO_2; SEM, standard error of mean; $\dot{V}O_2$, oxygen consumption rate; ww, wet weight

* To whom offprint requests should be sent

Since hypoxia is a common feature in the northern Adriatic Sea, mainly due to eutrophication and high temperatures (Degobbis 1989), it is assumed that this species can rely on aerobic metabolism even at very low O_2 levels when other species must switch to anaerobic metabolism.

This study compares the $\dot{V}O_2$ of *Scapharca inaequivalvis* over a wide range of PO_2 with some parameters of anaerobic metabolism. A metabolic transition occurs between 1.5 and 2.5 ppm (5 and 8 kPa) O_2 where anaerobic metabolism becomes activated. At the same point there is a marked change in PFK inhibition by ATP, indicating that the glycolytic rate becomes depressed. However, aerobic metabolism remains the most important energy source even at the lowest measured oxygen level (0.5 ppm, 1.4 kPa) despite the depressed metabolic rate. In addition, there was a significant increase in $\dot{V}O_2$ during a 12-h hypoxia exposure period. Thus, it appears that *Scapharca inaequivalvis* depresses its metabolic rate at low O_2 levels, while energy production remains mainly aerobic.

Materials and methods

Conditioning. Animals were caught by a local fisherman by trawling from the sand bottom a few miles offshore near Cesenatico in the Adriatic Sea from August 11 to September 11 1988. The animals were kept in (sand-filtered) running sea water at 20 °C for more than 2 weeks before use. The seawater was pumped in from an inlet about 300 m offshore into a large concrete basin for sedimentation prior to filtering and thermostatting. The animals were cleaned and selected on arrival, divided into groups of 40–50 animals and placed in open baskets suspended in 3000-l polyester tanks. The water was well aerated and thermostatted by equilibration with the air-conditioned aquarium room. The illumination was fixed at the daily cycle of mid-August.

Oxygen consumption measurements. For $\dot{V}O_2$ measurements the respirometer described in Fig. 1 was used. With this apparatus the $[O_2]$ can be maintained at a constant level while $\dot{V}O_2$ is calculated from the water flow which is registered continuously on a datalogger (van den Thillart and Verbeek 1991), allowing continuous $\dot{V}O_2$ measurements. Apart from the continuous $\dot{V}O_2$ measurement, slope measurements were made at the beginning and the end of each experimental series.

Blank respiration. Although the respirometer was thoroughly cleaned, the blanks stayed at about 10% of the normoxic $\dot{V}O_2$ and increased two- to three-fold during the experiment (20 h). At low O_2 levels ($O_2 < 2.5$ ppm), when the clams had a depressed metabolic rate, blanks sometimes accounted for 50% of total $\dot{V}O_2$. Preliminary experiments revealed that this increase in blank respiration was due to the presence of the clams in the respirometer. Blanks stayed constant for hours after the animals were taken out, but increased linearly with time and biomass as long as the animals were in the chamber; apparently, the animals excrete a substance that serves as a substrate for micro-organisms. For the calculation of the animal $\dot{V}O_2$ the actual blank value was calculated by interpolation of the blanks measured before and after each experiment.

Protocol of hypoxia experiments. Groups of 35 animals were exposed consecutively to two periods of 1.5 h (A and B), and one period of 12 h (C). Before and after each of these periods a blank was measured for at least 30 min. The $\dot{V}O_2$ measurements of all six blanks and the exposures A and B were calculated from a declining $[O_2]$. Depending on the $\dot{V}O_2$, the $[O_2]$ was raised half-way in order

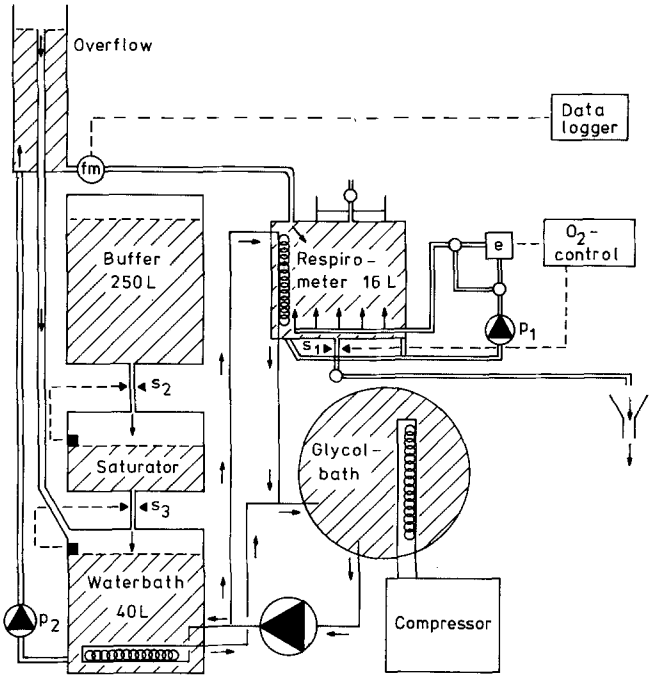

Fig. 1. Schematic diagram of respirometer and water control apparatus. The two respirometer chambers have a volume of 16 l and are kept at 20±0.1 °C by a quartz heater regulated by a thermostat. The respirometer chambers and the waterbath are continuously cooled by a closed cool-water circuit which allows a very accurate temperature control. The water in the respirometer chamber is circulated by pump (*P1*) at a rate of 2 l · min^{-1} and flows past an EIL (Electronic Instrument Ltd) temperature-compensated electrode (*e*) connected to an EIL O_2 monitor/controller, type 9401. The controller activates solenoid value *S1* when the $[O_2]$ falls below a set point, then air-saturated water flows from the overflow into the respirometer chamber until the preset level is reached. The flow is measured by a Rhodes low-flow meter (*fm*) coupled to a datalogger which registers the counts over a preset interval and stores the data on an exchangable EPROM. The water consumption is compensated by a series of storage tanks (250 l) connected to the saturator tank. The water level in the saturator and the waterbath are both set by electronic regulators connected to solenoid values S_2 and S_3. The water in the waterbath is thermostatted to 20±0.5 °C and circulated by pump P_2 to the overflow

to keep the $\Delta[O_2] < 0.7$ ppm (< 2.0 kPa). During exposure C the flowmeter method was employed. Exposure A was always at about 6 ppm (16 kPa) O_2, while B and C were at hypoxia levels ranging from 0.5 to 6 ppm (1.4–16 kPa) O_2. Hypoxia was reached by bubbling with N_2 gas, and during exposure C the PO_2 was kept at a constant level by the controlling device described in Fig. 1.

At the end of the hypoxia period the last blank was measured (by the slope method), while some animals were processed for metabolites and PFK kinetics, and others for determination of ww. The electrodes were calibrated before the beginning of each experiment with 10% sulphite and 100% air-saturated water. The O_2 concentration of the water in the animal chambers, and of the inflowing (saturated) water, was determined by the Winkler method during the normoxia and hypoxia period on every experimental day.

Enzyme and metabolite methods. The preparation of samples and the assays for PFK activity in the foot muscle were carried out as described before (Brooks et al. 1991). Succinate and propionate measurements were carried out on whole animals which were removed from the shells, blotted on tissue paper and rapidly frozen

with aluminum blocks cooled with liquid N_2. Extraction and assays were carried out as described elsewhere (Brooks et al. 1991).

Statistics. All data groups were tested for significant differences by the parameter-free Wilcoxon *Q*-test.

Results

Oxygen consumption pattern

In order to analyse the daily cycle and the variability of $\dot{V}O_2$, eight groups of 30 clams each were monitored over a period of 24 h, while four groups were followed over 48 h (Fig. 2). For pattern analysis the $\dot{V}O_2$ of each experiment was normalized at 100% at 20:00 hours (10 h after onset), then all groups were compared and tested (Wilcoxon dual tail). All experiments showed a daily pattern with a significant difference between the peak and valley points ($P<0.01$). The lowest values were always found shortly after the dark period. The mean $\dot{V}O_2$ for eight experiments over 24 h was 75 ± 25 µg $O_2 \cdot h^{-1} \cdot g\ ww^{-1}$; for each experiment there was a difference between the valley and the peak of about 30%.

Oxygen consumption during hypoxia

In a series of 14 experiments, groups of 35 animals were exposed to different O_2 concentrations (see protocol). The same group was exposed to a short hypoxia period (2 h), and after blank measurements to a long period (12 h) at the same O_2 concentration. In Fig. 3 the results show a clear-cut metabolic depression of about 15-fold occurring between 2.5 and 1.5 ppm (7 and 4 kPa) O_2. In addition, an adaptational response (dashed lines in Fig. 3) was observed in *all* experiments below 2.5 ppm (7 kPa). During the 12-h exposure to hypoxia, 9 out of 14 experiments showed a significant ($P<0.01$) increase in $\dot{V}O_2$ of $320\pm100\%$; the remaining 5 (normoxic) experiments gave values of $98\pm8\%$. From observations it appeared that a large number of clams kept their shells closed after the first transfer, sometimes for more than 1 h. This had a marked effect on the initial (normoxic) measurement: the values were between 30 and 40% lower, and were ignored since they could not be used as controls. Values returned to control levels within 2 h; no handling effect was discernable thereafter.

$\dot{V}O_2$ was monitored in all experiments during the 12-h hypoxia exposure. The patterns of a few typical experiments are shown in Fig. 4. The increase of $\dot{V}O_2$ occurred at the higher O_2 concentrations during the first 2 h but at the lower O_2 levels it was a slow process that lasted 12 h.

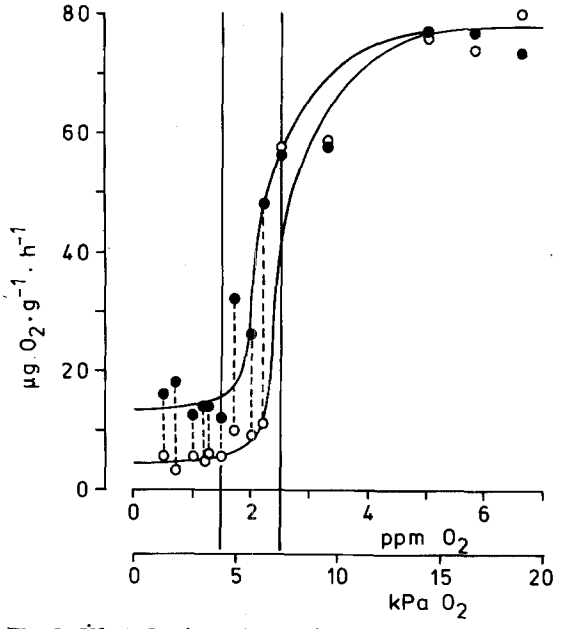

Fig. 3. $\dot{V}O_2/PO_2$ dependence of *Scapharca inaequivalvis* measured at the beginning and the end of a 12-h hypoxia exposure period. The data are from a series of 14 independent experiments, each with a group of 35 animals. The $\dot{V}O_2$ of the animals was monitored over a period of 12 h, while PO_2 was kept at a constant level. In all experiments below 2.5 ppm (7 kPa) O_2 a marked increase of $\dot{V}O_2$ was found during the exposure period *(dashed lines)*. The values are expressed as µmol $\cdot h^{-1} \cdot g\ ww^{-1}$. Symbols: ○, $\dot{V}O_2$ over t=0–2 h; ●, $\dot{V}O_2$ over 10–12 h

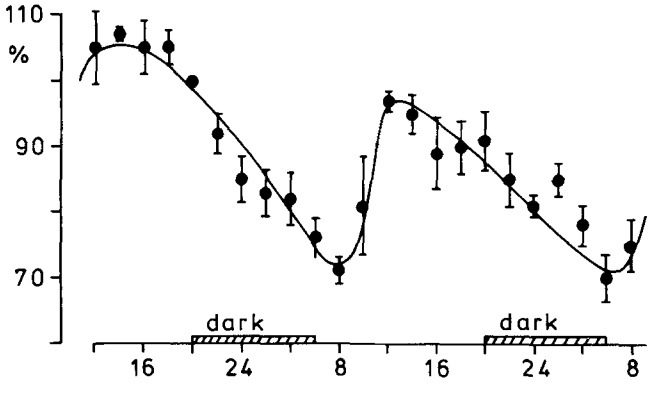

Fig. 2. The daily pattern in O_2 consumption of *Scapharca inaequivalvis;* a series of four independent experiments at 6 ppm (16 kPa) O_2 measured over 48 h. The curves were normalized by setting the $\dot{V}O_2$ at 20:00 hours at 100%. Means±SEM over 2-h periods are presented

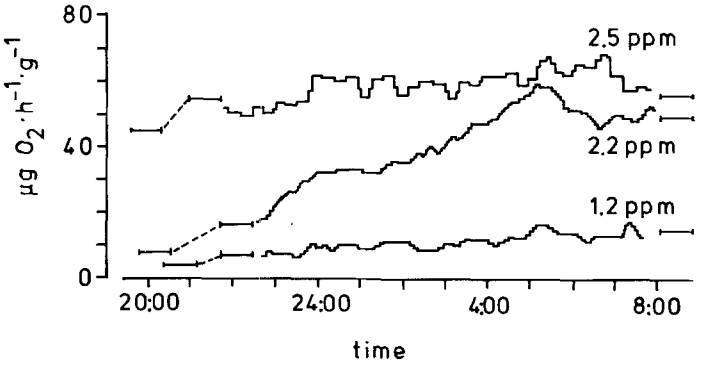

Fig. 4. Oxygen consumption of *Scapharca inaequivalvis* during a 12-h hypoxia exposure. The *bars* indicate the slope measurements, and in between are the waterflowbased measurements. In all experiments below 2.5 ppm (7 kPa) O_2 a marked (>3-fold) increase of $\dot{V}O_2$ was found. The values are expressed as µmol $\cdot h^{-1} \cdot g\ ww^{-1}$

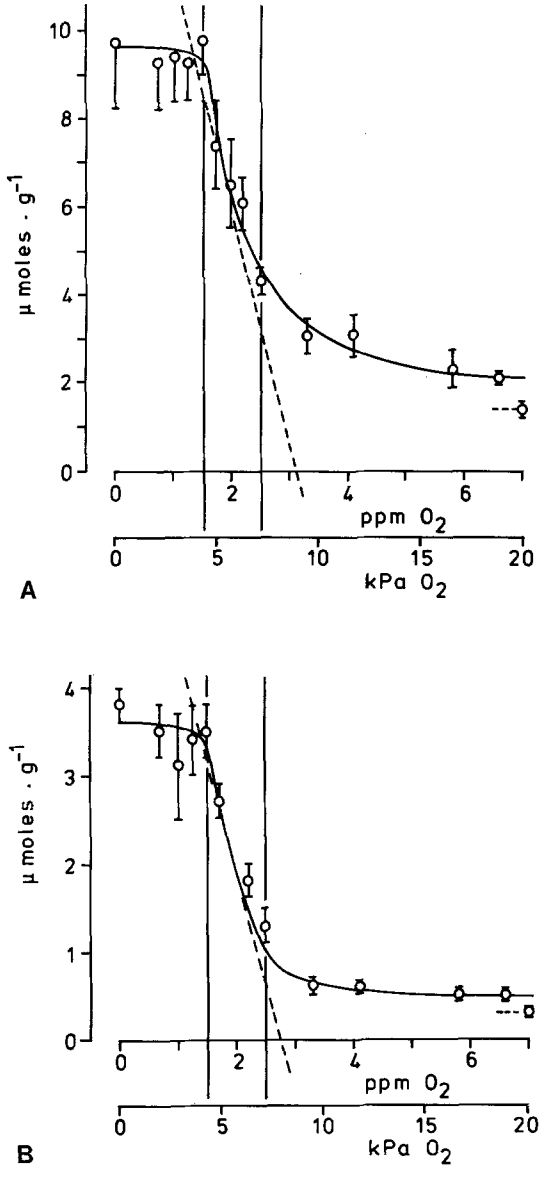

Fig. 5A, B. Whole-body succinate (**A**) and propionate (**B**) concentrations measured after a 12-h exposure period to 14 different ambient O_2 levels. Oxygen levels are expressed as ppm and kPa. Means ± SEM are given for each condition ($n=5$); under the curve at 7 ppm the reference value of animals taken from the storage tanks is shown. The values are expressed as µmol · h^{-1} · g ww^{-1}

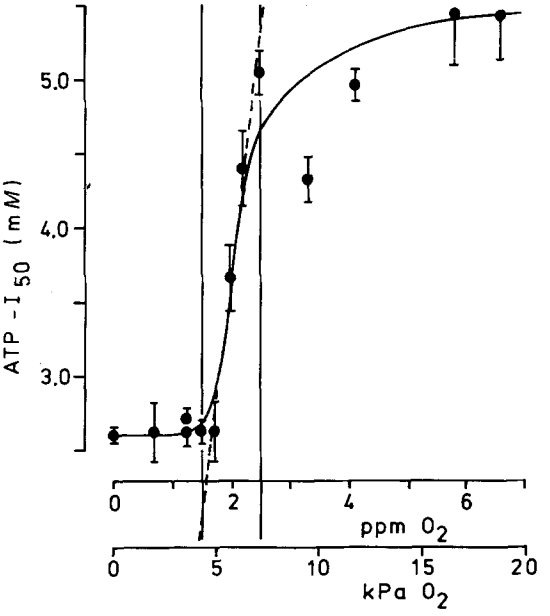

Fig. 6. ATP inhibition (I_{50}) of phosphofructokinase from the foot tissue shifts to lower values during exposure to $[O_2] < 2.5$ ppm. Each point represents an independent experiment at a different hypoxia level. The oxygen levels are expressed as ppm and kPa. Means ± SEM are given for each condition ($n=5$)

Anaerobic metabolism

The activation of anaerobic metabolism by hypoxic exposure was measured by succinate and propionate accumulation in whole animals. After a 12-h exposure period five animals were analysed from each group. In Fig. 5A, B, the metabolite levels (means ± SEM) are shown for the experimental animals, as well as data for animals taken from the storage tanks. For both metabolites there was a significant difference between the groups at O_2 concentrations below 1.5 ppm (4 kPa) and those groups above 2.5 ppm (7 kPa) O_2 ($P<0.01$). From Fig. 5A, B it is evident that anaerobic metabolism is activated at 2.5 ppm O_2 and that at $[O_2] \leq 1.5$ ppm the rate of accumulation of anaerobic end products is constant. No succinate or propionate was found in the incubation water of the hypoxic group after 12 h of exposure; also a test with anoxic exposure (in a small water volume) indicated no excretion of end products during 12 h of anaerobiosis.

Phosphofructokinase-inhibition by ATP

Phosphofructokinase inhibition by ATP was measured in the foot muscle. Fig. 6 shows the means ± SEM from groups of five animals after a 12-h exposure to different O_2 concentrations. The values for I_{50} at $O_2 < 2$ ppm (< 6 kPa) were all significantly different from those at $O_2 \geq 2.5$ ppm (7 kPa) ($P<0.01$), indicating a sharp change in PFK kinetics.

Discussion

Oxygen consumption. The O_2 consumption of *Scapharca inaequivalvis* showed a 24-h pattern with an increase in $\dot{V}O_2$ between 06:00 and 12:00 hours, followed by a decline over the remaining 18 h. As shown in Fig. 2, the increase in $\dot{V}O_2$ occurs 1–2 h after the lights were switched on. It is not clear what determines this pattern; a "startle" response is certainly not likely, since only a short-term response would be expected immediately after the stimulus. Also, the light conditions in the holding tanks were identical as those in the respirometer. The pattern is likely to be imposed by the dark/light cycle since there is no other 24-h cycle; therefore, the animals must have some kind of photoreceptor.

The change in $\dot{V}O_2$ is about 30%, which is small in comparison with more active animals where the difference between active and resting rates is over ten-fold. In this case observations were made on a group of 35, which means that the individual variation due to locomotion might be much higher. Groups of *Scapharca inaequivalvis* were also kept in aquaria with a water-infiltrated sand bed, where the animals left clearly visible tracks as a result of their locomotory activity. From these tracks it could be seen that there was indeed a large individual variation. $\dot{V}O_2$ measurements by Bayne (1971) on individuals of three different bivalve species showed a similar large individual variability.

Effects of long-term hypoxia on $\dot{V}O_2$ in bivalves have been studied by Livingstone and Bayne (1977), who investigated the effects of long-term exposure to 30% and 50% air saturation (6.3 and 10.2 kPa) on *Mytilus edulis*. At these levels there was a transient increase in succinate levels at 22 °C and none at 10 °C; in addition, there was a temporal reduction in $\dot{V}O_2$. However, the succinate disappeared and $\dot{V}O_2$ returned to normal values, so the animals were clearly able to acclimate to the hypoxic conditions and change from being oxyconformers to oxyregulators.

Most $\dot{V}O_2$ measurements with bivalves are based on a declining PO_2; this method has several disadvantages: (1) environmental conditions are changing, which may obscure short-term respiratory regulation; (2) long-term recovery and acclimation effects can not be observed; and (3) variability and cyclic patterns can not be observed. The effect of declining O_2 levels on the $\dot{V}O_2$ in bivalves is not very consistent; large differences between individuals are observed with respect to the so-called critical O_2 concentration as well as to absolute $\dot{V}O_2$ values (Herreid 1980). One reason for this large variability may be the sensitivity of the animals to handling. An extreme example is the response to *Venus gallina* (unpublished results) to transfer from the holding tank to the respirometer chamber. In several occasions O_2 consumption was zero for more than 3 h at 20 °C, in contrast to the sessile *Mytilus galloprovincialis*, in which this behaviour was not observed. *Scapharca inaequivalvis* showed a moderate response, from direct observations it was obvious that shell closure lasted up to 10 min; $\dot{V}O_2$ values obtained from the first normoxic exposure of 1.5 h (A) were all 30–40% lower than the controls. These effects may be related to the way of life; infaunic species like clams are more sensitive to handling than sessile epifaunic species like *Mytilus*.

Exposure of *Scapharca inaequivalvis* to $[O_2] \leq 1.5$ ppm (≤ 4 kPa) produced a significant metabolic depression to about 5% of the normoxic $\dot{V}O_2$ values. The rather sudden reaction suggests an almost complete shutdown of all activity. The pattern is certainly different from other bivalve species, which show a slowly decreasing $\dot{V}O_2$ with falling $[O_2]$ (Bayne 1971; Widdows et al. 1989; Zwaan et al. 1991). This drop in $\dot{V}O_2$ is unlikely to be due to the presence of haemoglobin; in fact, $\dot{V}O_2$ would be expected to be largely independent of PO_2 since the affinity for oxygen is rather low [$P_{50} = 0.8$ kPa; Weber (1990)]. A more likely explanation is a depression of circulation and ventilation, suggesting a general shutdown as a prelude to a long-term hypoxia survival strategy.

A remarkable feature of the respiration of *Scapharca* (Fig. 4) is the increase of $\dot{V}O_2$ during the 12-h hypoxia exposure, mainly observed at $[O_2] < 2.5$ ppm (< 7 kPa), where the initial $\dot{V}O_2$ is far below normoxic values. Although the increase is about three-fold, $\dot{V}O_2$ never reaches the normoxic value, indicating that even after chronic exposure to hypoxia aerobic metabolism remains depressed. *Mytilus edulis* shows a complete acclimation of $\dot{V}O_2$ after an initial depression due to exposure to 6.3 kPa O_2 (Livingstone and Bayne 1977). However, below this level this species shows a typical oxyconformity (de Zwaan et al. 1991) and is not able to compensate. A possible explanation for the compensatory response of *Scapharca* is a shift in the O_2-binding curve of the haemoglobin. The haemoglobin of *Scapharca inaequivalvis* is ATP sensitive; reduced ATP levels increase the O_2 affinity (Weber 1990). Recently, de Vooys et al. (1991) showed that ATP levels in erythrocytes decline slowly during anaerobiosis. Therefore, the slow increase in $\dot{V}O_2$ during a 12-h exposure to $PO_2 < 7$ kPa may be related to an increased haemoglobin sensitivity to O_2. The blood of the clam *Noetia ponderosa* (Freadman and Magnum 1976) contains a significant amount of haemoglobin-bound O_2 at very low ambient O_2 levels, indicating that haemoglobin at low PO_2 is still able to facilitate transport. Therefore, the combination of increased O_2 affinity with high haemoglobin content enables *Scapharca* to maintain a moderate $\dot{V}O_2$ under hypoxic conditions. Both shell closure and the handling response cannot explain the observed acclimation during the 12-h hypoxia exposure, since the latter is a typical long-term effect (Fig. 3), while the stress responses all disappeared within 1–2 h.

The pattern of $\dot{V}O_2$ versus $[O_2]$ for *Scapharca* resembles that of a regulator, in contrast to *Mytilus* which is more like a conformer. At low $[O_2]$, *Mytilus* is not able to extract O_2 (Bayne 1971; de Zwaan et al. 1991). It seems therefore that *Scapharca* is better equipped to stay aerobic under hypoxic conditions than *Mytilus* is.

Anaerobic metabolism. Figure 5a, b indicates that anaerobic metabolism is activated below 2.5 ppm (7 kPa) O_2, and that no further accumulation of end-products occurs between 1.5 and 0 ppm (< 4 kPa) O_2. This suggest that the rate of anaerobic metabolism increases gradually between 2.5 (7 kPa) and 1.5 ppm (4 kPa) O_2 and remains constant at $[O_2] \leq 1.5$ ppm. The $\dot{V}O_2$ curves (Fig. 4) indicate a persistent O_2 consumption, indicating that energy production is at least partly aerobic. Therefore, a significant difference with respect to metabolite accumulation at complete anoxia and at 0.5 ppm O_2 was expected. The fact that this is not the case indicates that the maximal level of anaerobic energy production is reached at $[O_2] \leq 1.5$ ppm.

In Table 1 the ATP production rate from the anaerobic end products is calculated based on the tissue levels of propionate and succinate. No anaerobic end products were detected in the incubation water of the hypoxic

Table 1. Anaerobic and aerobic ATP turnover of *Scapharca inaequivalvis* during a 12-h hypoxia exposure at $[O_2] \leq 1.5$ ppm (≤ 4 kPa)

Rate	μmol ATP · g ww^{-1}	μmol ATP · h^{-1} · g ww^{-1}	% of normoxic
Anaerobic metabolism			
Δ ATP+P-arg	2.34	0.20	1.4
Δ Succinate	4.15	0.35	2.5
Δ Propionate	2.13	0.18	1.3
Sum	8.62	0.73	5.2
Rate	μg O_2 · h^{-1} · g ww^{-1}	μmol ATP · h^{-1} · g ww^{-1}	% of normoxic
Aerobic metabolism			
Normoxia	75	14.1	100.0
Hypoxia begin	5.2	1.0	7.1
Hypoxia end	14.4	2.7	19.1

ATP and phospho-arginine data from Brooks et al. (1991); succinate and propionate to ATP conversion factors are 2.75 and 3.76, respectively (Kluytmans et al. 1983); dry to wet weight conversion factor is 5.3; for O_2 to ATP conversion the factor 0.188 is used

groups. Because flow-through conditions were applied during hypoxic exposure, it is possible that propionate was diluted below the level of detection and therefore underestimated as a source of energy production. The $\dot{V}O_2$ of animals exposed to $[O_2] < 2$ ppm was about 10 μg $O_2 \cdot g^{-1} \cdot h^{-1}$; including a blank of 50%, for 35 animals (5.3 g ww) the total $\dot{V}O_2$ in the respirometer was 33.4 mg over a period of 12 h. Since more than 90% of the O_2 in the inflowing water is consumed by the animals, the total water volume needed for repletion of oxygen was 4.9 l; thus, the dilution during hypoxia is rather small since the respirometer contains only 16 l. Furthermore, the biomass to water ratio employed in the respirometer (7.5 g ww · l^{-1}) was only half of the ratio used in the anoxia experiments described previously (Brooks et al. 1991). In the latter experiments 1.06 μmol propionate · g (ww)$^{-1}$ was excreted over 24 h of anoxia, so that half of the amount should certainly be detected after 12 h hypoxia. In addition, since the detection limit of propionate in water [100x concentrated; see Kluytmans et al. (1975)] is below 0.1 μmol · l^{-1}, the propionate excretion during hypoxia must have been below 0.01 μmol · g (ww)$^{-1}$. In view of the data in Table 1, the contribution of propionate excretion to anaerobic energy production is negligible.

Changes of ATP and phospho-arginine during anoxia are taken from Brooks et al. (1991). The total anaerobic ATP production would amount to 0.73 μmol ATP · g^{-1} · h^{-1}. The aerobic ATP production calculated from the O_2 consumption at $[O_2] \leq 1.5$ ppm is 1.0 μmol ATP · g^{-1} · h^{-1}, which initially is a little more than the anaerobic production. However, at the end of the 12-h exposure period the aerobic contribution was increased to 2.7 μmol ATP · g^{-1} · h^{-1}. Thus, the total ATP production at $[O_2] \leq 1.5$ ppm increases from 1.7 to 3.4 μmol · g^{-1} · h^{-1}, i.e. from 12% to 24% of the normoxic rate.

Anaerobic metabolism constitutes about 5% of the normoxic aerobic rate, close to the value of 4.5% found by Brooks et al. (1991) for *Scapharca* kept under anoxic conditions in N_2-bubbled seawater. The ATP turnover rate of anaerobic metabolism under hypoxic conditions was smaller than that of aerobic metabolism. Initially, energy production rates were 0.7 vs 1.0 μmol ATP · g^{-1} · h^{-1}; however, the contribution of aerobic metabolism increased about three-fold over the 12-h exposure period, while anaerobic metabolism did not change. So, the contribution of anaerobic metabolism to the overall metabolism becomes smaller during the exposure period.

The metabolic switchover point in *Scapharca* occurs at lower PO_2 levels than in *Mytilus*, for which a value of 3.4 ppm (9.5 kPa) O_2 was found (de Zwaan et al. 1991). The value of 2 ppm (5.7 kPa) O_2 for *Scapharca* cannot be considered to be low since *Arenicola marina* activates anaerobiosis at around 4.0 kPa O_2 at 8 °C (Schöttler et al. 1983). Furthermore, if data from *Scapharca* are compared with those from fish the differences are even more dramatic. For example, most cyprinids remain aerobic until 2.0 kPa O_2 at 20 °C, and some species perform even better (de Zwaan and van den Thillart 1985).

The anaerobic ATP production rate of a whole animal found in this study and in the study by Brooks et al. (1991) is about half that found for the posterior adductor muscle described by Isani et al. (1989), suggesting that this tissue has a much higher energy consumption that the other tissues. There appears to be another interesting difference: the posterior adductor muscle draws only 10% of the energy from ATP and phospho-arginine hydrolysis, whereas in the whole animal a 50% hydrolysis of high-energy phosphates was found. The adductor muscle is apparently able to conserve high energy phosphates by having a higher anaerobic capacity, or by depressing its ATP turnover rate. This certainly has survival value for the animals since they are protected as long as their shells remain closed.

Phosphofructokinase. The changes in inhibition of PFK by ATP during anoxia exposure are most likely due to phosphorylation of the enzyme (Storey 1985). During anoxia exposure the I_{50} value falls over a 48-h period to 1.8 mmol · l^{-1} ATP; 50% of the effect occurs over the first 12 h (Brooks et al. 1991). The 12-h period was therefore chosen as the shortest time period with the largest possible change of I_{50} values. The I_{50} of PFK was measured after exposure to hypoxia in order to see whether PFK changes correlate with the onset of anaerobic metabolism.

The results shown in Fig. 6 are self-explanatory: at $[O_2] < 2$ ppm the I_{50} values are about 50% of the initial values. Since PFK is probably the rate-limiting enzyme of glycolysis in bivalves (de Zwaan 1983), this reduction indicates that the glycolytic flux is depressed after a 12 h exposure to severe hypoxia.

To obtain the same amount of ATP, the anaerobic metabolism leading to succinate and propionate production needs 6–7 times more glycogen than the aerobic metabolism. Since O_2 consumption is reduced by about

10-fold (range 5- to 15-fold) and only a 5% compensation is observed by anaerobic metabolism, the net effect will be a reduction of glycolytic flux (negative Pasteur effect). As PFK becomes inhibited by the tissue ATP levels [which are about 2 m*M* (Brooks et al. (1991, de Vooys et al. (1991)], it is likely that the low anaerobic rate is determined by a depressed PFK activity.

Critical PO_2 *levels.* Studies with *Sipunculus nudus* showed that, depending on body weight, a critical PO_2 can be found below which the rate of anaerobic metabolism increases with a further decline in PO_2. For larger animals this value appeared to be 10 kPa (Pörtner et al. 1985). Below this level the decline of $\dot{V}O_2$ was only partially compensated by anaerobiosis (Hardewig et al. 1991), indicating a depression of the energy production rate. These animals showed an almost linear relationship between PO_2 and the total heat flux. A completely different picture is found with *Scapharca inaequivalvis,* where a rather sudden drop in metabolic rate occurred at 7 kPa O_2.

There is no general definition of the term "critical PO_2" (P_c), basically because it has no meaning without defining the function for which it is critical. Even if "activation of anaerobiosis" is considered as such a function, there is likely to be more than one P_c. Not only this study, but also that of Livingstone and Bayne (1977), show that at least some bivalves are able to acclimate to hypoxia and activate anaerobic metabolism for a restricted period. Therefore, P_c as an anaerobic threshold parameter should also be defined in terms of time, since animals may be able to increase their aerobic capacity over a certain period sufficiently to shut down anaerobiosis, thus shifting the P_c to lower values.

Conclusions

The effect of a 12-h hypoxia exposure on $\dot{V}O_2$, accumulation of metabolites and PFK kinetics was studied. Most remarkably, a very strong correlation between these three parameters was found. All changes apparently occur between 1.5 and 2.5 ppm O_2. The reduction of $\dot{V}O_2$ is initially 15-fold but $\dot{V}O_2$ subsequently increases so that at the end of the exposure period the reduction is 5-fold. This change is probably due to an increase in O_2 affinity of haemoglobin which is abundant in the erythocytes of these clams. Over the same O_2 range where $\dot{V}O_2$ is depressed the anaerobic end-products succinate and propionate begin to accumulate. It is found that the total energy production at O_2 concentrations ≤ 1.5 ppm is reduced to 19% of the normoxic rate. The ratio of aerobic to anaerobic metabolism increases over the 12-h exposure period from 1.4 to 3.7 due to increased O_2 uptake. PFK, the key enzyme for the glycolysis, is normally inhibited by high ATP concentrations. In this study I_{50} values were significantly reduced to physiological values of ATP after exposure to $[O_2] < 2$ ppm. These results indicate that glycolysis will be depressed, which corroborates with the observed rate of the anaerobic metabolism. Obviously, *Scapharca inaequivalvis* relies mainly on aerobic metabolism even under conditions of severe hypoxia.

Acknowledgements. This research project was funded by the Commission of the European Communities 4th Environment R&D programme under contract #EV4V–0122–NL, with additional support from an N.S.E.R.C. operating grant to KBS. Foreign travel and living expenses were funded by NATO International Collaboration Research Grant #0779 to AdZ, KBS, PC, and GvdT. We would like to thank Dr O. Cattani and G. Vitali for advice and assistance. Communication no. 562 Delta Institute for Hydrobiological Research, Yerseke, The Netherlands.

References

Bayne BL (1971) Oxygen consumption by three species of lamellibranch molluscs in declining oxygen tension. Comp Biochem Physiol 40A:955–970

Brooks SP, de Zwaan A, van den Thillart G, Cattani O, Cortesi P, Storey KB (1991) Differential survival of *Venus gallina* and *Scapharca inaequivalvis* during anoxic stress: covalent modification of phosphofructokinase and glycogen phosphorylase during anoxia. J Comp Physiol B 161:207–212

Degobbis DO (1989) Increased eutrophication of the northern Adriatic Sea. Mar Pollut Bull 20:452–457

De Vooys CGN, de Zwaan A, Roos J, Carpené E, Cattani O (1991) Anaerobic metabolism of erythrocytes of the arcid clam *Scapharca inaequivalvis:* effects of cadmium. Comp Biochem Physiol 98B:169–175

De Zwaan A (1983) Carbohydrate metabolism in bivalves. In: Wilbur KM (ed) The Mollusca, vol I, Academic Press, New York pp 137–175

De Zwaan A, van den Thillart G (1985) Low and high output modes of anaerobic metabolism of invertebrates and lower vertebrates. In: Gilles R (ed) Proc First Int Congr Comp Biochem Physiol. Springer, Berlin Heidelberg New York

De Zwaan A, Cortesi P, van den Thillart G, Roos J, Storey KB (1991) Differential sensitivities to hypoxia by two anoxia-tolerant marine molluscs: a biochemical analysis. Mar Biol 111:343–351

Freadman MA, Mangum CP (1976) The function of haemoglobin in the arcid clam *Noetia ponderosa:* I. Oxygenation in vitro and in vivo. Comp Biochem Physiol 53A:173–179

Hardewig I, Addink ADF, Grieshaber MK, Pörtner H-O, van den Thillart G (1991) Metabolic rates at different oxygen levels determined by direct and indirect calorimetry in the oxyconformer *Sipunculus nudus.* J Exp Biol 157:143–160

Herreid CF II (1980) Hypoxia in invertebrates; review. Comp Biochem Physiol 67A:311–320

Isani G, Cattani O, Tacconi S, Cortesi P (1989) Energy metabolism during anaerobiosis and recovery in the posterior adductor muscle of the bivalve *Scapharca inaequivalvis.* Comp Biochem Physiol 93B:193–200

Kluytmans JH, De Bont AM, Kruitwagen EC, Ravenstein HJ, Veenhof PR (1983) Anaerobic capacities and anaerobic energy production of some Mediterranean bivalves. Comp Biochem Physiol 75B:171–179

Livingstone DR, Bayne BL (1977) Responses of *Mytilus edulis* L. to low oxygen tension: anaerobic metabolism of the posterior adductor muscle and mantle tissues. J Comp Physiol 114:143–155

Pörtner H-O, Heisler N, Grieshaber MK (1985) Oxygen consumption and mode of energy production in the intertidal worm *Sipunculus nudus* L. Definition and characterization of the critical PO_2 for an oxyconformer. Respir Physiol 59:361–377

Schöttler U, Wienhausen G, Zebe E (1983) The mode of energy production in the lugworm *Arenicola marina* at different oxygen concentrations. J Comp Physiol 149:547–555

Schöttler U, Wienhausen G, Westermann J (1984) Anaerobic metabolism in the lugworm *Arenicola marina* L.: the transition

from aerobic to anaerobic metabolism. Comp Biochem Physiol 79B:93–103

Storey KB (1984) Phosphofructokinase from the foot muscle of the whelk *Busycotypus canaliculatum:* evidence for covalent modification of the enzyme during anaerobiosis. Arch Biochem Biophys 235:665–672

Storey KB (1985) A re-evaluation of the Pasteur effect: new mechanisms in anaerobic metabolism. Mol Physiol 8:439–461

Theede H, Ponat A, Hiroki K, Schlieper C (1969) Studies on the resistance of marine invertebrates to oxygen deficiency and hydrogen sulphide. Mar Biol 2:325–337

Van den Thillart G (1982) Adaptations of fish energy metabolism to hypoxia and anoxia. Mol Physiol 2:49–61

Van den Thillart G, Verbeek R (1991) Anoxia induced oxygen debt of goldfish (*Carassius auratus* L.). Physiol Zool 64:525–540

Weber RE (1980) Functions of invertebrate haemoglobins with special reference to adaptations to environmental hypoxia. Am Zool 20:79–101

Weber RE (1990) Effects of mercury on the functional properties of haemoglobins from the bivalve mollusc *Scapharca inaequivalvis*. J Exp Mar Biol Ecol 144:39–48

Widdows J, Newell RI, Mann R (1989) Effects of hypoxia and anoxia on survival, energy metabolism, and feeding of oyster larvae (*Crassostrea virginica,* G.). Biol Bull 177:154–166