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VENTILATORY AND METABOLIC RESPONSES TO HYPOXIA AND SULPHIDE IN THE LUGWORM *ARENICOLA MARINA* (L.)

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Summary

We examined the effects of hypoxia and sulphide levels on the ventilatory activity of *Arenicola marina* and determined whether ventilation compensates for oxygen deficiency and affects the mode of energy provision. *A. marina* ventilated intermittently, irrespective of ambient P_{O_2} and sulphide concentration. The ventilation rate was 28.5±16 ml h^{-1} g^{-1} wet mass during normoxia, but increased to 175±60 % of this value during moderate hypoxia, during which aerobic energy metabolism was maintained. Below a P_{O_2} of 6.2 kPa, *A. marina* reduced the ventilated volume to 54±16 % of the normoxic value and became anaerobic, as indicated by the accumulation of succinate and strombine. Incubation with 27 μmol l^{-1} ambient sulphide had no effect on the normoxic and hypoxic ventilation rates or on the P_{O_2} below which anaerobiosis started (P_{cM}). Increased sulphide concentrations reduced the ventilation rate and shifted the P_{cM} towards a higher P_{O_2} below 10.7 kPa. Sulphide diffused into the body and was at least partially detoxified to thiosulphate when oxygen was present. Under normoxia, sulphide accumulated in the body wall tissue and coelomic fluid when ambient sulphide levels exceeded 117 μmol l^{-1} and 216 μmol l^{-1}, respectively. A decrease in P_{O_2} in the presence of 27 or 117 μmol l^{-1} ambient sulphide had no significant effect on sulphide accumulation.

Key words: ventilation, sulphide, hypoxia, metabolism, lugworm, anaerobiosis, *Arenicola marina*.

Introduction

The lugworm *Arenicola marina* (Polychaeta) is a dominant member of the macrobenthos of many intertidal flats of the North Atlantic (Reise et al., 1994), of adjacent sublittoral areas and of the shallow coastal waters of the Baltic Sea (Groth and Theede, 1989). It lives in L-shaped burrows, which extend approximately 20 cm into the oxygen-free zone of the sediment (Theede et al., 1969; Krüger, 1971). When the tide is in, the burrow is ventilated with a current of sea water produced by rhythmic, peristaltic muscular movements of the animal's body wall. The animal extracts both oxygen and nutrients from the water (Wells, 1945, 1949b; Krüger, 1964, 1971) and releases waste products into it. During low tide, when no water covers the sediment, ventilation ceases (Toulmond, 1987), and the oxygen concentration of the burrow water declines (Jones, 1955; Watling, 1991). As a consequence, less sulphide becomes oxidised than during high tide and may accumulate within the burrow. Thus, *A. marina* will experience, amongst other factors, changes in oxygen and sulphide concentrations with the tidal rhythm, and it is conceivable that the worm may react by changing its ventilatory behaviour to prevent any detrimental effects.

Ventilatory activity is a major and obvious physiological response of many water-breathing invertebrates. The ventilatory response to the presence of various abiotic factors has been investigated previously. In general, ventilation rate is increased during moderate hypoxia and reduced during severe hypoxia. Crayfish, shore crabs and shrimps, for example, are able to increase their ventilation rate by as much as 2.5 to three or even five times the resting level at normoxia when they are exposed to reduced oxygen partial pressures (for reviews, see Taylor, 1982; McMahon, 1988; Taylor, 1988). Studies of the respiratory responses of molluscs and polychaetes to hypoxia have shown that some species, such as *Arctica islandica* and *Hyalinoecia tubicola*, rely on a continuous increase in their ventilatory rate over a wide range of reduced oxygen concentrations (Dales et al., 1970; Taylor and Brand, 1975). In addition, other abiotic factors, such as changes in salinity and temperature, affect ventilatory behaviour (Seymour, 1972; Shumway and Davenport, 1977; Kristensen, 1983) and therefore influence oxygen extraction and uptake.

Ventilation and respiratory gas exchange have also been investigated in tube-dwelling *Arenicola marina* as a function of ambient P_{O_2}, confirming the general mode of P_{O_2}-dependent ventilation and oxygen uptake: i.e. an increase in ventilation rate during moderate hypoxia and a reduction during severe hypoxia (Toulmond and Tchernigovtzeff, 1984). However, the influence of ventilation on the mode of energy production during oxygen deficiency has not been investigated.

Furthermore, the effects of sulphide on the ventilation rate and energy metabolism of *A. marina* have not been examined.

Our aim in this study was, therefore, to determine the P_{O_2}-dependent ventilation rate and the effects of sulphide on ventilation. In particular, we examined whether ventilation would compensate for a sulphide-induced oxygen deficiency and what influence the P_{O_2}- and sulphide-induced rate of ventilation would have on the mode of energy provision. We also investigated whether the uptake of sulphide into the animal and the detoxification of sulphide to thiosulphate were affected by the ventilation of *A. marina*.

Materials and methods

Animal collection and maintenance

Specimens of *Arenicola marina* (L.) were collected between 1994 and 1996 from an intertidal flat near Zierikzee, The Netherlands. The animals had a mean fresh mass of 1.7±0.4 g (N=70) and were kept in the laboratory for at most 6 weeks at 15±1 °C in darkened tanks containing aerated artificial sea water (435 mmol l^{-1} NaCl, 28 mmol l^{-1} $MgSO_4$, 24 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} KCl, 2 mmol l^{-1} HCO_3^-, salinity 35 ‰). For the experiments carried out in a sediment tank, lugworms were collected from an intertidal flat on the Isle of Cumbrae, Scotland. Immediately after being transported to the laboratory, animals were transferred into an aquarium containing sediment from the collecting site and filled with recirculating sea water (salinity 35 ‰, 10±1 °C). After stocking the aquarium, the lugworms burrowed into the sediment. No mortalities were observed during the course of the experiments.

Incubation media

Artificial sea water was used in all experiments. It was prepared and aerated 24 h prior to each experiment. When sulphide (the term 'sulphide' refers here to total dissolved sulphide, the sum of H_2S, HS^- and S^{2-}) was used in the experiments, a stock solution of sulphide was mixed with the artificial sea water before it flowed into the incubation chamber. The stock solution was prepared by adding washed crystals of $Na_2S{\cdot}9H_2O$ to N_2-saturated artificial sea water. The pH of the sulphide stock solution was adjusted to 8.0 with 1 mmol l^{-1} KOH.

Preliminary experiments revealed that the use of buffered sea water (20 mmol l^{-1} Hepes) caused the cessation of the ventilatory activity of the lugworms. Thus, unbuffered sea water was used. To keep the pH constant at 8.0, a pH-stat system was applied which consisted of a sulphide-insensitive pH electrode (InLab 412, Mettler Toledo, Steinheim, Germany) connected to a pH meter (PHM 82 Standard pH meter, Radiometer, Kopenhagen, Denmark) and an automatic titrator (TTT 80 titrator and ABU 80 autobürette, Radiometer, Kopenhagen, Denmark).

Flow-through apparatus

The ventilation measurements were carried out at 15±1 °C in a flow-through system to keep P_{O_2} and sulphide concentrations constant. The artificial sea water within a 10 l reservoir was equilibrated at a constant P_{O_2} and a normocapnic P_{CO_2} using a Wösthoff gas-mixing pump (Digamix 2KM 303/a-F, Wösthoff, Bochum, Germany). A peristaltic pump drew the sea water from the reservoir into the incubation chamber *via* a mixing chamber (volume 35 ml). In experiments with sulphide, a second peristaltic pump was used to introduce the sulphide stock solution into the mixing chamber, where the two solutions were thoroughly stirred. After the addition of sulphide, the P_{O_2} in the sea water declined because of the rapid oxidation of sulphide. To obtain the desired P_{O_2} in the sulphide-containing incubation medium, the sea water in the reservoir had to be equilibrated with a higher P_{O_2}.

The incubation medium was pumped at a flow rate of 1.4 l h^{-1} from the mixing chamber into the glass incubation chamber (volume 700 ml). In addition to the in- and outflow ports, it contained further openings: one for the pH electrode, a second for the P_{O_2} electrode (Cameron Instrument Company, Port Aransas, Texas, USA) and a third for the cable of the flow transducer (see below), the pH-titration tubing and an additional tube. This additional tube was connected to a syringe, which served to withdraw seawater samples out of the incubation chamber every 30 min for determinations of the sulphide concentration of the incubation medium.

Ventilation measurements

In the field, lugworms respire by irrigating their burrow with sea water. To obtain quantitative information about the effects of abiotic factors on ventilation and gas exchange in the laboratory, defined conditions must be used. Nevertheless, the experimental apparatus must resemble conditions in nature as closely as possible. Therefore, a worm was placed in a glass tube (length 15 cm, internal diameter 5 mm), which was open at both ends, located within the incubation chamber, as described above. Preliminary experiments using different kinds of tubes revealed that the lugworms also required a rough inner glass surface on which the setae could meet some resistance when the body wall muscles performed ventilatory movements. Thus, small pieces of glass were melted onto the inner surface of the glass tube.

The ventilation current produced by the animal was detected using an electromagnetic flowmeter (SP 2202, Gould Statham, Oxnard, USA) equipped with a 3 mm (i.d.) cannulating flow transducer (Hugo Sachs Elektronik, March, Germany). Data were continuously recorded using both an analogue pen recorder and a computer. The flow transducer was connected to one end of the artificial burrow and could measure the flow of water in both directions, i.e. tail-to-head as well as head-to-tail. To calibrate the flow transducer prior to an experiment, defined volumes of sea water were pumped through the empty glass tube and the flow transducer connected to it. The area between the recorded signal and the baseline (the signal when no sea water was passing the flow transducer) was integrated to calculate the volume of irrigated sea water (ml). On the basis of this calibration, the actual seawater volume ventilated by the

lugworm during a ventilation event was calculated. For the calculation of the mass-specific ventilation rate $\dot{V}_w$ (ml h^{-1} g^{-1} wet mass), the volume of the sea water ventilated during the entire incubation was summed and divided by the duration (h) of the incubation and the wet mass of the animal.

Experimental protocol and calculation of the normalised ventilation rate

Twelve hours prior to an experiment, a lugworm was placed within the glass tube, the flow transducer was connected and the apparatus was inserted into the incubation chamber. The experiment was started with a normoxic incubation period followed by hypoxic, normoxic/sulphidic or hypoxic/sulphidic incubations. Each incubation period lasted for 8 h. After the experiment, the animal was removed and weighed, and the tissues were prepared for the determination of levels of sulphur compounds and anaerobic end-products as described below.

To estimate the effects of hypoxic and/or sulphidic treatments on the ventilation volume, the normalised ventilation rate was calculated. For this calculation, the ventilation rate during the normoxic incubation without sulphide in every experiment (which will henceforth be referred to as the 'control' rate) was given a value of 100 %. The ventilation rate measured during the subsequent normoxic/sulphidic, hypoxic or hypoxic/sulphidic incubation was calculated as a percentage of the preceding normoxic control value. This data treatment was chosen because of the highly variable normoxic ventilation rates of *A. marina*.

Experiments in a sediment tank

To determine the extent to which the ventilation pattern of *A. marina* kept in a glass tube resembles that of a worm ventilating in a natural burrow within the sediment, experiments were performed with lugworms kept in a sediment tank. After placing the animal onto the sediment, it burrowed into the mud. At least 1 day later, the location of the newly established burrow was indicated by a funnel-shaped depression at one end and a faecal pile at the other end. To measure ventilatory activity, a glass funnel to which a flow transducer had been connected was pressed into the sediment above the funnel-shaped depression. The sea water irrigated by the animal within the sediment burrow had to pass through the glass funnel, and the flow rate could then be measured by the flow transducer.

Preparation of tissue samples

At the end of each experiment, the coelomic fluid of the lugworm was rapidly collected from a dorsal incision in the body wall. For the determination of concentrations of thiol compounds, a piece of body wall tissue (10–30 mg) was also dissected from between the sixth segment and the beginning of the tail. Visually, this region of the body wall tissue does not vary morphologically along its length. The remaining body wall tissue was freeze-clamped (Wollenberger et al., 1960) and stored in liquid nitrogen for the determination of levels of anaerobic end-products.

Determination of thiol compounds

The concentrations of thiol compounds in the coelomic fluid and in the body wall tissue were analysed using high-performance liquid chromatography (HPLC) following derivatization with monobromobimane (Fahey et al., 1981; Newton et al., 1981; Vetter et al., 1989), as modified by Völkel and Grieshaber (1994). For the determination of thiol levels in the coelomic fluid, a volume of 50 µl was mixed with a buffer solution consisting of 10 µl of monobromobimane (48 mmol l^{-1}, Sigma), 50 µl of acetonitrile and 50 µl of Hepes/EDTA (160 mmol l^{-1} Hepes, 16 mmol l^{-1} EDTA, pH 8.0). The freshly dissected piece of tissue was homogenised in a buffer solution containing 10 µl of monobromobimane (48 mmol l^{-1}; Sigma), 50 µl of acetonitrile and 100 µl of Hepes/EDTA (160 mmol l^{-1} Hepes, 16 mmol l^{-1} EDTA, pH 8.0) using a glass homogenizer. After a 30 min incubation in the dark at room temperature (20–22 °C), the fluorescent derivatization products were stabilised by the addition of 100 µl of methanosulphonic acid (65 mmol l^{-1}) and stored at –80 °C until measured.

Sulphide and thiosulphate in the tissues were separated using a Merck/Hitachi L-6200 intelligent pump (Merck, Darmstadt, Germany) combined with a Merck LiChrospher 60 RP-select-B reversed-phase column (5 µm, 125 mm×4 mm). The thiols were detected with a Merck/Hitachi F-1050 fluorescence spectrophotometer (excitation wavelength 380 nm, emission wavelength 480 nm) and analysed using a software package (D-6000 HPLC Manager; Merck, Darmstadt, Germany). The calibration curve was linear between 1 and 100 µmol l^{-1} of thiol compound. Samples containing more than 100 µmol l^{-1} were diluted prior to measurement. For statistical analyses, measured thiol concentrations of less than 1 µmol l^{-1} were taken to be 1 µmol l^{-1}. The sulphide concentration in the incubation medium was determined spectrophotometrically using the Methylene Blue method described by Cline (1969), as modified by Gilboa-Garber (1971). The calibration curve was linear between 5 and 100 µmol l^{-1} sulphide. Samples containing more than 100 µmol l^{-1} were diluted prior to the measurement.

Determination of levels of anaerobic end-products

For the determination of anaerobic end-products, the body wall tissue was extracted according to Beis and Newsholme (1975). Succinate was measured spectrophotometrically, as described by Beutler (1985). Alanopine and strombine were determined by HPLC, as originally described by Siegmund and Grieshaber (1983) but using a DX-100 ion chromatograph (Dionex, Idstein, Germany) without a suppressor unit. The opines were separated isocratically using a 45 °C heated ARH-601 aromatic acids cation-exchange column (100 mm×6.5 mm; Interaction, San Jose, USA) with 3.75×10^{-5} mol l^{-1} H_2SO_4 as solvent. The flow rate was 0.6 ml min^{-1}, and the pressure was 8×10^3 kPa. The opines were detected using a conductivity cell (Dionex, Idstein, Germany) and analysed using a software package (Maestro Software, Chrompack, Frankfurt/Main, Germany). The lower detection limits of succinate and opines

(alanopine and strombine) were 2 nmol and 0.1 nmol, respectively. The volume of tissue homogenate used was 10–100 µl in the enzymatic succinate assay (approximately 2–20 mg tissue wet mass, respectively) and 40–200 µl in the opine determination (approximately 3–15 mg tissue wet mass, respectively), depending on the expected metabolite content. Therefore, the lower detection limits were 0.1 µmol g^{-1} wet mass for succinate and 0.033 µmol g^{-1} wet mass for alanopine and strombine.

Statistical analyses

Data are given as mean ± one standard deviation (S.D.). Significant differences between means were evaluated by one-way analysis of variance (ANOVA) using the Student–Newman–Keuls *post-hoc* test at the $P \leqslant 0.05$ level with a statistical software package (SigmaStat version 1.01, Jandel Scientific, Corte Madera, USA).

Results

Ventilation pattern

A. marina kept in the glass tubes (artificial burrows) showed an intermittent but regular long-term pattern of ventilatory activity, i.e. periods of ventilation alternating with periods of rest (Fig. 1A). Even when the P_{O_2} of the sea water was reduced or sulphide was added, the pattern remained the same. In the particular experiment shown in Fig. 1A, the worm changed the direction of the water flow once during each ventilatory bout. In some experiments, no changes in the direction of the water flow occurred, and in others completely irregular patterns could occasionally be observed. The ventilation pattern of worms living in sediment burrows was similar (Fig. 1B), including changes in the direction of water flow (data not shown). It was not, however, as regular and consistent as that of animals kept in glass tubes.

P_{O_2}- *and sulphide-dependent ventilation*

Under normoxic (control) conditions, *A. marina* kept in an artificial burrow ventilated for 50 % of the entire incubation period, with a mean ventilation duration of 22.4±15.4 min (N=35 worms). The whole-animal ventilation rate ($\dot{V}$) was directly proportional to body mass and followed a logarithmic relationship [$\dot{V}=aW^b$, where a is the intercept, W is wet mass (g) and b is the slope of the regression line; with a=24.24 and b=1.02 (r^2=0.834, P<0.05)]. The mass-specific ventilation rate ($\dot{V}_w$) of *A. marina* during normoxia was 28.5±16.0 ml h^{-1} g^{-1} wet mass (N=35 worms), and this increased significantly to 175±60 % and 169±103 % of the control value when the P_{O_2} was reduced to 10.7 and 6.2 kPa, respectively (Fig. 2A, circles). Under anoxic conditions, *A. marina* reduced its ventilation rate significantly to 54±16 % of the control level.

The P_{O_2}-dependent ventilation rate of *A. marina* was not changed by the addition of 27 µmol l^{-1} sulphide (Fig. 2A, triangles), whereas ventilation rate was decreased for most P_{O_2} values in the presence of 117 µmol l^{-1} sulphide (Fig. 2A, squares). Compared with the values in the absence of sulphide, 117 µmol l^{-1} sulphide decreased the ventilation rate significantly at P_{O_2} values of 20.8, 10.7 and 0.1 kPa.

A. marina reduced $\dot{V}_w$ to 20–45 % of the control value when exposed to sulphide concentrations greater than 27 µmol l^{-1} under normoxia (Fig. 3A). The ventilation rates measured in the presence of 52 and 117 µmol l^{-1} sulphide were significantly lower than the control value.

Energy metabolism in ventilating lugworms

To determine the P_{O_2} value below which anaerobiosis started (P_{cM}) in ventilating lugworms under both hypoxic and sulphidic conditions, the levels of the anaerobic end-products succinate, alanopine and strombine were measured in the body wall muscles. With decreasing oxygen partial pressure and in the absence of sulphide, the succinate content remained at the control level (0.47±0.35 µmol g^{-1} wet mass) until the P_{O_2} was reduced from 6.2 kPa to anoxia (Fig. 2B, circles). Therefore, the P_{cM} is below 6.2 kPa. The presence of 27 µmol l^{-1} sulphide did not change the P_{cM}, but the succinate content after exposure to anoxia in the presence of 27 µmol l^{-1} sulphide was significantly higher than the value after exposure to anoxia in the absence of sulphide (Fig. 2B, triangles). In the presence of 117 µmol l^{-1} sulphide, however, the succinate content increased significantly below 10.7 kPa P_{O_2} (Fig. 2B, squares) compared with both the incubations with and without 27 µmol l^{-1} sulphide.

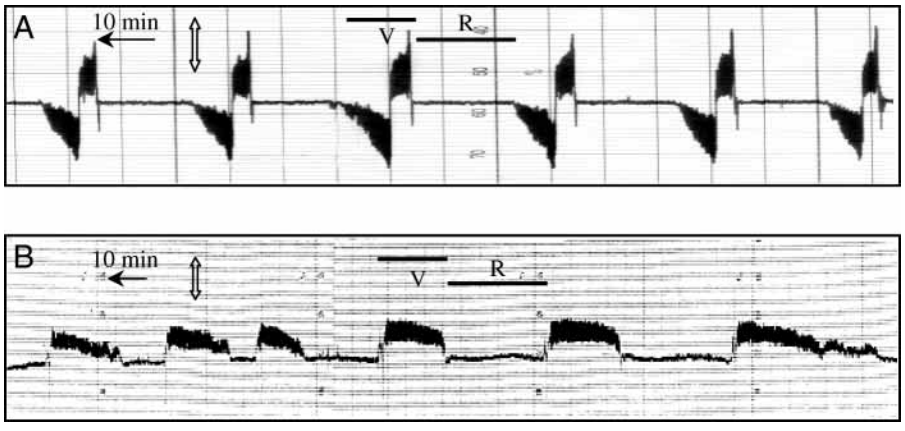

Fig. 1. Two examples of intermittent ventilation patterns of *Arenicola marina* kept in an artificial burrow (a glass tube) (A) and a sediment burrow (B). Periods of ventilation (V) alternated with resting periods (R). Vertical open arrows correspond to ventilation rates of 7 ml min^{-1} (A) and 0.7 ml min^{-1} (B).

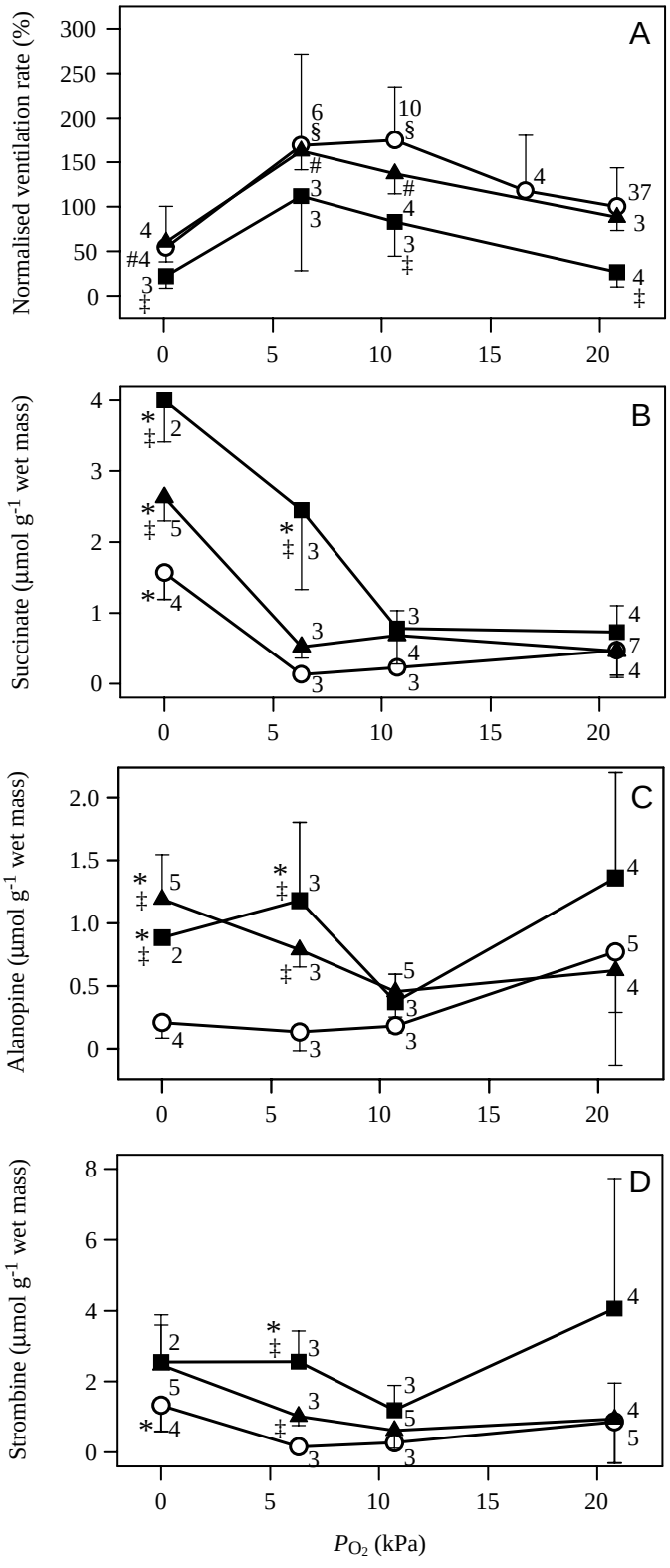

Fig. 2. Ventilation rate during, and the levels of anaerobic end-products after, 8 h of incubation of *Arenicola marina* in an artificial burrow at various levels of P_{O_2} (kPa) in the absence of sulphide (circles) and in the presence of 27 and 117 μmol l^{-1} sulphide (triangles and squares, respectively). (A) Ventilation rate, given as a percentage of the normoxic control value, and levels of succinate (B), alanopine (C) and strombine (D) in the body wall tissue (μmol g^{-1} wet mass). Values are given as means ± S.D. The numbers next to the symbols give the number of experiments. #, significantly different from the value at normoxia (20.8 kPa); §, significantly different from the value at normoxia and anoxia; ‡, significantly different from the corresponding value without sulphide; *, significantly different from the adjacent, higher P_{O_2}, value.

The levels of alanopine and strombine in the body wall tissue of the control animals were 0.77±0.90 and 0.86±1.16 μmol g^{-1} wet mass, respectively (Fig. 2C,D, circles). Reduction of oxygen partial pressure had no effect on alanopine concentration when sulphide was absent (Fig. 2C, circles). Exposure to 27 and 117 μmol l^{-1} sulphide resulted in a significant increase in alanopine content compared with the hypoxic controls when the P_{O_2} was reduced to below 10.7 kPa (Fig. 2C, triangles and squares). In the absence of sulphide, the strombine content increased significantly during anoxia (Fig. 2D, circles). Sulphide concentrations of 27 and 117 μmol l^{-1} caused an increase in strombine content compared with the hypoxic controls only at a P_{O_2} of 6.2 kPa (Fig. 2D, triangles and squares, respectively).

To isolate the effects of sulphide on the mode of energy production, we measured the accumulation of anaerobic end-products in the body wall of lugworms incubated with various sulphide concentrations under normoxic conditions. Sulphide concentrations up to 216 μmol l^{-1} did not alter the succinate or alanopine contents compared with controls (Fig. 3B,C). The succinate and alanopine contents were significantly higher than the control values only when the sulphide concentration in the normoxic sea water was increased to 506 μmol l^{-1}. Strombine appeared to follow the same pattern as succinate and alanopine, but strombine levels were very variable and the increase was not significant (Fig. 3D).

Accumulation of sulphide and thiosulphate within A. marina

The concentrations of sulphide in the body wall tissue and the coelomic fluid of actively ventilating *A. marina* were determined under various oxygen partial pressures and ambient sulphide concentrations. Sulphide was detected in the body wall tissue of control animals (27.2±13.5 μmol l^{-1}). This background value, which is probably due to the detection of the mercapto-groups of body wall proteins instead of free sulphide (Hauschild and Grieshaber, 1997), was subtracted from all subsequent body wall tissue values. In the presence of 27 and 117 μmol l^{-1} ambient sulphide, the sulphide concentration in the body wall tissue and in the coelomic fluid did not increase significantly above the control value (Fig. 4A,B), although there was a slight increase in sulphide levels in the coelomic fluid when 117 μmol l^{-1} sulphide was added to anoxic sea water. The mean sulphide concentration in the body wall tissue after normoxic exposures with 117 μmol l^{-1} sulphide showed large variability. Of the five animals in this data set, three animals accumulated between 50 and 75 μmol l^{-1} sulphide, whereas the remaining two accumulated 160 and 260 μmol l^{-1} sulphide. Interestingly, the ventilation rates of the two animals with the highest sulphide

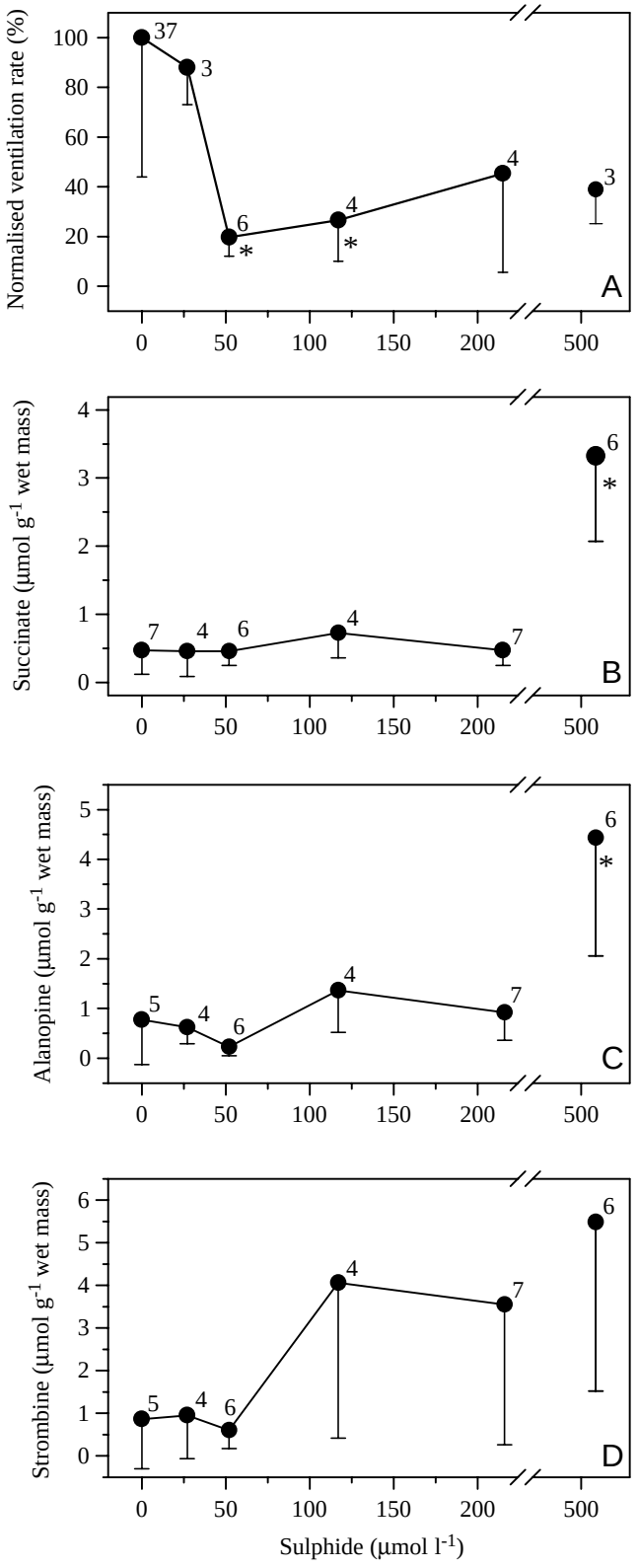

Fig. 3. Ventilation rate during, and levels of anaerobic end-products after, 8 h of incubation of *Arenicola marina* in an artificial burrow at normoxia in the absence of sulphide and in the presence of various sulphide concentrations (μmol l^{-1}). (A) Ventilation rate, given as a percentage of the control (no sulphide) value, and levels of succinate (B), alanopine (C) and strombine (D) in the body wall tissue (μmol g^{-1} wet mass). Values are given as means ± S.D. The numbers next to the symbols give the number of experiments. *, significantly different from the control value.

concentrations were approximately three times those of two of the animals with the lower sulphide concentration (ventilation data could not be obtained from the fifth animal), suggesting that an increase in ventilation rate increases sulphide exposure.

In *A. marina*, sulphide is oxidised mainly to thiosulphate (Völkel and Grieshaber, 1994). The concentrations of thiosulphate in the coelomic fluid and in the body wall tissue were therefore determined. In the absence of sulphide, a thiosulphate concentration of 0.90±0.78 μmol l^{-1} was recorded in the coelomic fluid, whereas it could not be detected in the body wall tissue. After incubations with 27 and 117 μmol l^{-1} ambient sulphide, the thiosulphate concentration in both tissues did not increase above control values over the whole range of P_{O_2} (Fig. 4C,D). However, thiosulphate concentrations between 3000 and 70 μmol l^{-1} in the coelomic fluid and between 1100 and 80 μmol l^{-1} in the body wall were detected with decreasing P_{O_2} and exposure to 117 μmol l^{-1} sulphide.

To investigate the influx of sulphide and its oxidation to thiosulphate in the presence of a normoxic P_{O_2}, levels of both compounds were measured in the tissues after normoxic incubations with various ambient sulphide concentrations. At a normoxic P_{O_2} and in the presence of sulphide concentrations above 27 μmol l^{-1}, the sulphide concentrations in the body wall tissue reached values of 50–112 μmol l^{-1}. The increase in the sulphide concentration in the body wall tissue was significant after exposure to 216 μmol l^{-1} ambient sulphide (Fig. 5A, squares), whereas it increased significantly in the coelomic fluid only when the animals were incubated with 506 μmol l^{-1} sulphide (Fig. 5A, circles). The sulphide concentrations in the coelomic fluid were significantly lower than in the body wall tissue at every ambient sulphide concentration.

The thiosulphate concentration in the body wall tissue after normoxic exposure increased significantly in the presence of 216 μmol l^{-1} ambient sulphide (Fig. 5B, squares). In the coelomic fluid, it was significantly elevated at 117 μmol l^{-1} ambient sulphide and remained elevated with further increases in sulphide concentration (Fig. 5B, circles). When the ambient sulphide concentration was raised from 216 to 506 μmol l^{-1}, the thiosulphate concentrations in the coelomic fluid and the body wall tissue were significantly reduced to 2.3±1.1 mmol l^{-1} and 1.0±0.7 mmol l^{-1}, respectively.

Discussion

Ventilation pattern

The tube-dwelling polychaete *Arenicola marina* ventilates its burrow with a water current produced by piston-like pumping movements of the body wall muscles (van Dam, 1937; Wells, 1945). van Dam (1937) investigated lugworms within glass tubes and reported a pattern of active ventilation that was regularly interrupted by periods of inactivity, a pattern

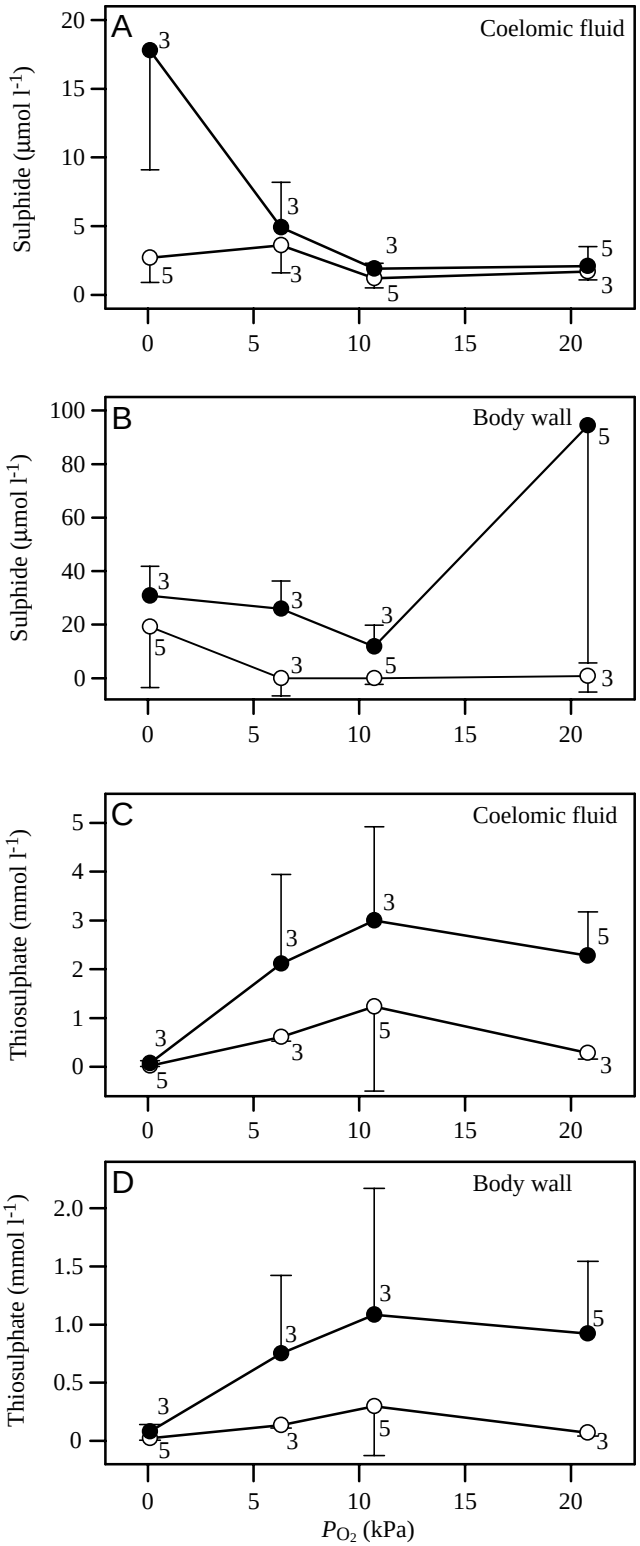

Fig. 4. Concentrations of sulphide (A,B) (μmol l^{-1}) and thiosulphate (C,D) (mmol l^{-1}) in the coelomic fluid (A,C) and the body wall tissue (B,D) of *Arenicola marina* after 8 h of incubation at various P_{O_2} values (kPa) in the presence of 27 and 117 μmol l^{-1} ambient sulphide (open and filled circles, respectively). Values are given as means ± S.D. The numbers next to the symbols give the number of experiments.

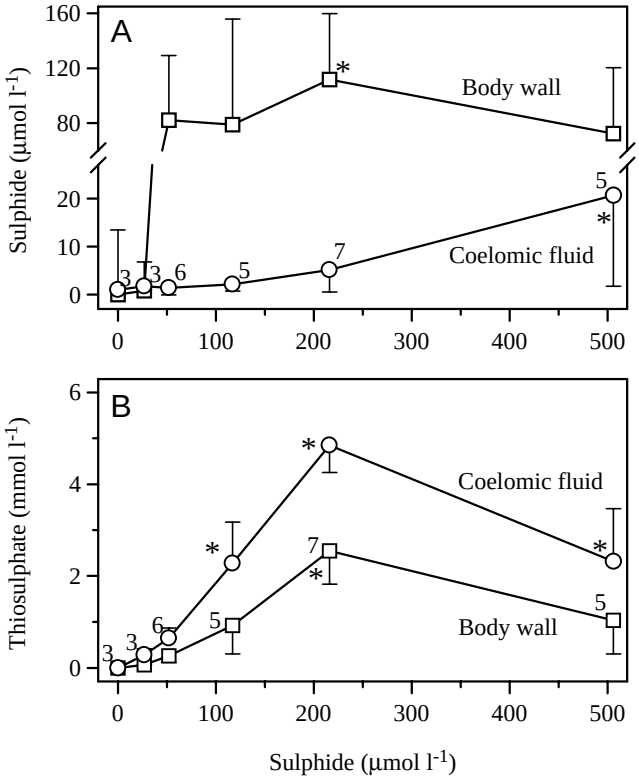

Fig. 5. Concentrations of (A) sulphide (μmol l^{-1}) and (B) thiosulphate (mmol l^{-1}) in the coelomic fluid (circles) and the body wall tissue (squares) of *Arenicola marina* after 8 h of normoxia in the absence of sulphide and in the presence of various concentrations (μmol l^{-1}) of ambient sulphide. Values are given as means ± S.D. The numbers next to the symbols give the number of experiments. *, significantly different from the control (no sulphide) value.

subsequently confirmed by others (Wells, 1949a; Wells and Albrecht, 1951; Davey et al., 1990). Whether the observations were relevant to animals under natural conditions was questioned, however, because the lugworms had been incubated in artificial tubes. Krüger (1964) investigated lugworms both in artificial tubes and within sediment burrows in their natural habitat. He also recorded an intermittent ventilation pattern when the worms were situated in the artificial tube, but the worms ventilated continuously in their natural sediment burrows. In contrast, Wells (1949b) measured an intermittent ventilation pattern in both artificial tubes and natural sediment burrows. In the present study, we monitored the ventilation pattern of lugworms kept in a sediment tank. *A. marina* showed an intermittent pattern of ventilation in its burrow within this sediment tank (Fig. 1B).

Since the aim of the present study was to measure the ventilation of lugworms in the laboratory under defined and constant conditions, such as varying ambient P_{O_2} and sulphide concentrations, the use of sediment was disadvantageous because sulphide reacts rapidly with iron ions present within the sediment (Jørgensen, 1988). The worms were therefore placed in an inert, artificial burrow made from a glass tube. The

ventilation pattern of lugworms situated in such a tube was also intermittent and was similar to, and even more regular than, the pattern we recorded in worms ventilating in sediment burrows (Fig. 1A). In both the glass tube and the sediment burrow, *A. marina* could pump water either in a tail-to-head or in the opposite direction. The direction of pumping was constant or could change within an incubation, which is consistent with the observations of van Dam (1937), Wells and Albrecht (1951) and Krüger (1964). No difference, such as that postulated by Krüger (1964), was seen between the ventilation patterns of lugworms in sediment burrows and in artificial tubes.

P_{O_2}-dependent ventilation

Under normoxic conditions *A. marina* ventilated for 50 % of the time recorded, corresponding with values reported in earlier publications (van Dam, 1937; Seymour, 1972; Davey et al., 1990), with a mean mass-specific ventilation rate, $\dot{V}_w$, during normoxia of 28.5±16.0 ml h^{-1} g^{-1} wet mass. The whole-animal ventilation rate was directly proportional to body mass. A similar correlation was also found in *Nereis succinea* (Kristensen, 1983). $\dot{V}_w$ was similar to previously reported values for lugworms of comparable size, which were found to ventilate at between 11 and 30 ml h^{-1} g^{-1} wet mass (Wells, 1949a; Mangum, 1976; Riisgård et al., 1996), and was comparable with that of other polychaetes, such as *Eupolymnia heterobranchia* and *Diopatra cuprea* (Dales, 1961; Dales et al., 1970).

A. marina increased $\dot{V}_w$ to 170 % of the normoxic control level at a P_{O_2} of 6.2 kPa and reduced $\dot{V}_w$ during anoxia. Even in the absence of oxygen, however, $\dot{V}_w$ was still 54 % of the normoxic value (Fig. 2A). Therefore, the endogenous pacemaker controlling ventilation, which was postulated by Wells (1949a,b), apparently does not respond to oxygen levels alone and, by maintaining water flow even under anoxia, it may also serve to ensure the removal of excretion products and to sample the P_{O_2} of the water overlying the burrow.

The effects of P_{O_2} on the ventilation rate in lugworms have previously been investigated by Toulmond and Tchernigovtzeff (1984), who found a comparable increase in ventilation rate to approximately 160 % of the control rate when P_{O_2} decreased to 10.7 kPa, a subsequent decrease with further reduction in oxygen availability, and a complete interruption below a P_{O_2} of 2.7 kPa. However, these authors measured the ventilation rate of the lugworms only over a period of 4 h. This might have been too brief a period, since Conti and Toulmond (1986) demonstrated the influence of acclimation to hypoxic conditions on the ventilation rate, revealing an enhanced ventilation rate when the animals were exposed to hypoxia for 25 h instead of 3 h. The different incubation times between the present study and that of Toulmond and Tchernigovtzeff (1984) could explain the differences between the estimations of the oxygen partial pressures below which the ventilation rate decreased or ceased.

Toulmond and Tchernigovtzeff (1984) also described a compensatory effect of the increased ventilation rate on the rate of oxygen consumption of *A. marina* during moderate hypoxia and determined a critical respiratory P_{O_2} (P_{cR}) of 16 kPa, below which the rate of oxygen consumption declined. In ventilating lugworms incubated in the experimental apparatus described in the present study, an increased ventilation rate prevented the rate of oxygen consumption from decreasing until the ambient P_{O_2} fell below 10.7 kPa (S. E. Wohlgemuth and M. K. Grieshaber, in preparation). Apart from the P_{cR}, which represents the transition from a regulatory to a conforming mode of respiration, a second variable, the P_{cM}, can be defined at which anaerobic metabolism starts (Pörtner and Grieshaber, 1993).

Energy metabolism when oxygen supply is limiting

We used the analysis of anaerobic end-products to determine the extent to which a modified ventilation rate affected the mode of energy production in hypoxia-exposed *A. marina* ventilating under simulated natural conditions. The anaerobic metabolism of many marine invertebrates has been studied in detail (for a review, see Grieshaber et al., 1994). In *A. marina*, the glycolytic end-product alanopine is mainly produced during increased muscular work (Siegmund et al., 1985), while strombine is produced during environmental hypoxia (Kreutzer et al., 1989). Succinate can serve as a particularly sensitive indicator of anaerobic mitochondrial metabolism because its steady-state levels increase by approximately five- to tenfold as soon as oxygen becomes limiting in these organelles (Grieshaber et al., 1988).

Anaerobic metabolism in ventilating *A. marina* did not commence until the ambient P_{O_2} fell below 6.2 kPa, as indicated by an approximately threefold increase in body wall succinate content during anoxia compared with a P_{O_2} value of 6.2 kPa. Since the increased ventilation rates that occurred at P_{O_2} values above 6.2 kPa were produced by enhanced muscular work, *A. marina* could have relied on glycolytic energy provision, which is analogous to a functional anaerobiosis and would be indicated by an elevated content of alanopine. Also, strombine would have been produced as soon as the enhanced ventilation rate could no longer compensate for the oxygen deficiency. However, the alanopine content of the tissue remained at the control level over the whole range of P_{O_2}, and the strombine content was not elevated until conditions became anoxic, corroborating the mode of energy provision indicated by the accumulation of succinate. Thus, the P_{cM} under these experimental conditions is below 6.2 kPa. In contrast to these findings, Hauschild and Grieshaber (1997) recently gave a P_{cM} of between 16 and 10 kPa. However, in that study the lugworms were incubated in an open respirometer through which sea water flowed, and they were not, therefore, able to change the flow by changing their ventilatory behaviour. Moreover, the animals may have been stressed by the flow of sea water through the chamber. It seems reasonable to suggest that increased ventilatory activity during moderate hypoxia could compensate for the oxygen deficiency and allow aerobic metabolism to be maintained down to lower ambient oxygen tensions.

The effects of sulphide on ventilating lugworms

Many marine sediments are characterised not only by temporary oxygen deficiency but also by the presence of

sulphide, which may naturally accumulate as a metabolic end-product of sulphate-reducing bacteria (Jørgensen and Fenchel, 1974; Nedwell, 1982). Sulphide is toxic to aerobic respiration, mainly because of its reversible binding to cytochrome *c* oxidase at nanomolar to low micromolar concentrations, poisoning the electron transport chain (Nicholls, 1975; National Research Council, 1979). Despite the detrimental effects of sulphide, several animal species can exist in sulphide-rich habitats. *A. marina* (Groenendaal, 1980; Völkel and Grieshaber, 1992) and many other invertebrates (for a review, see Grieshaber and Völkel, 1998) withstand exposure to sulphide mainly by oxidising it to less harmful sulphur compounds, primarily to thiosulphate, and by their ability to rely on anaerobic metabolism when sulphide influx exceeds their capacity for detoxification.

Ventilation

The effects of sulphide on ventilatory functions have been investigated for only a few animals. The hydrothermal vent crab *Bythograeа thermydron*, for example, which may be exposed to sulphide concentrations as high as 320 μmol l^{-1} in the presence of oxygen, maintains its heart and ventilation rates when exposed to sulphide concentrations up to 1.4 mmol l^{-1} (Vetter et al., 1987; Childress and Fisher, 1992). This ability is probably linked to the efficient oxidation of sulphide to thiosulphate within the animal's hepatopancreas (Vetter et al., 1987). Excised gills of the sulphide-adapted, symbiont-harbouring clam *Solemya reidi* maintained their ciliary activity when exposed to ambient sulphide concentrations up to 250 μmol l^{-1}, although ciliary activity declined during exposure to higher sulphide concentrations (Anderson et al., 1987). Since sulphide oxidation in *S. reidi* is coupled to oxidative phosphorylation (Powell and Somero, 1986; O'Brien and Vetter, 1990), the maintenance of ciliary activity (which produces ventilation) at low sulphide concentrations and its subsequent decline at higher concentrations may be correlated with sulphide-supported ATP production, which is inhibited by mitochondrial sulphide concentrations exceeding 20 μmol l^{-1} (Powell and Somero, 1986).

In the present study, we found that ventilation rate decreased at sulphide concentrations above 27 μmol l^{-1}. While moderate hypoxia induces hyperventilation, sulphide does not. Ventilation was not affected by 27 μmol l^{-1} sulphide. Sulphide that had entered the body must have been effectively oxidized so that it did not affect aerobic metabolism (see below) and, hence, no oxygen deficiency occurred that could have induced hyperventilation. In fact, when the rate of oxygen consumption of *A. marina* was recorded using an open respirometer, sulphide concentrations up to 25 μmol l^{-1} had no effect, but the rate of oxygen consumption declined rapidly at higher sulphide concentrations (Hauschild and Grieshaber, 1997). These authors calculated the amount of oxygen necessary for sulphide oxidation in the presence of 25 μmol l^{-1} ambient sulphide to be just 1.4 % of the total oxygen consumed at normoxia and 3.4 % of the total oxygen consumed at 5 kPa P_{O_2}. Consistent with these results, the ventilation rate in *A. marina* with 27 μmol l^{-1} sulphide in the present study did not differ from that of controls even when the P_{O_2} of the sea water was reduced. This sulphide concentration is similar to average sulphide concentrations in the burrows of *A. marina* in the field, which range from 0 to 32 μmol l^{-1} (Völkel et al., 1995), and with the temporary levels of sulphide in stagnant water columns (Rethmeier, 1995). However, when the ambient sulphide concentrations exceeded 27 μmol l^{-1}, *A. marina* reduced its ventilation rate under both normoxic and hypoxic conditions compared with controls, so that no sulphide-induced compensatory increase in ventilation rate was evident. Furthermore, at higher sulphide concentrations, the main response was apparently to reduce or to avoid the influx of sulphide into the tube, which is particularly important when sulphide not only diffuses from the surrounding sediment into the burrow but is also present in the overlying water and would reach the animal during the course of ventilation.

Accumulation of sulphide and thiosulphate within A. marina

Biological membranes are highly permeable to sulphide (Beerman, 1924), and the influx of sulphide into the tissues has been demonstrated for *A. marina* (Groenendaal, 1981; Völkel and Grieshaber, 1992, 1994) and several other invertebrates (Vismann, 1990; Julian and Arp, 1992). In the present study, sulphide was taken up by ventilating *A. marina* exposed to 27 μmol l^{-1} sulphide, and it was oxidised to thiosulphate. Sulphide did not accumulate in the tissues until the ambient sulphide concentration exceeded 117 μmol l^{-1}. The efficient oxidation of sulphide probably explains the maintenance of ventilatory activity by *A. marina* in the presence of 27 μmol l^{-1} sulphide. When 117 μmol l^{-1} sulphide was added, sulphide was detected in the tissues by HPLC, albeit at very low concentrations. However, these concentrations may have caused the reduced ventilation rate. Inadequate sulphide detoxification at this ambient sulphide concentration was also indicated by the accumulation of anaerobic end-products during moderate hypoxia and anoxia.

Mode of energy production in ventilating lugworms exposed to sulphide

A wide range of sulphide tolerance has been described for marine invertebrates. The echiuran worm *Urechis caupo* (Encomio and Arp, 1995) and the oligochaete *Limnodrilus hoffmeisteri* (Schneider, 1994), for example, became anaerobic at sulphide concentrations between 37 and 60 μmol l^{-1}, whereas the symbiont-harbouring mussel *Solemya reidi* (Anderson et al., 1990) and the oligochaete *Tubificoides benedii* (Dubilier et al., 1994, 1995) tolerated sulphide concentrations up to 250 μmol l^{-1} and 300 μmol l^{-1}, respectively, before anaerobic metabolites could be detected.

In the presence of sulphide and normoxia, ventilating *A. marina* accumulated neither succinate nor opines until the sulphide concentration exceeded 216 μmol l^{-1}. Although 27 μmol l^{-1} sulphide did not affect the ventilation rate or the mode of energy production of *A. marina*, the animals reduced their ventilation rate at higher sulphide concentrations and

therefore avoided exposure to sulphide. This response apparently prevented the tissues from being exposed to sulphide concentrations that would have induced anaerobiosis, whereas *A. marina* kept in an open respirometer (in which they were passively irrigated with sea water) became anaerobic even under normoxic conditions in the presence of 25 μmol l^{-1} sulphide (Hauschild and Grieshaber, 1997).

The P_{O_2}-dependent accumulation of succinate in the body wall tissue (i.e. the P_{cM}) was not affected by the addition of 27 μmol l^{-1} sulphide. This result confirms the assumption that, in the presence of 27 μmol l^{-1} sulphide, even at reduced P_{O_2}, oxygen delivery within the tube is sufficient for both aerobic metabolism and complete sulphide detoxification. However, the accumulation of opines in the tissues at lower oxygen tensions indicates that at least some cells are supporting their energy provision by anaerobic glycolysis. In contrast, when the sulphide concentration was increased to 117 μmol l^{-1}, succinate began to accumulate at P_{O_2} values below 10.7 kPa. The reduced ventilation rate in the presence of 117 μmol l^{-1} ambient sulphide apparently reduced both the oxygen supply and exposure to sulphide and, as a result, anaerobic metabolism had to contribute to energy provision at a higher P_{O_2}.

Does sulphide serve as an energy source in A. marina*?*

Sulphide-supported ATP formation and subsequent energy exploitation occur *in vitro* in gills excised from the sulphide-adapted mussel *Geukensia demissa* (Gaschen, 1997; Doeller et al., 1999). Similarly, in mitochondria isolated from the body wall muscle of *A. marina* and some other sulphide-adapted invertebrates, the oxidation of low sulphide concentrations to thiosulphate is coupled to oxidative phosphorylation (for a review, see Grieshaber and Völkel, 1998). In isolated body wall mitochondria from *A. marina*, sulphide-stimulated ATP production reached a maximal rate in the presence of 8 μmol l^{-1} sulphide. Cytochrome *c* oxidase, which appears to be the only coupling site of sulphide-stimulated ATP production, was inhibited by sulphide concentrations greater than 10 μmol l^{-1}, concomitant with a decrease in ATP production, which was completely inhibited at 50 μmol l^{-1} sulphide (Völkel and Grieshaber, 1996, 1997).

The conditions under which sulphide-supported energy production in animal tissues occur should therefore meet the following conditions: (i) the ventilation rate during exposure to low sulphide concentrations should be maintained in order to supply a sufficient amount of oxygen for both aerobic metabolism and sulphide oxidation; (ii) to prevent the inhibition of cytochrome *c* oxidase, and therefore the production of ATP, the sulphide concentration in the tissue must be below the inhibitory concentration; and (iii) energy metabolism should remain aerobic since the oxidation of sulphide provides additional ATP. In the present study, we have provided evidence that each of these prerequisites is satisfied and, therefore, that sulphide at low concentrations may serve as an inorganic energy source in *A. marina*, as has been proposed by Völkel and Grieshaber (1997). For *A. marina* exposed to 27 μmol l^{-1} sulphide, we confirmed that the ventilation rate was maintained, that sulphide was absent from the animal's tissues, and that aerobic energy production was maintained. This suggests that sulphide-driven energy production in *A. marina* at low sulphide concentrations, previously recorded only in isolated mitochondria, may also occur in whole animals.

Environmental relevance of oxygen availability and sulphide exposure in A. marina

What are the implications of this study for the animal's life in anoxic and sulphidic environments? The lugworm inhabits marine sediments that are usually stratified, with an upper oxidised layer and an anoxic deeper layer. Oxygen at concentrations sufficient for aerobic processes is present only in the top few millimetres, whereas the deeper layers are oxygen-free (Jørgensen, 1988; Giere, 1992). Furthermore, during low tide, the ventilatory activity of *A. marina* ceases and the burrow water is no longer renewed and becomes increasingly hypoxic. *A. marina* may therefore be exposed intermittently to hypoxia. A different situation occurs in shallow waters, e.g. of the Baltic Sea, that lack a pronounced tidal rhythm. The water here may remain stagnant for prolonged periods, resulting in a decline in oxygen concentration both in the sediment and in the overlying water column (Ehrhardt and Wenck, 1983). *A. marina* in this habitat may compensate for moderate hypoxia by increasing its ventilation rate. However, during low tide or when the overlying water column is extremely oxygen-deficient, the ventilation rate declines and the animal has to rely on anaerobic energy provision.

Apart from oxygen deficiency, *A. marina* may also be affected by sulphide, which can reach concentrations in the micromolar to millimolar range in the sediment and will even diffuse into the overlying water column if the concentration in the sediment is high enough (Groenendaal, 1979; Erhardt and Wenck, 1983; Völkel and Grieshaber, 1992; Rethmeier, 1995). In the presence of low sulphide concentrations in the burrow water, *A. marina* maintains ventilatory activity and thereby removes sulphide from the burrow. Even if low sulphide concentrations are already present in the overlying water column, and subsequently in the ventilated water, ventilatory activity can be sustained. Sulphide that enters the animal's tissues is then efficiently detoxified to thiosulphate. However, when the sulphide level in the sediment or the overlying water column increases, the higher ambient sulphide concentration results in a reduced ventilation rate. With the resulting reduction in O_2 availability, sulphide detoxification is no longer sufficient and *A. marina* relies increasingly on anaerobic metabolism.

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