Vol. 143: 131–139, 1996 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published November 14

Recovery from hypoxia with and without sulfide in *Saduria entomon*: oxygen debt, reduced sulfur and anaerobic metabolites

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ABSTRACT: The Baltic brackish water isopod *Saduria entomon* was exposed (8 h; *T*: 11°C; S: 10 ppt) to hypoxia (0.1 kPa) and to hypoxia (0.7 kPa) with sulfide (41 µM). Oxygen consumption was measured using computer-controlled intermittent respirometry. The routine oxygen consumption was 0.109 mg O_2 h^{-1} g^{-1} at normoxia (19.5 kPa). The recovery from hypoxia with and without sulfide was studied in terms of oxygen debt, reduced sulfur concentration and anaerobic metabolites. In hypoxia, the metabolism of *S. entomon* was reduced by 70% compared to the normoxic level and the oxygen debt was 0.57 mg O_2 g^{-1}. Normal respiration rate was re-established after 8 h of recovery. Lactate oxidation accounted for 44% of the oxygen debt. In hypoxia with sulfide, the metabolism was reduced by 38% compared to the normoxic level and the oxygen debt was 2.74 mg O_2 g^{-1} (5 times the oxygen debt at hypoxia). Normal respiration level was not restored until 28 h after exposure. Lactate oxidation accounted for only 20% of the oxygen debt. Sulfide and detoxification products accounted for as little as 0.04% (1 µg O_2 g^{-1} wet wt) of the oxygen debt. The majority (80%) of processes that occurred during the recovery from sulfide exposure are inferred to be involved in restoring normal cell function. Removal of internal sulfide and reoxidation of anaerobic metabolites play secondary roles.

KEY WORDS: Sulfide · Hypoxia · Recovery · Oxygen debt · Anaerobic metabolites · *Saduria entomon*

INTRODUCTION

Marine animals from sulfidic habitats have some degree of sulfide tolerance (reviewed by Somero et al. 1989, Vismann 1991a and Bagarinao 1992). In the literature, studies of sulfide tolerance have focussed on processes occurring during sulfide exposure. Little attention has been given to processes occurring after the exposure. Surviving animals may be moribund or recovering. Hence, the processes occurring during and after exposure to sulfide are equally important for the survival. Knowledge of the physiological mechanisms at work during recovery from sulfide exposure will improve our understanding of the physiology of animals exposed to sulfide.

Most species from sulfidic habitats detoxify sulfide by oxidation to thiosulfate and turn to an anaerobic metabolism if the sulfide concentration at the mitochondrial level exceeds approximately 20 µM (Powell & Somero 1986, Bagarinao & Vetter 1990, Oeschger & Vismann 1994), even if sufficient oxygen to permit aerobic metabolism is present (Oeschger & Vetter 1992, Völkel & Grieshaber 1992, Hagerman & Vismann 1993, 1995). The toxic effect of sulfide is primarily a reversible inhibition of cytochrome *c* oxidase (National Research Council 1979). As most animals turn to an anaerobic metabolism during sulfide exposure, the need for detoxification is not obvious. However, sulfide has been shown to affect the survival of animals relying on anaerobic energy metabolism for protection (Theede 1969, Vismann 1990). The need to detoxify sulfide during anaerobiosis, therefore, potentially exists.

A successful recovery from sulfide poisoning depends on a quick return to an aerobic metabolism. This is possible only if cytochrome *c* oxidase is not inhibited

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by sulfide. Regardless of metabolic state, detoxification of sulfide will facilitate the recovery from sulfide poisoning by keeping the internal sulfide concentration as low as possible. In order to restore the internal environment, numerous processes takes place during recovery (e.g. oxidation of anaerobic metabolites, removal of detoxification products, osmotic and ionic regulation, protein synthesis, regeneration of phosphogen, regeneration of ATP stores, and replenishment of physically dissolved and pigment-bound oxygen). Oxygen used in these processes is referred to as oxygen debt (Herreid 1980, Ellington 1983). Oxygen debt is seen as a temporary increase above the normal level of normoxic oxygen consumption. The significance of the different processes occurring during the recovery can be stoichiometrically evaluated in terms of oxygen equivalents.

The objective of the present study was to elucidate the physiological differences between the recovery from anaerobiosis induced by sulfide and recovery from lack of oxygen in the sulfide-tolerant isopod *Saduria entomon*. Emphasis is on the temporal progression of oxygen debt, reoxidation of anaerobic metabolites and reduced sulfur.

MATERIALS AND METHODS

Material. *Saduria entomon* were collected in the Åland archipelago, Finland, off the Husö Marine Biological Station, and in the Gulf of Gdansk, Poland, during 1995. The isopods were transported to the Marine Biological Laboratory, Helsingør, Denmark. Prior to experiments, *S. entomon* were kept in large tanks with natural sediment and fully aerated water (T = 7°C; S = 8 ppt). They were fed *Mytilus edulis* once a week.

Exposure experiments. ***Experimental set-up:*** All exposures were made using a set-up in which pH, sulfide, oxygen and temperature were monitored and controlled by a computer. Oxygen tension was measured with an oxygen electrode (Radiometer, Copenhagen, Denmark; E5046, modified according to the principles of Revsbech & Ward 1983) and a reference electrode (Radiometer K401) connected to a pH/blood gas monitor (Radiometer PHM73). The pH was measured with an electrode (Radiometer pHC2401) connected to the pH/blood gas monitor. Sulfide potential was measured with an ion selective Ag-Ag_2S electrode (laboratory constructed) and a reference electrode (Radiometer K401) connected to an ion analyzer (Radiometer Ion 85). Temperature was measured with a thermistor probe (Yellow Springs Instrument Co., OH, USA; 700 series) connected to a thermistor panel (Cole-Parmer Instrument Co., IL, USA; 700 series). The signals from the meters measuring pH, sulfide, oxygen and temperature were fed into the computer at a frequency of 0.5 Hz (Fig. 1: signals). The computer compared the signals with preset set-points. Signals outside their respective hysteresis caused the computer to turn on the appropriate pumps or magnetic valves (Fig 1: output). The sulfide potential was continuously converted by the computer to sulfide concentration according to the appropriate equations (Vismann 1996). The water in the aquarium (vol = 6 l) was mixed by 2 centrifugal pumps (Eheim GmbH, Deizisau, Germany; 1048 and 1005; Fig. 1: pumps D and E). The 2 pumps had a total flow rate of 14.5 l min^{-1}. Data for post experimental analysis were saved on disk with a frequency of 8.3×10^{-3} Hz (for further details see Vismann 1996). Temperature was only monitored by the computer because it was controlled by a constant thermobath (Hetofrig, Lab Equipment AS, Allerød, Denmark). Before and after each exposure salinity was measured with a refractometer (Atago Co., Tokyo, Japan).

Experimental conditions: In a series of experiments, *Saduria entomon* was exposed to normoxia, severe hypoxia and hypoxia with sulfide (the latter condition is referred to as sulfide in the following text). The hypoxic level in the sulfide exposure would, if sulfide was not present, allow *S. entomon* to use aerobic metabolism

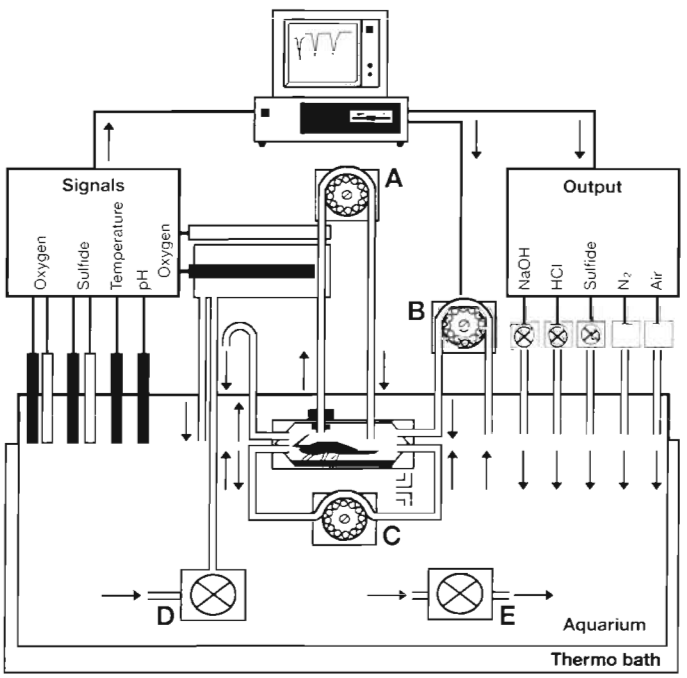

Fig. 1 Experimental set-up for exposure to hypoxia with and without sulfide and intermittent respirometry. See text for further details

(Hagerman & Szaniawska 1988). In the hypoxia and sulfide exposures, the protocol was: normoxia for the first 10 h (acclimation period), followed by 8 h of hypoxia with or without sulfide (exposure period) and then normoxia until routine respiration was restored (recovery period). The water in the aquarium was renewed before the recovery period. The protocol was produced by the computer by defining different set-points for the 3 experimental periods. The experimental conditions in the exposures are given in Table 1. It was not possible to renew the water completely and accordingly in the sulfide experiments a small amount of residual sulfide was initially present in the recovery period (Table 1). In a series of pilot experiments, the rate of chemical oxidation of residual sulfide was determined. The rate of oxygen use in residual sulfide oxidation was best fitted to an exponential equation [$MO_2 = 0.6632e^{-0.4302t}$ where t = time (h); $R^2 = 0.88$; data not shown].

Respirometry. ***Experimental set-up:*** The oxygen consumption was measured using intermittent respirometry (see, e.g., Forstner 1983). In this method, the respiration chamber (vol = 42 ml) changed between being a closed system and being flushed with water from the aquarium as explained below. During the flush period, the respiration chamber was flushed (23.5 ml min^{-1}) with water from the aquarium by a peristaltic pump (Gilson Medical Electronics, Middleton, USA; minipuls 3, Fig. 1: pump B). The oxygen tension in the respiration chamber was measured via a shunt using an oxygen electrode (Radiometer E5046, modified according to the principles of Revsbech & Ward 1983) installed in a constant temperature cell (Radiometer D616) and a reference electrode (Radiometer K401) both connected to a pH/blood gas monitor (Radiometer PHM73). The shunt (flow rate = 3.7 ml min^{-1}) was driven by a peristaltic pump (Ismatec Sa, Zürich, Switzerland; Mini-S 840; Fig. 1: pump A). The flow of cooling water (10 l min^{-1}) through the constant temperature cell was delivered by a centrifugal pump (Eheim 1048; Fig. 1: pump D). Additional mixing of the respiration chamber was provided by a peristaltic pump (Ismatec Mini-S 840; flow rate = 3.7 ml min^{-1}; Fig. 1: pump C). Every 20 min the computer stopped flushing for 10 min (Fig. 1. inactivation of pump B), turning the respiration chamber into a closed system. In these periods, the computer sampled the chamber oxygen tension with a frequency of 8.3×10^{-3} Hz and stored it on a disk in a separate file. As the chamber was closed during these periods, the consumption of oxygen caused the chamber oxygen tension to decrease. The decrease in oxygen tension in closed systems is linear if oxygen consumption is constant. The oxygen consumption was defined as the slope of the linear regression line and the coefficient of determination (R^2) provided a measure of the constancy of oxygen consumption (see Fig. 2). Oxygen consumption rates with $R^2 < 0.80$ were regarded as not being constant and they were excluded from the data-set (less than 3% of more than 600 measurements). The oxygen consumptions used in calculations had a mean R^2 of 0.96 ± 0.04. Immediately before and after each experiment, the blank oxygen consumption of the system was measured. The blank oxygen consumption as a function of time was regarded as linear.

Oxygen debt: Oxygen consumption was measured during the recovery period following hypoxia and sulfide exposures. The oxygen consumption was also measured during the normoxic control experiments. For each recovery period, the total oxygen consumption was calculated by numerical integration (Simpson's approximation). The oxygen debt was calculated by subtracting the integrated mean routine oxygen consumption of a time period equal to the recovery period from total oxygen consumption. The oxygen used for chemical sulfide oxidation (see above) at the start of the recovery period was subtracted from the total oxygen consumption in the sulfide experiments.

Table 1. Experimental conditions. All data are mean values ± SEM

Experimental period	Oxygen (kPa)[a]	pH	Sulfide (µM) Total	Sulfide (µM) H_2S	Temperature (°C)	Salinity (ppt)	n
Normoxia							
Normoxic control	19.47 ± 0.46	8.09 ± 0.04	0.0 ± 0.0	0.0 ± 0.0	11.1 ± 0.4	10 ± 1	6
Severe hypoxia							
Adaptation	19.00 ± 0.25	8.09 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	11.1 ± 0.4	10 ± 1	6
Exposure	0.12 ± 0.02	8.83 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	11.0 ± 0.5	10 ± 1	6
Recovery	18.62 ± 0.20	8.39 ± 0.09	0.0 ± 0.0	0.0 ± 0.0	11.1 ± 0.4	10 ± 1	6
Hypoxia and sulfide							
Adaptation	20.26 ± 0.27	8.07 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	11.4 ± 0.6	10 ± 1	6
Exposure	0.68 ± 0.09	8.11 ± 0.03	41.2 ± 6.5	3.5 ± 0.5	11.1 ± 0.6	10 ± 1	6
Recovery	20.03 ± 0.36	8.01 ± 0.04	1.1 ± 1.1	0.1 ± 0.1	10.6 ± 0.4	10 ± 1	6

[a]Oxygen partial pressure; 1 kPa = 7.5 Torr

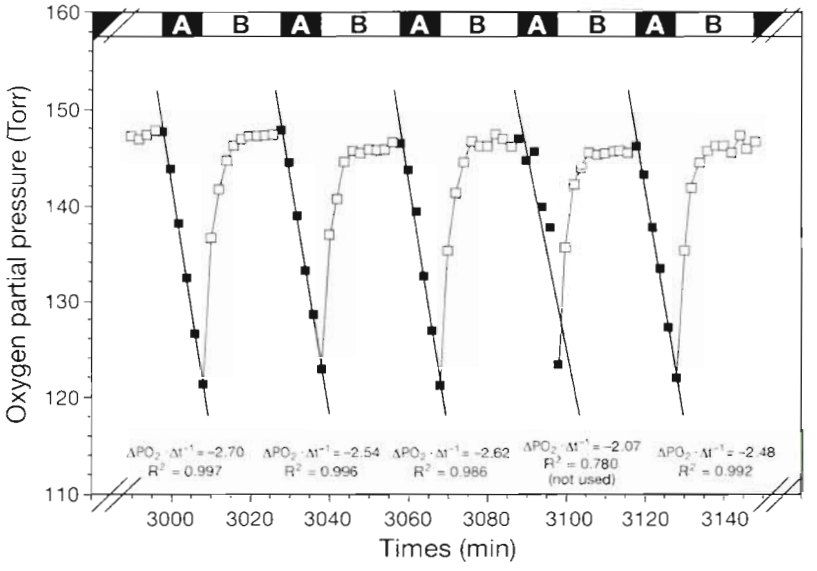

Fig. 2. Example of intermittent respirometry registered by computer (A) 10 min of measuring followed by (B) 20 min of flushing. (■) Data points used in regression analysis. Regression equations and R^2 shown for each line. The oxygen partial pressure 150 Torr is equivalent to 20 kPa

Chemical analysis. In addition to the *Saduria entomon* in the respiration chamber a number of *S. entomon* were incubated in the aquarium (Fig. 1). Half of these were taken for chemical analysis immediately after the exposure period and the rest at the end of the recovery period. From these *S. entomon*, the concentrations of anaerobic metabolites in the hemolymph and of reduced sulfur in the hemolymph, tissue and midgut gland were determined. Lactate and alanine were the only anaerobic metabolites considered in the present study following the results of Hagerman & Szaniawska (1990) and Hagerman & Vismann (1993).

Lactate: Hemolymph samples were taken with a hypodermic syringe (Hamilton, Bonaduz AG, Switzerland) inserted from a posterodorsal direction into the pericardium. The hemolymph samples (50 µl) were immediately combined with 100 µl 0.6 N perchloric acid and centrifuged at 3500 × *g* for 10 min. Lactate was then analyzed using a lactate kit (Boehringer-Mannheim, Germany; no. 139 084) and a spectrophotometer (Pharmacia LKB Biotechnology, Uppsala, Sweden; Ultraspec plus) set at 340 nm. Lactate analysis was made in all exposures.

Alanine: Hemolymph samples (10 µl) were taken as above and analyzed for alanine by HPLC (Jasco, Tokyo, Japan; 880 PU) by pre-column reaction with orthophthalaldehyde and fluorescence detection (Jasco 820 FP), according to Gardner & Miller (1980). Alanine analysis was made only in the sulfide exposures.

Reduced sulfur species: In the sulfide exposures a hemolymph sample (50 µl) was taken as described above. The carapace was then removed in an argon atmosphere and samples of the midgut gland and muscle tissue were removed. The samples were immediately (in the argon atmosphere) homogenized on ice in deoxygenated buffer [200 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid) and 5 mM EDTA; pH 8.0] using a sonicator (Sonics and Materials, CT, USA; Vibra Cell VC50T). The homogenates were analyzed for reduced sulfur compounds using the monobromobimane technique (Fahey & Newton 1987, Vetter et al. 1989). Sample treatment and HPLC analysis were performed according to Vismann (1991b). Elemental sulfur analysis was not made; it has earlier been shown that *Saduria entomon* does not produce S^0 (Vismann 1991b).

Allometric calculations. All results were converted using the equations below and are presented as mol g^{-1} wet wt. It was assumed that lactate and alanine were homogeneously distributed throughout the soft parts of the *Saduria entomon*.

Oxygen: The oxygen consumption data were calculated for a 1 g isopod according to the allometric relation: $MO_2 = MO_2' \cdot W^{-0.709}$ where MO_2' is the observed oxygen consumption corrected for blank (mg O_2 h^{-1}) and W is the wet weight (g) (data from Hagerman & Szaniawska 1988).

Lactate, alanine and reduced sulfur: Concentrations of lactate, alanine and reduced sulfur were calculated for a 1 g isopod using allometric relations for the midgut gland, the remaining tissue (exoskeleton and gut excluded) and the hemolymph as described below.

Midgut gland and remaining tissue: The total wet weights of a number of *Saduria entomon* were determined. The midgut gland was dissected out and weighed. The gut was then removed and the remains were dried at 100°C for 2 d. After 1 h in a desiccator, the dry weight was determined. The specimen was then heated at 455°C for 8 h. After 1 h in a desiccator, the ash free dry weight was determined.

Hemolymph. Saduria entomon were first dried with Kleenex® and total wet weight was determined. The isopods were then cut open and after bleeding they were reweighed to determine hemolymph volume.

The *Saduria entomon* used were within the size range of 0.911 to 5.322 g total wet weight. Best fit was obtained using linear relationships. For the hemolymph, the midgut gland and the remaining tissue the slope of the line (with SD) and R^2 were 0.235 ± 0.017 (R^2 = 0.89), 0.042 ± 0.007 (R^2 = 0.42) and 0.655 ± 0.007 (R^2 = 1.00), respectively.

RESULTS AND DISCUSSION

Oxygen consumption

Normoxia. The normoxic oxygen consumption as a function of time is seen in Fig. 3. The oxygen consumption is elevated due to initial stress during the first 3 h. Routine oxygen consumption was therefore calculated after excluding the first 3 h data. Regression analysis shows a slope which is not significantly different from zero ($R^2 = 0.09$; $p = 0.093$) and routine oxygen consumption was calculated as the mean value. Mean routine oxygen consumption (± SEM) was 0.109 ± 0.003 mg O_2 g^{-1} h^{-1}. Hagerman & Szaniawska (1988) found a routine oxygen consumption of 0.13 mg O_2 g^{-1} h^{-1} in *Saduria entomon* buried in sand, and when not buried (i.e. active or stressed) oxygen consumption increased to 0.25 mg O_2 g^{-1} h^{-1}. Although sand was not used in the present study, the routine (and the stressed) oxygen consumption corresponds well with values found by Hagerman & Szaniawska (1988).

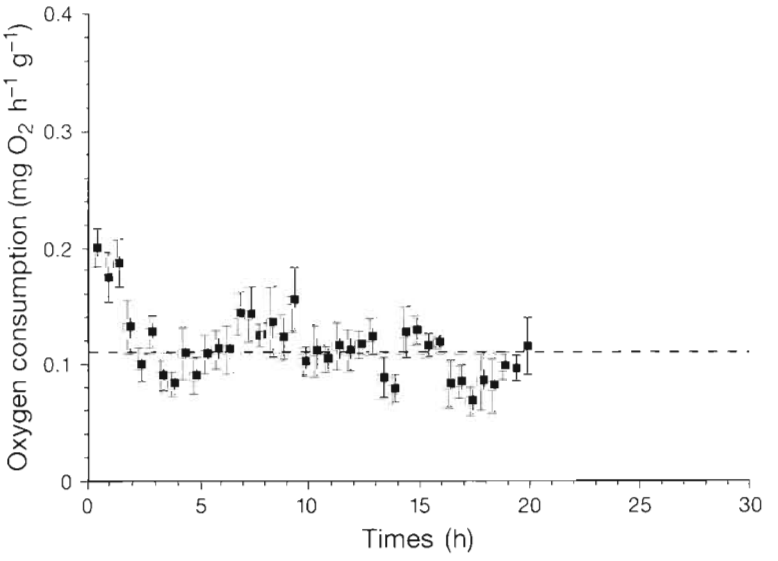

Fig. 3. *Saduria entomon* oxygen consumption during normoxia (19.5 kPa; *T*: 11°C; S: 10 ppt). Data presented as mean values ± SEM (n = 6). (– – –) Mean normoxic oxygen consumption rate

Severe hypoxia

The oxygen consumption of *Saduria entomon* recovering from severe hypoxia is shown in Fig. 4 The maximum oxygen consumption was 0.21 ± 0.01 mg O_2 g^{-1} h^{-1}. This point was reached after 1 h of recovery. The oxygen consumption returned to the normoxic level after 8 h of recovery. The oxygen debt was 0.57 ± 0.05 mg O_2 g^{-1} (17.8 ± 1.6 µmol O_2 g^{-1}). The time to recover from hypoxia is about the same as the exposure period; this has previously been found for other burrowing crustaceans (Bridges & Brand 1980).

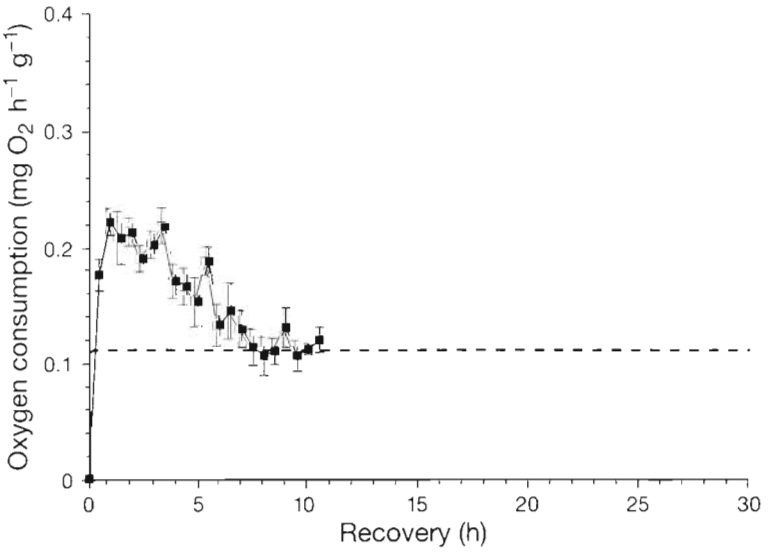

Fig. 4. *Saduria entomon* oxygen consumption during recovery from 8 h exposure to severe hypoxia (0.1 kPa; *T*: 11°C; S: 10 ppt). Data presented as mean values ± SEM (n = 6). (– – –) Mean normoxic oxygen consumption rate

Hypoxia with sulfide

The oxygen consumption of *Saduria entomon* recovering from hypoxia with sulfide is shown in Fig. 5. The maximum oxygen consumption was 0.33 ± 0.02 mg O_2 g^{-1} h^{-1} and this point was reached after 7 to 8 h of recovery. The oxygen consumption returned to the normoxic level only after about 28 h of recovery. The oxygen debt was 2.74 ± 0.29 mg O_2 g^{-1} (i.e. 85.6 ± 9.1 µmol O_2 g^{-1}) or about 5 times the oxygen debt at hypoxia. The time to recover from sulfide exposure was significantly prolonged compared to the recovery from severe hypoxia. This contrasts with the finding of Oeschger & Pedersen (1994), that the recovery period in the clam *Scrobicularia plana* was unaffected by the presence of sulfide.

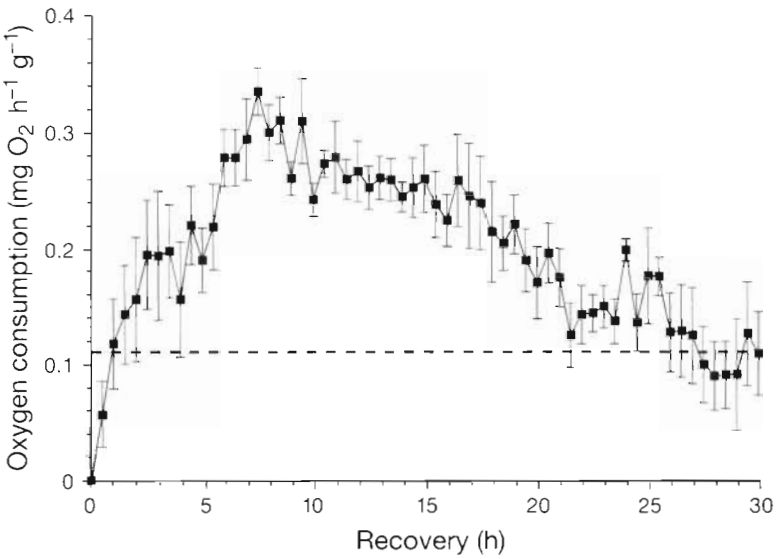

Fig. 5. *Saduria entomon* oxygen consumption during recovery from 8 h exposure to combined hypoxia (0.7 kPa; *T*: 11°C; S: 10 ppt) and sulfide (50 µM total sulfide). Data presented as mean values ± SEM (n = 6). (---) Mean normoxic oxygen consumption rate

Anaerobic metabolites

Normoxia. Normoxic lactate concentration in *Saduria entomon* was 0.18 ± 0.09 µmol g^{-1} (n = 3) in accordance with Hagerman & Szaniawska (1990) and Hagerman & Vismann (1993).

Severe hypoxia. After exposure to severe hypoxia, the lactate level (n = 5) was 15.5 ± 4.7 µmol g^{-1}. After recovery, the lactate returned to the normoxic level (0.13 ± 0.04 µmol g^{-1}; n = 3). During recovery, 15.4 ± 4.7 µmol g^{-1} lactate was removed.

Oxygen to lactate ratio in severe hypoxia. Species which produce lactate during anaerobiosis depend on rapid lactate removal during recovery in order to avoid toxic effects (Hochacka & Somero 1973). Removal of lactate can involve gluconeogenesis in which lactate is converted back to glucose (i.e. glucose-6-phosphate). Lactate accumulation and subsequent gluconeogenesis have been shown to be higher in species adapted to hypoxic conditions compared to species rarely exposed to hypoxic conditions (Hochacka & Somero 1973, Pritchard & Eddy 1979) Two molecules of lactate that have entered into gluconeogenesis use 6 high energy phosphate bonds to form 1 molecule of glucose-6-phosphate (Stryer 1975, Lehninger 1976). The aerobic metabolism produces about 6 ATP modules per O_2 molecule consumed. The theoretical oxygen to lactate ratio is therefore 0.5. In the present study, the oxygen debt was 17.8 ± 1.6 µmol g^{-1} and the lactate removed in *Saduria entomon* recovering from severe hypoxia was 15.4 ± 5.1 µmol g^{-1}; giving an oxygen to lactate ratio of 1.16 ± 0.49. An oxygen to lactate ratio >0.5 indicates that oxygen is used not only in the oxidative removal of lactate. Oxygen will also be used in the replenishment of physically dissolved oxygen and of hemocyanin-bound oxygen. Using a total oxygen carrying capacity of the hemolymph of 0.56 mmol l^{-1} (Hagerman & Vismann 1997) this can only account for about 0.13 µmol O_2 g^{-1}. Regeneration of ATP and phosphogen stores are other possible processes using oxygen during the recovery from hypoxia (Herreid 1980). Using the oxygen to lactate ratio of 0.5 the lactate removed during recovery is equivalent to 7.7 µmol O_2 g^{-1} (0.25 mg O_2 g^{-1}) and other processes consume 10.1 µmol O_2 g^{-1} (0.32 mg O_2 g^{-1}).

Following Hagerman & Szaniawska (1990), the alanine concentration after 8 h exposure to anoxia is not significantly different from the normoxic level. Although not measured in the present study, alanine was certainly not accumulating in the severe hypoxia exposure. This can be seen from the fact that not even during 8 h of sulfide exposures did alanine accumulate (see below and Hagerman & Vismann 1993).

Hypoxia with sulfide. After sulfide exposure the lactate level (n = 10) was 36.1 ± 5.0 µmol g^{-1} and after recovery the lactate level (n = 3) had returned to the normoxic level (0.18 ± 0.01 µmol g^{-1}). During recovery, 35.9 ± 5.0 µmol lactate g^{-1} was removed. The oxygen to lactate ratio during sulfide exposure was 2.38 ± 0.59, showing an increased importance of other processes. Using the above oxygen to lactate ratio of 0.5, the lactate removed is equivalent to 18.0 ± 2.5 µmol O_2 g^{-1} (0.58 mg O_2 g^{-1}) used, and other processes consume 67.7 µmol O_2 g^{-1} (2.16 mg O_2 g^{-1}).

After exposure to severe hypoxia, the alanine level (n = 5) was 0.04 ± 0.01 µmol g^{-1} and after recovery, the alanine (n = 3) was still 0.04 ± 0.01 µmol g^{-1} The normoxic alanine concentration is 0.07 ± 0.05 µmol g^{-1} (data from Hagerman & Szaniawska 1990) and starts to accumulate only after approximately 20 h of exposure to sulfide (Hagerman & Vismann 1993).

Reduced sulfur species

Concentrations of sulfide, thiosulfate and sulfite at the start of recovery from the sulfide exposure and at the end of recovery are shown in Table 2. Of the total 50.5 nmol g^{-1} reduced sulfur present at the start of

Table 2. Reduced sulfur in different parts of *Saduria entomon* during the recovery from sulfide exposure. All results are mean values ± SEM (n = 6)

	Sulfide (nmol g^{-1})	Thiosulfate (nmol g^{-1})	Sulfite (nmol g^{-1})	S atoms (nmol g^{-1})	(% of total)
Start of recovery					
Hemolymph	12.0 ± 0.2	9.1 ± 0.9	0.0 ± 0.0	30.2 ± 2.0	60
Midgut gland	3.0 ± 0.1	5.1 ± 1.1	0.0 ± 0.0	13.2 ± 2.3	26
Tissue	1.1 ± 0.2	3.0 ± 0.6	0.0 ± 0.0	7.1 ± 1.4	14
Total	16.1 ± 0.5	17.2 ± 2.6	0.0 ± 0.0	50.5 ± 5.7	100
End of recovery					
Hemolymph	0.3 ± 0.0	2.6 ± 0.8	0.3 ± 0.2	5.8 ± 1.8	28
Midgut gland	1.5 ± 0.3	2.9 ± 0.7	1.8 ± 1.6	9.1 ± 3.3	44
Tissue	1.0 ± 0.2	2.5 ± 1.0	0.0 ± 0.0	6.0 ± 2.2	28
Total	2.8 ± 0.5	8.0 ± 2.5	2.1 ± 1.8	20.9 ± 7.3	100
Balance					
Hemolymph	−11.7 ± 0.2	−6.5 ± 1.7	0.3 ± 0.2	−24.4 ± 3.8	82
Midgut gland	−1.5 ± 0.4	−2.2 ± 1.8	1.8 ± 1.6	−4.1 ± 5.6	14
Tissue	−0.1 ± 0.4	−0.5 ± 1.6	0.0 ± 0.0	−1.1 ± 3.6	4
Total	−13.3 ± 1.0	−9.2 ± 5.1	2.1 ± 1.8	−29.6 ± 13.0	100

recovery, 29.6 nmol g^{-1} disappeared during the 28 h of recovery. At the start of recovery, 60% of the reduced sulfur was present in the hemolymph. At the end of recovery, 44% of the remaining reduced sulfur was present in the midgut gland. During recovery, reduced sulfur concentration and composition in the tissue did not change significantly. Sulfite was not found at the start of recovery, but eventually accumulated to concentrations of 1.3 nmol g^{-1} in the midgut gland and 0.3 nmol g^{-1} in the hemolymph. The accumulation of H_2S and $S_2O_3^{2-}$ in the midgut gland of *Saduria entomon* found by Vismann (1991b) was not found in the present study. When exposed to sulfide for 24 h, the midgut gland turned black (Vismann 1991b). In the present study, no such color change was seen. Whether the difference is due to different exposure time or different handling prior to experiments (i.e. difference in sediment conditions in the storage tank) is not known, but the normoxic sulfide levels seem to indicate that the latter is the case (see below).

During recovery, 29.6 nmol reduced sulfur g^{-1} disappeared and were assumed to be oxidized to SO_4^{2-} (Table 2). This plus the 2.1 nmol SO_3^{2-} g^{-1} require 59.2 nmol O_2 g^{-1} to be oxidized. The 9.2 nmol $S_2O_3^{2-}$ g^{-1} that disappeared contained 13.8 nmol O_2 g^{-1}. Total oxygen needed for sulfide oxidation during recovery was calculated to be 45.4 nmol O_2 g^{-1} (= 1 µg O_2 g^{-1}). Whether the removed sulfide was oxidized or excreted is not known. However, the formation of sulfite during the recovery shows that some oxidation occurs. After recovery, the reduced sulfur in the hemolymph and in the midgut gland of *Saduria entomon* is similar to normoxic levels found by Vismann (1991b). However, the normoxic level of reduced sulfur in the tissue found by Vismann (1991b) is significantly higher than the reduced sulfur level in the tissue found after recovery in the present study. The higher tissue sulfide levels seems to indicate that *S. entomon* in Vismann (1991b) were exposed to some sediment sulfide in the storage tank prior to experiments.

Energetics of recovery

Severe hypoxia. An *Saduria entomon* uses 0.87 mg O_2 g^{-1} during 8 h when routine respiration (0.109 mg O_2 g^{-1} h^{-1}) is assumed. The oxygen debt paid by *S. entomon* during the 8 h of recovery was 0.57 ± 0.05 mg O_2 g^{-1} and lactate accounted for 0.25 mg O_2 g^{-1} of that (i.e. 44% of the oxygen debt). The oxygen debt after severe hypoxia is 'subnormal' (Herreid 1980). The metabolic rate, evaluated in terms of lactate accumulation, in *S. entomon* during severe hypoxia compared to the normoxic rate was thus reduced by approximately 70% Metabolic shutdown is a well-known phenomenon in animals periodically exposed to hypoxic conditions. However, most crustaceans will not show metabolic shutdown; they react to hypoxia by showing escape responses. *S. entomon* is unusual because it stays and awaits the return of normoxia. In general, the oxygen debt can also appear 'subnormal' when anaerobic metabolites are excreted or only partly oxidized (Herreid 1980). In the present study, however, the lactate was measured and the oxygen to lactate ratio did not indicate that any significant excretion took place.

Hypoxia with sulfide. The oxygen debt in *Saduria entomon* after sulfide exposure was 2.74 mg O_2 g^{-1}. The part of the oxygen debt which was due to lactate removal was 0.54 mg O_2 g^{-1} (i.e. 20% of the oxygen debt). The oxygen debt after sulfide exposure is 'supernormal' (Herreid 1980). The metabolic rate (evaluated in terms of lactate) of *S. entomon* in hypoxia with presence of sulfide compared to that of normoxia decreased by approximately 38%. In contrast to the 70% metabolic shutdown seen in hypoxia the metabolism decreases significantly less when sulfide was present. This accords with the increased consumption of energetic reserves seen in *S. entomon* exposed for 48 h to an oxygen tension of 4.0 to 6.7 kPa and 150 µM total sulfide (Hagerman & Vismann 1993). The phenomenon of 'supernormal' oxygen debt has also been found in other crustaceans (Taylor et al. 1977, Butler et al. 1978). In these studies, the supernormal oxygen debt is related to

succinate accumulation and regeneration of blood oxygen.

The part of the oxygen debt related to removal of reduced sulfur is as little as 0.04%. Thus, sulfide oxidation during recovery does not contribute significantly to the oxygen debt. Using the data of Vismann (1991b), in which the midgut gland was found to accumulate reduced sulfur, the oxygen demand for sulfide oxidation during recovery was calculated to have been about 10 times higher than that of the present result. Using these data, the sulfide oxidation would, however, still contribute less than 0.5% of the total oxygen consumption during recovery. The oxidation of sulfide during recovery has also been investigated in the bivalve *Scrobicularia plana*, for which Oeschger & Pedersen (1994) found that it did not contribute significantly to the oxygen debt.

The remaining 80% (2.2 mg O_2 g^{-1}) of the observed oxygen debt cannot be explained in terms of oxidation of reduced sulfur or anaerobic metabolites. Alternatively, it could be caused by an increased locomotory activity, as seen during the first 3 h of the normoxic experiments (Fig. 3). However, in the present study, no locomotory activity was seen in *Saduria entomon* recovering from sulfide exposure. Processes such as, e.g., restoring ionic and osmotic levels, regulation of cellular ionic composition, protein synthesis, regeneration of phosphogen, regeneration of ATP stores and restoration of enzyme levels are obvious candidates for explaining the remaining oxygen debt. These processes are likely to have been arrested during sulfide exposure as shown for hemocyanin synthesis in *S. entomon* exposed to sulfide (Hagerman & Vismann 1993). Replenishment of physically dissolved oxygen and hemocyanin-bound oxygen can only account for approximately 0.2% of the 2.2 mg O_2 g^{-1}.

In conclusion, the oxidation of anaerobic metabolites and reduced sulfur can only explain 20% of the oxygen debt seen in *Saduria entomon* recovering from sulfide exposure. Since no locomotory activity was seen, the majority of the oxygen debt is related to some not yet identified activities. These take place during recovery and are most likely concerned with restoring arrested processes. This suggests that some effects of sulfide on animal physiology have hitherto been overlooked. Future studies will address the identification of these physiological mechanisms.

Acknowledgements. We are indebted to Dr Eva Sandberg and Prof. Anna Szaniawska for supplying the experimental animals and to Merete Allerup for technical assistance. This study was supported by the Danish Science Research Council, grant no. 11-8391 and by EC, MAST-2, grant no. MAS2-CT93-0058.

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This article was presented by T. Fenchel (Senior Editorial Advisor), Helsingør, Denmark

Manuscript first received: August 9, 1996
Revised version accepted: September 3, 1996