The Journal of Experimental Biology 204, 3755–3764 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3498

Chemolithoheterotrophy in a metazoan tissue: thiosulfate production matches ATP demand in ciliated mussel gills

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Accepted 10 August 2001

Summary

The ribbed mussel *Geukensia demissa* inhabits sulfide-rich coastal sediments with a distribution that suggests a preference for exposure to sulfide. Although sulfide is a respiratory poison, it is also a potent reductant. *Geukensia demissa* gill mitochondria can use sulfide as a respiratory substrate for ATP production, and the gills of this species exhibit sulfide-supported oxygen consumption that matches the energy demand of ciliary beating. Here, we demonstrate (i) that the major product of *G. demissa* gill sulfide oxidation is thiosulfate and (ii) that the rate of sulfide oxidation also matches the cellular energy demand, resulting in a ratio near unity of oxygen consumed to sulfide oxidized at both low and high ciliary beat frequencies. A value for this ratio of unity is consistent with electrons from sulfide oxidation entering the mitochondrial electron transport chain. In the gills of the blue mussel *Mytilus edulis* from sulfide-free conditions, this ratio is 3–5 times higher, indicating an uncoupling of oxygen consumption from sulfide oxidation. Whereas *M. edulis* gills exhibit anaerobic metabolism during sulfide exposure, *G. demissa* gills do not, indicating a difference in sulfide tolerance between the two mussel species.

Key words: sulphide, gills, sulphide oxidation, bromobimane HPLC, succinate, mussel, *Geukensia demissa*, *Mytilus edulis.*

Introduction

The ribbed mussel *Geukensia demissa* inhabits sulfide-rich coastal sediments (Lee et al., 1996), with a distribution that suggests a preference for sulfide exposure. Sulfide is a potent reductant and source of energy that is exploited by sulfide-oxidizing bacteria. In addition, mitochondria isolated from a number of invertebrate species living in sulfidic habitats have been shown to oxidize sulfide as a respiratory substrate (Grieshaber and Völkel, 1998), although it is a potent toxin of aerobic respiration (Nicholls, 1975; National Research Council, 1979). Mitochondria isolated from the gills of *G. demissa* oxidize sulfide and produce ATP with an ADP/O ratio of 1 (Parrino et al., 2000). This sulfide-supported chemolithoheterotrophic production of ATP matches the energy demand of ciliary beating (Doeller et al., 1999). Thus, despite sulfide toxicity, mitochondrial sulfide oxidation can support cellular work in the gills of *G. demissa*.

Although the biochemistry of sulfide oxidation in metazoan tissue is only partially understood, thiosulfate has been shown to be the major product of sulfide oxidation in the animals studied thus far [for a review, see Grieshaber and Völkel (Grieshaber and Völkel, 1998)]. In the present study, the products of sulfide oxidation in *G. demissa* gills were determined as a function both of ambient sulfide concentration and of tissue ATP demand. The rates of sulfide oxidation and oxygen consumption were measured in the same gill preparation to determine the relationship between sulfide oxidation and ATP demand. In addition, the gill contents of succinate were estimated as an indication of anaerobic metabolism: when cytochrome *c* oxidase can no longer transfer electrons to oxygen because of either limiting ambient oxygen tensions or sulfide inhibition, fumarate is reduced to succinate (Grieshaber et al., 1994) and steady-state levels of succinate increase. Gills from the blue mussel *Mytilus edulis*, from intertidal low-sulfide habitats, were examined for comparative purposes.

Materials and methods

Animal collection and maintenance

Geukensia demissa Dillwyn 1817 were collected from intertidal *Spartina* sp. grass beds located on Dauphin Island, Alabama, USA, with sediment sulfide levels routinely near 1 mmol l^{-1} but as high as 8 mmol l^{-1} (Lee et al., 1996). Animals were shipped by overnight freight to Düsseldorf, Germany, and maintained in large Nalgene tubs with aerated artificial sea water [ASW; ingredients except for Hepes as listed in Hauschild et al. (Hauschild et al., 1999), with salinity adjusted accordingly] at 20‰ salinity. Animals were partially buried

within sulfide-generating sediment maintained at a depth of 15 cm in the tubs. Bacterial sulfide production was enhanced by inserting pieces of the brown alga (*Fucus* sp.) into the sediment. Sediment sulfide levels, measured using the 2,2′-dipyridyl disulfide (PDS) assay (Svenson, 1980), were 200–500 μmol l^{-1}. Sulfide-stimulated mass-specific gill oxygen consumption rate remained nearly constant for 4 months, during which time the animals were used for experimentation. *Mytilus edulis* Limé were collected from intertidal mussel beds of the Dutch Wadden Sea, near Zierikzee in the Netherlands, and maintained in sediment- and sulfide-free aerated ASW in Nalgene tubs at 35‰ salinity. Bacterial sulfide production and/or animal nutrition were supplemented by adding pulverized fish food (Tetra) twice weekly to the tanks. The animal room was maintained at 17±1 °C.

Experimental protocol

Identification of thiol compounds and determination of their production and release kinetics

Whole gills were excised from two living mussels and placed in Millipore-filtered (0.45 μm) ASW at maintenance salinity and room temperature (18–20 °C) for at least 30 min prior to experimentation to allow removal of excess mucus. Gills were then cut into small pieces; mean tissue wet mass was 13.6±3.7 mg (mean ± S.D., *N*=42). Individual pieces, randomly mixed from the two animals, were placed in incubation vials, one piece per vial, containing 5 ml of aerated sea water with either 0.5 μmol l^{-1} (low) or 10 μmol l^{-1} (high) 5-hydroxytryptamine (5-HT) to stabilize ciliary beating at 5–10 Hz or 20–25 Hz, respectively (Doeller et al., 1999). 5-Hydroxytryptamine, also known as serotonin, is an endogenous neurotransmitter in the bivalve gill that stimulates beating of the lateral cilia (Clemmesen and Jørgensen, 1987) probably *via* cAMP signal-transduction pathways [for a review, see Satir and Sleigh (Satir and Sleigh, 1990)]. At time zero, 100 μmol l^{-1} Na_2S (25 μl of 20 mmol l^{-1} Na_2S stock) was added to the incubation vials. Stock solutions of 20 mmol l^{-1} Na_2S were prepared by dissolving washed crystals of $Na_2S.9H_2O$ in N_2-saturated filtered ASW, with pH adjusted to 8.0 with 0.1 mol l^{-1} HCl (Wohlgemuth et al., 2000). Stock solutions of 0.01 mmol l^{-1} and 1 mmol l^{-1} 5-HT were prepared by dissolving crystals in filtered ASW. At 0, 5, 10, 20, 30 and 50 min, six tissue pieces were removed; three pieces were prepared immediately and three were frozen in liquid nitrogen for high-performance liquid chromatography (HPLC) determination of thiol compounds (see below). All pieces were prepared individually. Ambient seawater samples were taken at the same times and prepared for thiol HPLC. Control gill pieces placed in incubation vials containing only either a low or a high 5-HT concentration in aerated seawater were removed after 50 min.

Determination of the ratio of oxygen consumed to sulfide oxidized and of anaerobic end-products

Gill pieces, weighing approximately 50 mg, were placed in respirometer chambers and exposed to either 0.5 μmol l^{-1} or 10 μmol l^{-1} 5-HT and to 0–1000 μmol l^{-1} Na_2S at time zero. After 10 min, during which time a stable oxygen consumption rate was recorded (see below), a small piece of gill tissue, weighing 10–20 mg, was excised from the experimental tissue and frozen in liquid nitrogen for thiol HPLC (see below). Another gill piece was treated in the same way for determination of succinate levels (see below). The remaining gill tissue was weighed, dried for at least 48 h at 70 °C and reweighed. Ambient seawater samples were taken at the same time and frozen in liquid nitrogen for thiol HPLC. These experiments were repeated three times, each time with a different animal.

Measurement of thiol compounds by HPLC

Levels of thiol compounds in gill tissue homogenates and ambient sea water were measured using the monobromobimane HPLC method (Fahey et al., 1981; Newton et al., 1981; Vetter et al., 1989) as described by Völkel and Grieshaber (Völkel and Grieshaber, 1992; Völkel and Grieshaber, 1994), with the following modification: tissues that had previously been frozen in liquid nitrogen were then homogenized while thawing in the bimane reaction mixture. Thiol levels determined in previously frozen tissue were not significantly different from thiol levels determined in tissue processed immediately for HPLC (*t*-test; data not shown). The compounds of interest were the inorganic thiols sulfide, thiosulfate and sulfite, as possible components of the sulfide oxidation reaction, and the organic thiols glutathione and cysteine, as possible components involved in cellular oxidation/reduction reactions and sulfur storage, respectively. This method produced linear results for all thiol standards within the concentration range 2–100 μmol l^{-1} in the assay volume (data not shown). Blank tissue thiol levels were measured in the absence of added sulfide, and blank ambient seawater thiol levels were measured in the absence of tissue.

Measurement of gill oxygen consumption rate

Gill oxygen consumption rate was measured in a dual closed-chambered respirometer (Oroboros Oxygraph, model 67097; Paar, Graz, Austria) as described by Lee et al. (Lee et al., 1996). Briefly, a section of excised gill was placed on a stainless-steel screen shelf inside a respirometer chamber containing 5 ml of stirred (500 revs min^{-1}) ASW at 20 °C. The second chamber containing identical apparatus minus gill served as a control for blank oxygen consumption rates. During experiments, microliter additions of 5-HT and Na_2S stock solutions were made through an injection port in the stopper of each chamber using a Hamilton syringe; rates of oxygen consumption were measured within 10 min following additions.

Measurement of succinate levels

Tissue was extracted according to the method of Beis and Newsholme (Beis and Newsholme, 1975), and succinate was

measured spectrophotometrically according to the method of Beutler (Beutler, 1985).

Data presentation and statistical analyses

Data are presented as means ± S.D. (number of repetitions). Two-sample comparisons were made using paired or unpaired one-tailed *t*-tests assuming equal variance (Microsoft Excel). Multiple comparisons were made using analysis of variance (ANOVA) using the Bonferroni *post-hoc* test (SAS Institute Inc.; StatView). Significance was accepted at the 5 % level.

Results

Thiol compound production and release in mussel gills

Previous respirometric work with intact mussel gills showed that exposure to sulfide caused a rapid increase in oxygen consumption rate (Lee et al., 1996). To match this measured oxygen consumption response with sulfide oxidation in *G. demissa* gills, experiments were designed to determine the levels of thiol compounds after exposure to sulfide. Although the maximum oxygen consumption rate occurred within 10 min of sulfide exposure (Lee et al., 1996), these experiments were carried out for up to 50 min to capture more fully the kinetics of the sulfide oxidation response.

Thiol levels in *G. demissa* gills during a 50 min incubation in sea water injected with 100 μmol l^{-1} Na_2S at time zero are shown in Fig. 1 (see time course of ambient seawater Na_2S concentration in Fig. 2). At both 0.5 μmol l^{-1} and 10 μmol l^{-1} 5-HT, the main product of sulfide oxidation was thiosulfate; control tissue in the absence of sulfide did not produce thiosulfate during the 50 min incubation time. In low-5-HT gills, thiosulfate levels increased significantly at 10 and 20 min to near 1000 μmol kg^{-1} wet mass, then declined (Fig. 1A) following the drop in ambient sulfide level. In high-5-HT gills, thiosulfate levels increased significantly within 5 min and reached a maximum of approximately 2000 μmol kg^{-1} wet mass at 20 min, then declined (Fig. 1B) following the drop in ambient sulfide level. Sulfite showed patterns in both low- and high-5-HT gills that resembled those of thiosulfate, although at less than 5 % of the thiosulfate concentration (Fig. 1). At 10 min, levels of both thiosulfate and sulfite in high-5-HT gills, those with a higher ciliary beat frequency, were significantly higher than levels in low-5-HT gills. In contrast to thiosulfate and sulfite, levels of sulfide, glutathione and cysteine remained relatively constant throughout the incubation.

In *G. demissa* gills at both low and high 5-HT concentrations, the near linear rise in thiosulfate concentration during the first 5 min was not accompanied by release of thiosulfate from the tissue; detectable release occurred at 10 min and continued somewhat linearly throughout the 50 min incubation period (Fig. 2). The mathematical addition of levels of tissue thiosulfate plus released thiosulfate produced a near linear rise in thiosulfate concentration during the first 10 min of incubation, allowing the sulfide oxidation rate for that period to be calculated reliably. Sulfide levels in the ambient sea water, starting with 100 μmol l^{-1} at time zero, exhibited a rapid decline (Fig. 2), perhaps resulting in limited substrate for thiosulfate production. On the basis of these results, gill exposure time in all further experiments was limited to 10 min, and calculations of sulfide oxidation rate were made using the added quantities of thiosulfate in the tissue and that released into the ambient sea water. There were no significant changes in seawater levels of sulfite, cysteine or glutathione 10 min after the addition of 100 μmol l^{-1} Na_2S in the presence of either a low or a high 5-HT concentration (data not shown).

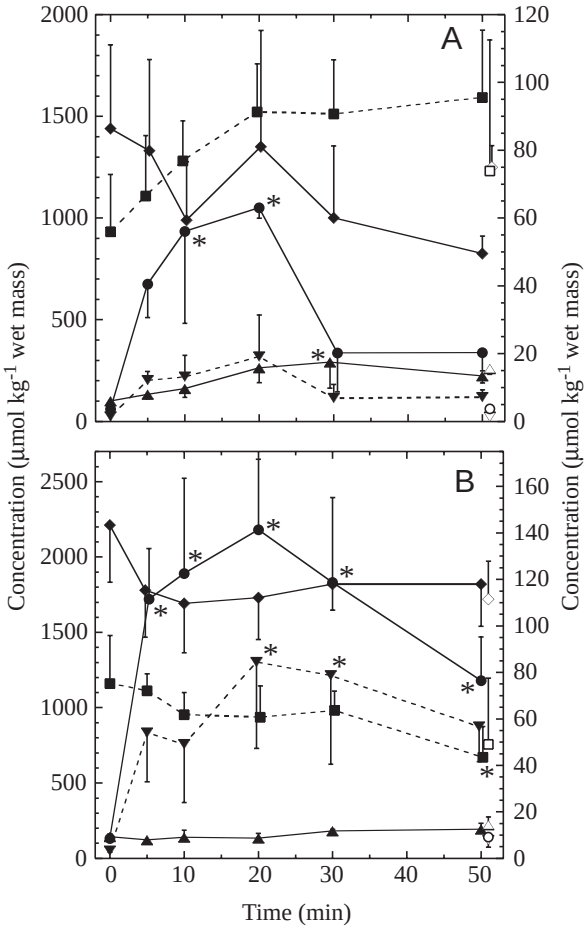

Fig. 1. Thiol levels in *Geukensia demissa* gills as a function of time. Gills were exposed to 100 μmol l^{-1} Na_2S at time zero. Thiols pertaining to the left *y*-axis are thiosulfate (filled circles), glutathione (filled diamonds) and cysteine (filled upright triangles), connected with solid lines. Thiols pertaining to the right *y*-axis are sulfide (filled squares) and sulfite (filled inverted triangles), connected with broken lines. Lines serve only to connect data points. Open symbols shown near the 50 min mark depict levels of the same thiols measured in control tissue after 50 min. Values are means ± S.D. (N=6). To eliminate overlap of error bars, some points have been offset by 0.25–0.5 min. Asterisks designate a significant difference from the value at 0 min (Bonferroni test). (A) Gills exposed to 0.5 μmol l^{-1} 5-HT. (B) Gills exposed to 10 μmol l^{-1} 5-HT.

Thiol levels in Geukensia demissa *gills versus* Mytilus edulis *gills*

A comparison of initial levels of thiol compounds in gills of

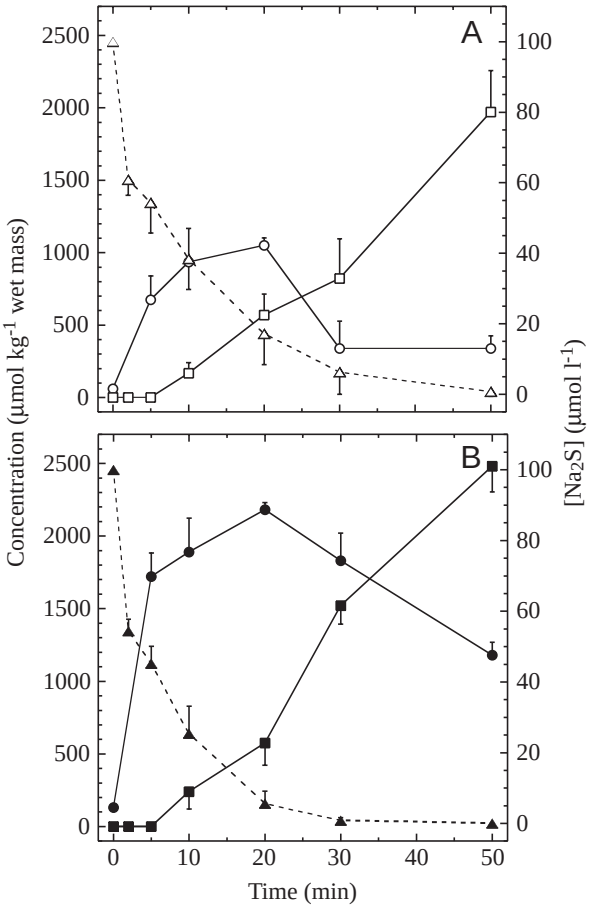

Fig. 2. The kinetics of thiosulfate production and release in *Geukensia demissa* gills. Gills were exposed to 100 µmol l^{-1} Na_2S at time zero. Thiols pertaining to the left *y*-axis are thiosulfate measured in tissue (circles) and thiosulfate measured in the ambient sea water (squares), connected with solid lines. The thiol pertaining to the right *y*-axis is sulfide in the sea water (upright triangles), connected with a broken line. Lines serve only to connect data points. Values are means ± S.D., shown as upper or lower error bars to reduce overlap (*N*=6). (A) Gills exposed to 0.5 µmol l^{-1} 5-HT. (B) Gills exposed to 10 µmol l^{-1} 5-HT.

Table 1. *Initial concentrations of thiol compounds in mussel gills*

	Concentration (µmol g^{-1} wet mass)	
Compound	*Geukensia demissa* (*N*=19)	*Mytilus edulis* (*N*=6)
Thiosulfate	94.1±32.3*	47.6±26.1
Glutathione	1720±441*	483±39
Sulfide	82.6±27.8*	17.6±4.1
Cysteine	99.6±36.3	88.6±18.0
Sulfite	2.60±5.05	0

An asterisk indicates a significant difference ($P \leqslant 0.05$) between mussel species.

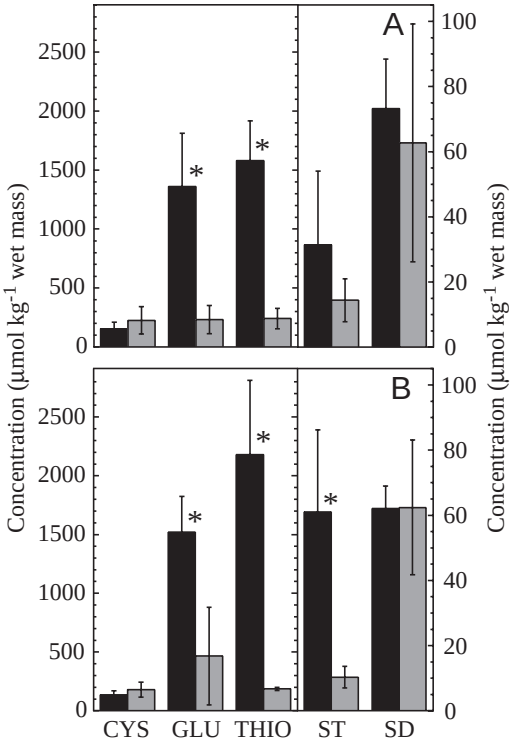

Fig. 3. Thiol levels in *Geukensia demissa* gills (black columns) and *Mytilus edulis* gills (grey columns). Gills were exposed to 100 µmol l^{-1} Na_2S at time zero for 10 min. Thiols pertaining to the left *y*-axis are cysteine (CYS), glutathione (GLU) and thiosulfate (THIO). Thiols pertaining to the right *y*-axis are sulfite (ST) and sulfide (SD). Values are means ± S.D. (*N*=3–6). Asterisks designate significant difference between mussel species (paired *t*-test). (A) Gills exposed to 0.5 µmol l^{-1} 5-HT. (B) Gills exposed to 10 µmol l^{-1} 5-HT.

mussels taken directly from maintenance aquaria (Table 1) showed that thiosulfate, glutathione and sulfide levels were significantly greater in *G. demissa* gills than in *M. edulis* gills, whereas cysteine and sulfite levels were not significantly different. A comparison of inorganic thiol levels measured 10 min after injection of 100 µmol l^{-1} Na_2S at time zero in respirometer chambers (Fig. 3) indicated that thiosulfate in both low- and high-5-HT gills and sulfite levels in high-5-HT gills were significantly greater in *G. demissa* than in *M. edulis*; sulfide levels were not significantly different between the species in either low- or high-5-HT gills. A comparison of organic thiol levels showed that glutathione levels were significantly greater in *G. demissa* gills than in *M. edulis* gills and that cysteine levels were not significantly different (Fig. 3).

The ratio of oxygen consumed to sulfide oxidized in mussel gills

The sulfide oxidation rates of mussel gills at low and high 5-HT concentrations, calculated as the rate of thiosulfate production during the 10 min after injection of 100 µmol l^{-1} Na_2S at time zero (see Fig. 2) multiplied by 2 to take into account the reaction stoichiometry, and the oxygen

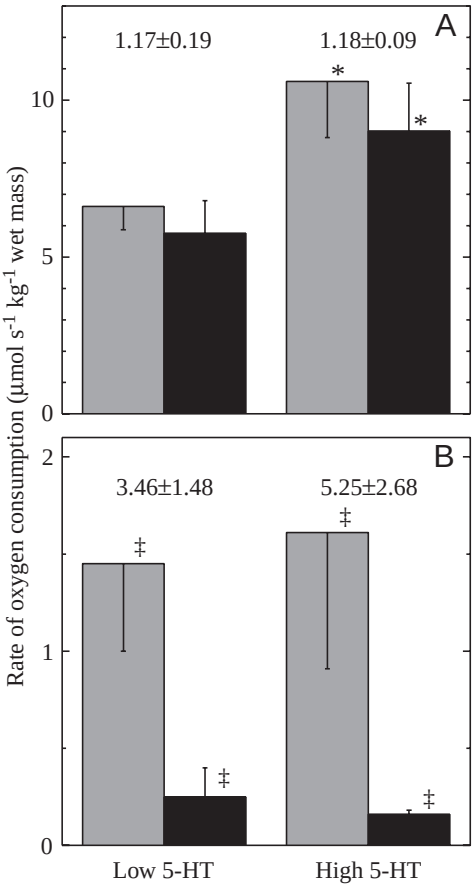

Fig. 4. Rates of oxygen consumption (grey columns) and sulfide oxidation (black columns) in mussel gills exposed to the low and high concentration of 5-HT. Numbers over each pair of columns refer to the oxygen/sulfide ratio for that measurement pair. Values are means ± S.D. (*N*=3–4). Asterisks designate significant difference between low- and high-5-HT treatments; double daggers designate significant difference between mussel species (Bonferroni test). (A) *Geukensia demissa* gills. (B) *Mytilus edulis* gills.

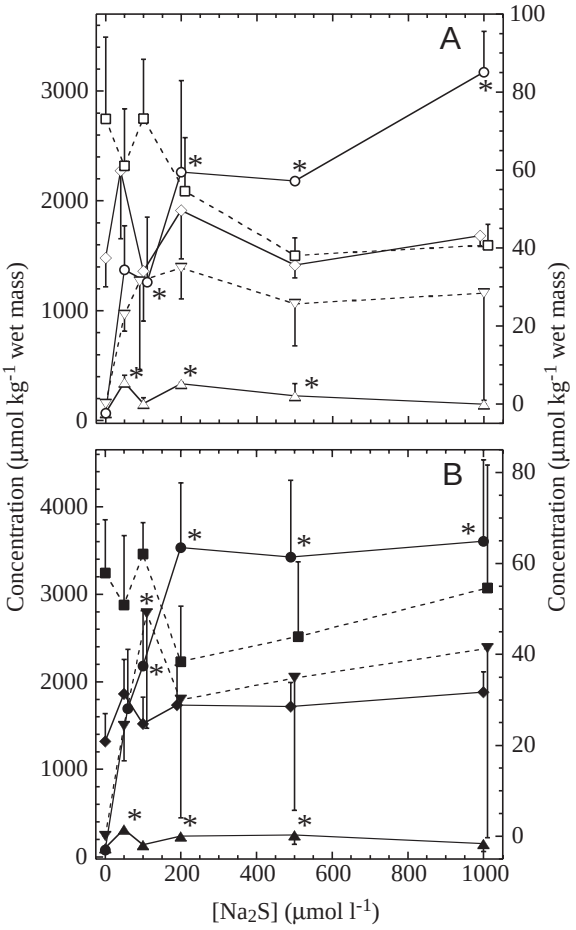

Fig. 5. Thiol levels in *Geukensia demissa* gills as a function of Na_2S concentration. Thiols pertaining to the left *y*-axis are thiosulfate (circles), glutathione (diamonds) and cysteine (upright triangles), connected with solid lines. Thiols pertaining to the right *y*-axis are sulfide (squares) and sulfite (inverted triangles), connected with broken lines. Lines serve only to connect data points. Values are means ± S.D. (*N*=3). To eliminate overlap of error bars, some points have been offset by 10 μmol l^{-1}. Asterisks designate significant difference from the value at 0 μmol l^{-1} Na_2S (Bonferroni test). (A) Gills exposed to 0.5 μmol l^{-1} 5-HT. (B) Gills exposed to 10 μmol l^{-1} 5-HT.

consumption rates of these same gills are shown in Fig. 4. The ratio of these two rates gives the ratio of oxygen consumed to sulfide oxidized, shown above the columns. For *G. demissa* gills, rates of both sulfide oxidation and oxygen consumption were significantly elevated at higher ciliary beat frequency and the resultant oxygen/sulfide ratios near 1.2 for both low- and high-5-HT gills were not significantly different (Fig. 4). For *M. edulis* gills, oxygen consumption rates were less than one-third of those of *G. demissa* gills [see Lee et al. (Lee et al., 1996)] and sulfide oxidation rates were less than one-tenth, representing significant differences (Fig. 4). The rates of sulfide oxidation were not significantly different in low- and high-5-HT *M. edulis* gills, nor were the rates of oxygen consumption or the resultant oxygen/sulfide ratios.

The effects of initial ambient sulfide concentration on thiol levels in *G. demissa* gills after a 10 min exposure are shown in Fig. 5. In both low- and high-5-HT gills, thiosulfate levels were increased significantly at 100 μmol l^{-1} ambient Na_2S and remained significantly elevated. Thiosulfate levels in high-5-HT gills were significantly higher than those in low-5-HT gills at 100 μmol l^{-1} Na_2S. Sulfite levels increased significantly at 100 μmol l^{-1} Na_2S in high-5-HT gills. Tissue sulfide levels did not change significantly, nor did glutathione levels. However, cysteine levels were significantly elevated at 50, 200 and 500 μmol l^{-1} Na_2S in both low- and high-5-HT gills.

The effects of initial ambient sulfide concentration on thiol levels in *M. edulis* gills after a 10 min exposure are shown in Fig. 6 (note the differences in the scales of the *x* and left *y* axes between Fig. 5 and Fig. 6). In low-5-HT gills, thiosulfate level was significantly increased to a maximum at 50 μmol l^{-1} ambient Na_2S, then decreased at higher sulfide concentrations. In high-5-HT gills, thiosulfate level was significantly increased at 50 μmol l^{-1} ambient Na_2S and remained significantly elevated. Thiosulfate levels in high-5-HT gills were not

significantly greater than those in low-5-HT gills at 100 μmol l^{-1} Na_2S. Sulfite showed patterns similar to thiosulfate in both low- and high-5-HT gills, but levels were not significantly different from controls. Tissue sulfide level was significantly increased at 50 μmol l^{-1} Na_2S and remained significantly elevated in high-5-HT gills. Cysteine levels were significantly increased at 200 μmol l^{-1} Na_2S in high-5-HT gills. Glutathione levels remained near 500 μmol kg^{-1} wet mass at all Na_2S concentrations in both low-and high-5-HT gills, although standard deviations approached 100 % in the presence of sulfide (not shown).

The ratio of oxygen consumed to sulfide oxidized as a function of Na_2S concentration in both low- and high-5-HT mussel gills is shown in Fig. 7. For *G. demissa* gills, the oxygen/sulfide ratio averaged 1.11±0.29 (*N*=10), ranging between 0.5 and 1.6. For *M. edulis* gills, the oxygen/sulfide ratio averaged 3.90±0.84 (*N*=6), ranging between 3 and 5.2. There were no significant differences in the ratios within the two species as Na_2S concentration was increased.

Both low- and high-5-HT mussel gills exhibited significant increases in succinate production under anaerobic conditions compared with aerated conditions (Fig. 8). In the presence of 100 μmol l^{-1} Na_2S under aerated conditions, *M. edulis* gills at the high 5-HT concentration produced significantly more succinate than the aerated controls, whereas *G. demissa* gills at both low and high 5-HT concentrations did not.

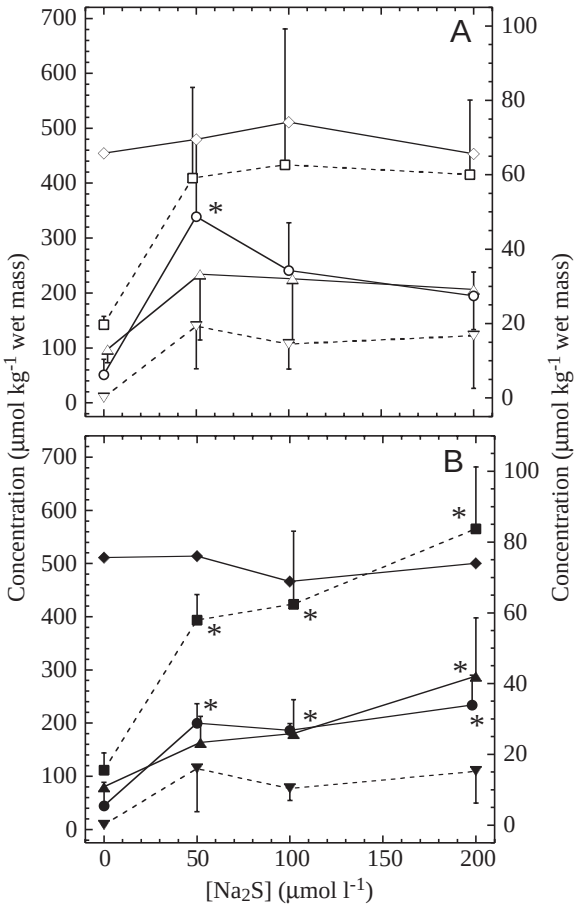

Fig. 6. Thiol levels in *Mytilus edulis* gills as a function of Na_2S concentration. Thiols pertaining to the left *y*-axis are thiosulfate (circles), glutathione (diamonds) and cysteine (upright triangles), connected with solid lines. Thiols pertaining to the right *y*-axis are sulfide (squares) and sulfite (inverted triangles), connected with broken lines. Lines serve only to connect data points. Values, except for glutathione, are means ± S.D. (*N*=3). Glutathione values are only the mean; standard deviations reach 71–99 % of the mean in gills exposed to any level of Na_2S and are therefore not included. To eliminate overlap of error bars, some points have been offset by 2 μmol l^{-1}. Asterisks designate a significant difference from the value at 0 μmol l^{-1} Na_2S (Bonferroni test). (A) Gills exposed to 0.5 μmol l^{-1} 5-HT. (B) Gills exposed to 10 μmol l^{-1} 5-HT.

Discussion

Sulfide is a potent toxin of cellular respiration, but it is also a potent reductant and is used by some chemolithotrophic prokaryotes as an energy source. We have recently reported that the gills of the mussel *Geukensia demissa* function metabolically as chemolithoheterotrophs, using sulfide as a source of energy to fuel ciliary beating (Doeller et al., 1999). Electrons from sulfide oxidation enter the mitochondrial electron transport chain, possibly at the level of cytochrome *c*, and travel to cytochrome *c* oxidase for the chemiosmotic production of ATP (Doeller et al., 1999; Parrino et al., 2000). Here, we report that, in *G. demissa* gills, the main product of sulfide oxidation is thiosulfate and that the rate of sulfide oxidation is matched to the rate of oxygen consumption.

Thiosulfate

Thiosulfate is the main sulfide oxidation product in a variety

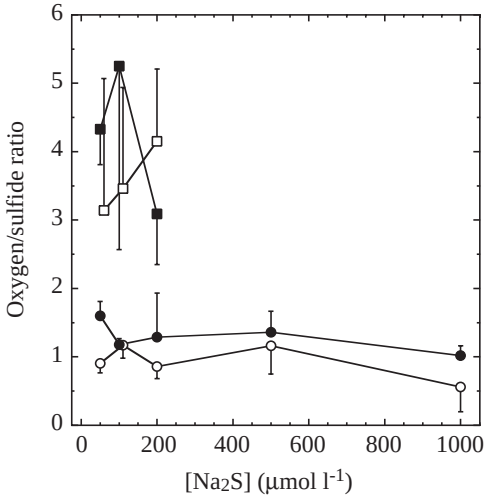

Fig. 7. Oxygen/sulfide ratio in *Geukensia demissa* gills (circles) and *Mytilus edulis* gills (squares) as a function of Na_2S concentration. Gills were exposed to 0.5 μmol l^{-1} 5-HT (open symbols) or 10 μmol l^{-1} 5-HT (filled symbols). Values are means ± S.D. (*N*=3). To eliminate overlap of error bars, some points have been offset by 10 μmol l^{-1}. Lines serve only to connnect data points.

of macrofaunal sulfide inhabitants, accumulating in the tissues and body fluids [for a review, see Grieshaber and Völkel (Grieshaber and Völkel, 1998)]. Thiosulfate has also recently been shown to be the sulfide detoxification product in rat cecal mucosa (Levitt et al., 1999). The peak thiosulfate concentrations reported here for low-5-HT and high-5-HT gills of *G. demissa* exposed to 100 μmol l^{-1} Na_2S in aerated sea water, 1000 and 2200 μmol kg^{-1} wet mass, respectively, are similar to values reported for body wall tissue of the lugworm *Arenicola marina* exposed to Na_2S in aerated sea water [1424 μmol kg^{-1} wet mass exposed to 200 μmol l^{-1} Na_2S (Hauschild and Grieshaber, 1997); near 1 mmol l^{-1} exposed to 117 μmol l^{-1} Na_2S (Wohlgemuth et al., 2000)]. In *A. marina*, sulfide is oxidized in the mitochondria to thiosulfate (Völkel and Grieshaber, 1996; Völkel and Grieshaber, 1997), which accumulates in the body wall, coelomic fluid and blood (Hauschild and Grieshaber, 1997). In the echiuroid worm *Urechis caupo* exposed to sulfide, thiosulfate, the product of sulfide oxidation primarily by hematin in coelomocytes (Powell and Arp, 1989), accumulates in the coelomic fluid (Julian et al., 1999). In *G. demissa* gills, sulfide is oxidized in gill mitochondria (Parrino et al., 2000), and we show here that thiosulfate accumulated in the gill tissue for a brief period before detectable release into the ambient sea water.

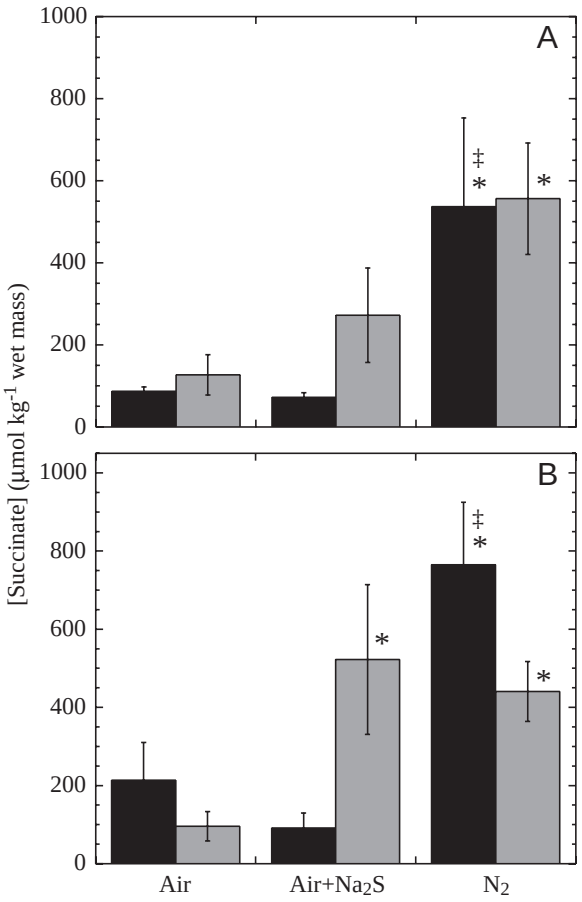

Fig. 8. Succinate levels in *Geukensia demissa* gills (filled columns) and *Mytilus edulis* gills (hatched columns) under aerated conditions (Air), aerated conditions with 100 μmol l^{-1} Na_2S added at time zero (Air+Na_2S) and anaerobic conditions produced by nitrogen gas (N_2), all for 10 min. Values are means ± S.D. (*N*=3). Asterisks designate a significant difference from the value under aerated conditions; double daggers designate a significant difference from the value under aerated conditions with 100 μmol l^{-1} Na_2S. (A) Gills exposed to 0.5 μmol l^{-1} 5-HT. (B) Gills exposed to 10 μmol l^{-1} 5-HT.

Biological sulfide oxidation in prokaryotes is thought to occur mainly by enzymes linked to the respiratory chain, such as the sulfide quinone oxidoreductase (Arieli et al., 1994; Schütz et al., 1997; Schütz et al., 1999) and the sulfide cytochrome *c* oxidoreductase, also called flavocytochrome *c* or sulfide dehydrogenase (Schneider and Friedrich, 1994; Visser et al., 1997; Sorokin et al., 1998). The putative products of these enzymes are elemental sulfur or sulfate (Schütz et al., 1997; Schneider and Friedrich, 1994; Visser et al., 1997; Sorokin et al., 1998). The gene from a yeast mitochondrial sulfide-oxidizing enzyme has recently been shown to have homology with genes from other prokaryotes and eukaryotes, extending the family of sulfur chemistry enzymes to the eukaryotes (Vande Weghe and Ow, 1999). The sulfide-oxidizing enzyme(s) in *G. demissa* gills may also belong to this enzyme family, as was suggested for *A. marina* (Völkel and Grieshaber, 1996). Mitochondrial enzymes such as thiosulfate thiotransferase (rhodanese) and sulfite oxidase may be responsible for thiosulfate production (O'Brien and Vetter, 1990).

The soluble, non-toxic thiosulfate may represent a favorable alternative to the other products of sulfide oxidation; in addition, its production requires less oxygen than sulfate production (O'Brien and Vetter, 1990; Grieshaber and Völkel, 1998). Sulfite was shown to be an intermediate in the production of thiosulfate in mitochondria from the sulfide-tolerant clam *Solemya reidi* (O'Brien and Vetter, 1990). In this reaction, a single sulfide molecule undergoes a six-electron oxidation to sulfite, and a second sulfide molecule is added with a further two-electron oxidation (O'Brien and Vetter, 1990). On the basis of the similar kinetic pattern between thiosulfate and sulfite [compare Fig. 1 in this paper with fig. 4 in O'Brien and Vetter (O'Brien and Vetter, 1990)], we suggest that sulfite is also an intermediate in *G. demissa* gills.

Since thiosulfate is a charged molecule, thiosulfate elimination would require cell and possibly mitochondrial membrane transport mechanisms; however, the nature of these mechanisms is unknown at the present. Whole-animal thiosulfate elimination has been shown to occur by passive diffusion in *A. marina* (Hauschild et al., 1999) and *U. caupo* (Julian et al., 1999), crossing the body wall and the hindgut, respectively. The estimated thiosulfate gradient, based on volume average cellular and ambient concentrations, suggests that thiosulfate release does not require active transport. Thiosulfate probably moves through epithelia *via* paracellular spaces following a concentration gradient in the outward direction (Hauschild et al., 1999; Julian et al., 1999). In *G. demissa* gills, thiosulfate accumulated for approximately 5–10 min before release was detectable. Because mussel

gill epithelia consist of two cell layers, cellular release of thiosulfate represents an important mechanism for thiosulfate elimination. Direct transport of thiosulfate into the ambient medium is proposed.

Glutathione

Glutathione is a major cellular thiol participating in cellular redox reactions and in the elimination of H_2O_2 and organic hydroperoxides [for a review, see Sies (Sies, 1999)]. Reduced glutathione is also essential for the activity of sulfur-oxidizing enzyme in several species of thiobacilli [for a review, see Kelly (Kelly, 1999)]. Our results indicate that glutathione levels in *G. demissa* gills are approximately three times greater than in *M. edulis* gills (Table 1; Fig. 3, Fig. 5, Fig. 6). Glutathione levels show initial, although non-significant, decreases upon sulfide exposure (Fig. 1), indicating a possible correspondence between glutathione and sulfide oxidation. Although Vismann (Vismann, 1991) states that glutathione does not play an important role in the detoxification of sulfide in the isopod *Saduria entomon*, we propose that the high glutathione level in *G. demissa* gills compared with *M. edulis* gills represents an adaptation to environmental sulfide exposure and, thus, that glutathione may function in sulfide detoxification.

Cysteine

Cysteine is a non-essential amino acid in mammals, synthesized from serine and homocysteine, a breakdown product of methionine; in plants and microorganisms, cysteine is synthesized from serine and sulfide (Cooper, 1983) and may therefore represent a storage form of sulfide. In the gills of both mussel species, cysteine levels show significant increases in response to changing ambient sulfide levels, indicating that cysteine production may be influenced by sulfide exposure.

Sulfide

The initial level of gill tissue sulfide for *M. edulis* taken from sulfide-free seawater aquaria was 18 μmol kg^{-1} wet mass (Table 1). Values reported for body wall tissue of *A. marina* under control conditions in the absence of ambient sulfide were 24–28 μmol l^{-1}, attributed to the presence of mercapto groups of body wall proteins not to free sulfide (Hauschild and Grieshaber, 1997; Wohlgemuth et al., 2000). This may also be the case for mussel gills. In *M. edulis* gills, tissue sulfide concentration rose to 60–80 μmol kg^{-1} wet mass in the presence of ambient Na_2S levels up to 200 μmol l^{-1} (Fig. 6). In contrast, the initial level of gill tissue sulfide for *G. demissa* taken from sulfide tanks was 83 μmol kg^{-1} wet mass (Table 1), and the value remained at 40–100 μmol kg^{-1} wet mass at ambient Na_2S concentrations up to 1000 μmol l^{-1} (Fig. 5). In the gills of both mussel species, tissue sulfide concentration appears to remain relatively low at the ambient Na_2S concentrations tested. However, even these relatively low values may be inhibitory in *M. edulis* gills, as demonstrated by significant succinate production under these conditions compared with *G. demissa* gills (see below).

Anaerobic metabolism

In the presence of toxic levels of sulfide, oxygen consumption could become limited as a result of cytochrome *c* oxidase poisoning (Nicholls, 1975), and animals may exhibit anaerobic metabolism to maintain ATP turnover [for a review, see Grieshaber and Völkel (Grieshaber and Völkel, 1998)]. Two animals, the symbiont-containing clam *S. reidi* and the symbiont-free lugworm *A. marina*, produce the anaerobic product succinate under aerated conditions in the presence of sulfide concentrations greater than 250 μmol l^{-1} (Anderson et al., 1990; Völkel and Grieshaber, 1994). We have shown that, in the absence of oxygen, the gills of both mussel species exhibit anaerobic heat dissipation (Doeller et al., 1990; Doeller et al., 1993; Doeller and Kraus, 1992) and a build-up of succinate (Fig. 8). However, under aerated conditions in the presence of 100 μmol l^{-1} Na_2S, only the gills of *M. edulis* produced significant levels of succinate, nearly as high as those seen in anaerobic gills; this is probably evidence of sulfide poisoning of aerobic metabolism. The gills of *G. demissa* did not produce significant levels of succinate. These data indicate that, in contrast to the gills of *M. edulis*, the gills of *G. demissa* do not undergo anaerobic metabolism at 100 μmol l^{-1} Na_2S. In fact, succinate levels in *G. demissa* gill were not significantly greater than those of the control until the gills were exposed to 500 and 1000 μmol l^{-1} Na_2S (data not shown).

Sulfide oxidation and energy demand

We have previously proposed that sulfide oxidation in *G. demissa* gills functions in cellular ATP production (Doeller et al., 1999; Parrino et al., 2000). The evidence includes (i) a turning on and off of gill ciliary beating by sulfide exposure and removal, respectively, in the presence of antimycin A, (ii) a fall in the ratio of gill ciliary beat frequency to oxygen consumption rate to a level that is quantitatively consistent with electrons from sulfide oxidation entering the mitochondrial electron transport chain at the level of cytochrome *c*, (iii) gill cytochrome *c* reduction in the presence of sulfide and (iv) sulfide-supported coupled respiration and ATP production by isolated gill mitochondria, with an ADP/O ratio of 1.

A further piece of evidence consistent with the hypothesis that *G. demissa* gills function in sulfide-supported chemolithoheterotrophy, presented here, is the near unity ratio of oxygen consumed to sulfide oxidized at both low and high 5-HT concentration or ciliary beat frequency (Fig. 4), which demonstrates a coupling between sulfide oxidation and ATP demand in gills and ATP production in mitochondria. We interpret this ratio as follows: when sulfide is oxidized to thiosulfate, eight electrons are released in the oxidation of two sulfide molecules, or four electrons per sulfide (O'Brien and Vetter, 1990). If these electrons were to enter the mitochondrial electron transport chain, four electrons would lead to the reduction of two atomic oxygens or one molecular diatomic oxygen to water; therefore, the oxidation of one sulfide molecule would lead to the reduction of one oxygen molecule.

Our data show that this ratio is the same at low and high ciliary beat frequency or ATP demand. If sulfide oxidation in *G. demissa* gills at 100 μmol l^{-1} Na_2S functions mainly to detoxify sulfide, then the rate of sulfide oxidation should be the same whether the tissue has a low or high ATP demand. Instead, the sulfide oxidation rate follows ATP demand. Additional evidence suggests that, if terminal oxidases suspected of participation in the sulfide oxidation pathway are inhibited with cyanide or salicylhydroxamic acid (SHAM), the gill exhibits decreases in both oxygen consumption rate and thiosulfate production (D. Kraus and J. Doeller, unpublished observations), as has been shown for *A. marina* (Völkel and Grieshaber, 1997).

In contrast to the gills of *G. demissa*, the gills of *M. edulis* show roughly one-fifth the rates of sulfide oxidation and oxygen consumption in the presence of sulfide. In addition, the difference in oxygen/sulfide ratios between *G. demissa* gills and *M. edulis* gills remains over a range of sulfide concentrations (Fig. 7). One interpretation of the high oxygen/sulfide ratios in the gills of *M. edulis* is that sulfide oxidation results in the production of sulfate or polythiols, which also requires oxygen. However, in most macrofaunal sulfide inhabitants studied thus far, thiosulfate was shown to be the major product of sulfide oxidation (Grieshaber and Völkel, 1998). We interpret the elevated oxygen/sulfide ratio in *M. edulis* gills as indicating a possible uncoupling of sulfide oxidation from oxygen consumption and energy demand. In contrast, the near unity oxygen/sulfide ratio in *G. demissa* gills up to 1000 μmol l^{-1} Na_2S indicates that electrons from sulfide oxidation probably enter the mitochondrial electron transport chain.

In conclusion, we have shown (i) that thiosulfate is the main product of sulfide oxidation in mussel gills, (ii) that the gills of *G. demissa* have much higher rates of thiosulfate production and levels of glutathione than the gills of *M. edulis* and (iii) that sulfide oxidation and mitochondrial oxidative phosphorylation appear to be coupled in *G. demissa* gills, with no input from anaerobic metabolism, and uncoupled in *M. edulis* gills. This latter conclusion further supports our hypothesis that *G. demissa* gills function in sulfide-supported chemolithoheterotrophy, able to use either carbon compounds or sulfide as respiratory substrate. How the gills make the choice between substrates is the subject of ongoing research.

This work was supported in part by National Science Foundation grants IBN9219658 and IBN9728409 to J.E.D. and D.W.K. and by the Deutsche Forschungsgemeinschaft GR 456/20 to M.K.G. Special thanks to Ms Lisa Kellogg at the Dauphin Island Sea Laboratory for the collection of *Geukensia demissa* from Alabama and shipment to Germany. Special thanks also to Dr Stephanie Wohlgemuth, Ms Silke Jacob and the rest of the staff and students of the Institute for Zoophysiology, Heinrich Heine University of Duesseldorf, Germany, for technical and other help during our (J.E.D. and D.W.K.) sabbatical stay.

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