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INORGANIC CARBON ACQUISITION BY THE HYDROTHERMAL VENT TUBEWORM *RIFTIA PACHYPTILA* DEPENDS UPON HIGH EXTERNAL P_{CO_2} AND UPON PROTON-EQUIVALENT ION TRANSPORT BY THE WORM

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Summary

Riftia pachyptila **is the most conspicuous organism living at deep sea hydrothermal vents along the East Pacific Rise. To support its large size and high growth rates, this invertebrate relies exclusively upon internal chemosynthetic bacterial symbionts. The animal must supply inorganic carbon at high rates to the bacteria, which are far removed from the external medium. We found substantial differences in body fluid total inorganic carbon ($\sum CO_2$) both within and between vent sites when comparing freshly captured worms from a variety of places. However, the primary influence on body fluid $\sum CO_2$ was the chemical characteristics of the site from which the worms were collected. Studies on tubeworms, both freshly captured and maintained in captivity, demonstrate that the acquisition of inorganic carbon is apparently limited by the availability of CO_2, as opposed to bicarbonate, and thus appears to be accomplished *via* diffusion of CO_2 into the plume, rather than by mediated transport of bicarbonate. The greatly elevated P_{CO_2} measured at the vent sites (up to 12.6 kPa around the tubeworms), which is a result of low environmental pH (as low as 5.6 around the tubeworms), and elevated $\sum CO_2$ (as high as 7.1 mmol l^{-1} around the tubes) speeds this diffusion. Moreover, despite large and variable amounts of internal $\sum CO_2$, these worms maintain their extracellular fluid pH stable, and alkaline, in comparison with the environment. The maintenance of this alkaline pH acts to concentrate inorganic carbon into extracellular fluids. Exposure to *N*-ethylmaleimide, a non-specific H^+-ATPase inhibitor, appeared to stop this process, resulting in a decline in extracellular pH and $\sum CO_2$. We hypothesize that the worms maintain their extracellular pH by active proton-equivalent ion transport *via* high concentrations of H^+-ATPases. Thus, *Riftia pachyptila* is able to support its symbionts' large demand for inorganic carbon owing to the elevated P_{CO_2} in the vent environment and because of its ability to control its extracellular pH in the presence of large inward CO_2 fluxes.**

Key words: tubeworm, *Riftia pachyptila*, inorganic carbon, hydrothermal vent, ion transport, carbon fixation, symbiosis, pH regulation, *N*-ethymaleimide.

Introduction

The giant hydrothermal vent tubeworm *Riftia pachyptila* was first found to be symbiotic with intracellular carbon-fixing sulfide-oxidizing bacteria in 1981 (Cavanaugh *et al.* 1981; Felbeck, 1981). *R. pachyptila* supplies its symbionts with inorganic carbon, oxygen, hydrogen sulfide and nitrate, which are taken up from the environment across the plume (Arp *et al.* 1985; Felbeck and Childress, 1988; Childress and Fisher, 1992; Lee and Childress, 1994). These substances are transported in the vascular blood to the bacteria located in an organ known as the trophosome (Jones, 1981). The trophosome is highly vascularized and surrounded by non-circulating coelomic fluid, which is in equilibrium with the circulating vascular blood for smaller molecules such as CO_2 (Childress *et al.* 1984, 1991). These worms have two extracellular hemoglobins in the blood that bind and transport both oxygen and hydrogen sulfide to the symbionts (Arp *et al.* 1985, 1987). Inorganic carbon accumulation and transport to the bacteria, however, apparently takes place without significant binding or buffering by blood proteins (Childress *et al.* 1993; Kochevar *et al.* 1993; Toulmond *et al.* 1994).

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Previous studies have shown that the symbionts in the trophosome fix CO_2, yet have a low affinity for it (K_m=0.021–0.035 mmol l^{-1}; Scott *et al.* 1994).

Inorganic carbon (C_i) uptake is problematic in the ocean because, although abundant in sea water, most C_i is in the form of bicarbonate, which bears a net negative charge and, thus, does not diffuse as readily through membranes as does CO_2. Because biological membranes are highly permeable to gaseous CO_2, inorganic carbon flux into aquatic organisms, including micro- and macroalgae, angiosperms and host animals, is often *via* passive diffusive uptake of CO_2, despite its low concentration in sea water (Badger, 1987; Weis *et al.* 1989; Raven, 1993; Riebesell *et al.* 1993). Active uptake of bicarbonate, *via* a number of different mechanisms, however, has also been shown to be a primary mode of carbon acquisition in many marine photoautotrophs and autotrophic symbioses. For instance, an electrogenic HCO_3^- pump has been proposed as the only mode of C_i uptake in some marine algae and cyanobacteria (Al-Moghrabi *et al.* 1996). It has been shown that the red alga *Chondrus crispus* supports 90 % of its photosynthesis by exogenous HCO_3^- uptake (Badger, 1987) and the macroalga *Ulva* sp. acquires all of its C_i *via* facilitated uptake of HCO_3^- (Drechsler *et al.* 1993). Marine cyanobacteria have also been shown to take up both CO_2 and HCO_3^- *via* a HCO_3^- exchanger with a carbonic-anhydrase-like moiety attached to the protein (Badger, 1987). In addition, it has recently been proposed that photosynthesis in *Galaxea fascicularis*, a scleractinian coral harboring dinoflagellate symbionts, depends entirely upon a HCO_3^- symport mechanism in the animal host (Al-Moghrabi *et al.* 1996). Thus, instances in which HCO_3^- is taken up from the medium *via* specific porters are widespread in the marine environment, where external HCO_3^- levels approach 2.5 mmol l^{-1} and dissolved CO_2 concentration only reaches 10–15 μmol l^{-1} (Kerby and Raven, 1985; Riebesell *et al.* 1993). The vent environment, however, can have very elevated CO_2 partial pressures due to increased total C_i and acidic water conditions (Childress *et al.* 1993).

R. pachyptila has extremely high vascular blood and coelomic fluid $\sum CO_2$ values because of the elevated external $\sum CO_2$ (Childress *et al.* 1993). These high vascular blood and coelomic fluid $\sum CO_2$ values in *R. pachyptila* are of particular note because the pH values of these fluids remain stable and alkaline even at extremely high $\sum CO_2$ concentrations, thus serving as an inorganic carbon-concentrating mechanism (Childress *et al.* 1993; Toulmond *et al.* 1994). The present study was directed towards elucidating the relationships between external and extracellular fluid C_i concentrations and pH and the mechanisms by which C_i uptake occurs. In particular, we were concerned with determining whether the external conditions could be inferred from measurements made on freshly captured animals, determining whether C_i uptake is *via* CO_2 diffusion or by mediated transport of bicarbonate, and investigating the relationship between extracellular pH regulation and inorganic carbon uptake.

These studies involved the measurement of environmental parameters around the worms in their natural habitats, the measurement of internal gas parameters of freshly captured worms, and the execution of experiments in which the worms were kept at different pH values and $\sum CO_2$ concentrations in pressure systems on board ship. An important tool for the shipboard studies was the use of specific inhibitors of transport processes. Sulfanilamide, an inhibitor of carbonic anhydrase, was tested to determine whether it affected the rate of C_i uptake into the worms. It has been postulated that carbonic anhydrase plays a significant role in CO_2 uptake by *R. pachyptila* (Kochevar *et al.* 1993), by converting CO_2 to HCO_3^-. The mechanisms of pH control and the effects of eliminating such control were tested using the ATPase inhibitors *N*-ethylmaleimide (NEM) and dicyclohexylcarbodiimide (DCCD). Inhibitors of anion (bicarbonate) exchange, specifically 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) and 4-acetamido-4′-isothiocyano-2,2′-disulphonic stilbene (SITS), were also tested for possible effects on ion exchange mechanisms, although they were not expected to have direct effects on inorganic carbon fluxes or concentrations.

Materials and methods

Collections

Riftia pachyptila Jones were collected at an average depth of 2600 m by submersible during research expeditions to 9° N (9°50′ N, 104°18′ W) and 13° N (12°48′ N, 103°57′ W) along the East Pacific Rise in 1992 and 1994. Hot venting water and warm water samples around the tubeworms were collected using titanium samplers from the *Alvin*. Animals were brought to the surface in a temperature-insulated container and transferred to cold sea water (5 °C) in a refrigerated van on board ship. Worms were then sorted to be used either for experiments on living animals or for immediate measurements of physiological variables. Live animal experiments were initiated within 2 h of surfacing. Worms labeled ‘freshly collected’ in this text were dissected less than 4 h after collection from the sea floor and analyzed for coelomic fluid and vascular blood P_{CO_2}, pH, $\sum CO_2$, $\sum H_2S$ (‘$\sum$’ is used to indicate total concentrations, including all species present), N_2, O_2, CH_4 and ammonia concentrations. All worm dissection techniques were similar to those described in Childress *et al.* (1991) with the exception of vascular blood, which was withdrawn from the ciliated area of the vestimentum, which is underlain by the ventral vessel, using a syringe equipped with a 26 gauge needle. The blood samples may therefore be considered as ‘arterial’ since this ventral vessel comes out of the plume and directs blood flow to the trophosome (Jones, 1988). Tissue and blood samples were also frozen (at –20 °C) in order to measure carbonic anhydrase activity.

An *in situ* analysis system (ISAS) for measuring water temperature and pH around the worms was successfully used from *Alvin* on some dives. This system employed a stainless-steel probe (1 m long) held by the *Alvin* manipulator. At the end of this probe was a thermistor and the inlet of a fine rubber tube

(Norprene tubing, 1.6 mm i.d.). Water was drawn through this tubing by a peristaltic pump driven by a d.c. motor running in hydraulic fluid. This water was drawn through a manifold containing solenoid valves. In the normal configuration, the water passed unaltered to pressure-compensated double-junction pH electrodes (Innovative Sensors). The solenoids were switched at least twice during each dive to allow buffer solutions to flow past the electrodes, allowing *in situ* calibration. Organic buffers with minimal pressure responses were used (Neuman *et al.* 1973). Tris was adjusted to near pH 7.5 and MES to pH 5.5 at the surface. The effect of temperature (the electrodes were always at deep sea bottom water temperatures of approximately 2 °C as verified by a thermistor immediately ahead of them in the flow stream) was estimated from the known effects of temperature on these buffers. The data were digitized and logged by a logging board (Onset Computers) with an add-on amplifier circuit (Derek Manov, University of Southern California, Geology Department).

Pressure aquaria

Animal maintenance, single time point and time course experiments were all conducted inside a refrigerated van maintained at 8 °C. Sea water was chilled to 8 °C by moving the water through approximately 60 m of polypropylene tubing (5 mm i.d.). Sea water was taken from two reservoir tanks, of 200 l and 75 l, and pumped by high-pressure, diaphragm metering pumps through the vessels at flow rates ranging from 4 to 12 l h^{-1} at pressures of approximately 21.5 MPa.

Vessels used for animal maintenance were made entirely of type 316 stainless steel with dimensions of 9.8 or 14.5 cm i.d. and 110 cm length. Vessels used as experimental chambers had outer cylinders made of type 304 stainless steel and inner cylinders of acrylic. This greatly increased the working pressures of our vessels over comparable cylinders made entirely of acrylic (burst pressures of approximately 14.9 MPa) (Quetin and Childress, 1980). The outer steel cylinders were between 91.4 and 101.6 cm in length, with a thickness of 1.2 cm and an inner diameter of 17.8 cm. The inner cylinders were the same length with an inner diameter of 9.3 cm and a wall thickness of 4.3 cm. Sixteen to twenty-four holes (2.5 cm diameter) were cut into the stainless-steel outer sleeve and located in two rows along the length of the vessel to allow viewing of animals during incubations. Type 316 stainless-steel plugs fitted with rubber O-rings sealed both the bottom and top of each vessel, and the top plug had holes for connecting high-pressure inlet and outlet fittings. Pressure gauges and sample ports were placed in-line immediately after flow through the vessel to allow monitoring of pressure and water conditions (see diagram in Quetin and Childress, 1980). Animals kept alive at the surface were maintained in these high-pressure vessels.

Single time point experiments

For single time point experiments (12–22 h), water $\sum CO_2$, pH and P_{CO_2} were varied by bubbling CO_2 gas directly into a gas equilibration column and out to the vessels *via* high-pressure pumps (see diagram in Kochevar *et al.* 1992). For experiments at higher $\sum CO_2$, the addition of CO_2 gas varied the pH sufficiently (5.6–6.6); however, for experiments at low $\sum CO_2$, it was necessary to vary the pH (5.6–8.3) by titration with hydrochloric acid. Sulfide concentrations were controlled by continuously pumping anaerobic solutions of sodium sulfide (30–50 mmol l^{-1}) into the gas equilibration column. To stabilize and control the pH, 10 mmol l^{-1} MOPS buffer was added to the sulfide solutions. In the experiments, concentrations of total inorganic carbon within the range 2–11 mmol l^{-1} and $\sum H_2S$ concentrations within the range 0–0.6 mmol l^{-1} were used. Oxygen concentrations were maintained between 0.1 and 0.5 mmol l^{-1} and nitrogen concentrations were maintained between 0.2 and 0.5 mmol l^{-1} by bubbling both gases into the gas equilibration column together with CO_2. At the end of each experiment, the animals were quickly removed from the pressure vessel and dissected.

Between experiments, including the time course experiments described below, both the vessels and high-pressure hose were cleaned by rinsing with sea water, followed by additional rinses with 10 ml l^{-1} chlorine bleach, approximately 36 mmol l^{-1} thiosulfate, and a final rinse with sea water. Bleach was used to sterilize the equipment, and thiosulfate was used to ensure that all chlorine from the bleach was removed before the next addition of worms. After all inhibitor experiments, also described below, methanol was added to the vessels before the initial seawater rinse in order to solubilize any remaining inhibitors.

Time course experiments

Control worms

All time course experiment worms were first kept in flowing-water maintenance aquaria, also at 8 °C and 21.5 MPa, supplied with surface sea water. Decreases in blood $\sum CO_2$ were monitored over periods of 2–4 days. Animals were then transferred to experimental vessels, described above for single time point experiments. Water conditions, $\sum CO_2$ between 4.6 and 5.5 mmol l^{-1}, $\sum H_2S$ between 0.1 and 0.6 mmol l^{-1} and pH between 5.9 and 6.2, were controlled in the same manner as for the single time point experiments. Methane was also supplied to the worms, in 18 of the 28 time course experiments, including some inhibitor experiments, through direct input of methane gas *via* the gas equilibration column. Final methane concentrations ranged from 0.1 to 0.3 mmol l^{-1}. Worms were killed at intervals up to 20 h after the start for tissue, vascular blood and coelomic fluid collection.

Inhibited worms

Worms used in inhibitor experiments were maintained in the same manner as for the control worms, and experimental conditions were similarly controlled. Once the worms had been placed in the experimental vessels, however, they were exposed to one of various inhibitors. Fourteen worms were exposed to NEM, a non-specific inhibitor of H^+-ATPases, at final concentrations of 1.1–1.6 mmol l^{-1} in the surrounding water (Marver, 1984; Stone *et al.* 1984), while four additional worms

were exposed to DCCD, an inhibitor specific for mitochondrial and plasma H^+-ATPases, at a final concentration of 1.2 mmol l^{-1} (Marver, 1984; Steinmetz *et al.* 1981). Eleven worms were exposed to sulfanilamide (1–1.4 mmol l^{-1}), an inhibitor of carbonic anhydrase (Holder and Hayes, 1965; Maren, 1967). NEM, DCCD and sulfanilamide were first dissolved in 5% ethanol in sea water (at 20 mmol l^{-1}). This stock was then mixed continuously with sea water and introduced into the vessels over a 1 h period *via* metering pumps. Thus, worms exposed to NEM, DCCD and sulfanilamide were also exposed to less than 1% ethanol. The flow rate determined the final concentration for each inhibitor. Worms were also exposed to anion exchange inhibitors, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid) and SITS (4-acetamido-4′-isothiocyano-2,2′-disulphonic stilbene) (four worms each) (Duranti *et al.* 1986; Schlue and Deitmer, 1988). Because amounts of these inhibitors were limited, both DIDS and SITS were mixed directly into the vessels containing 6 l of sea water, resulting in final concentrations of 0.08 mmol l^{-1} and 0.2 mmol l^{-1}, respectively. The worms in these two experiments were kept at atmospheric pressure for 1 h with no flow in order to ensure exposure at these concentrations. Like the control worms, those exposed to inhibitors were also removed from the vessels and killed at time points between 1 and 20 h.

Analytical methods

$\sum CO_2$, $\sum H_2S$ and CH_4 concentrations of both body fluids and water samples were measured in 0.5 ml samples using a Hewlett Packard 5880A gas chromatograph (Childress *et al.* 1984). Both body fluid and water pH values were measured with a thermostatted Radiometer BMS-2 blood pH analyzer equipped with a G299A capillary pH electrode and connected to a PHM73 pH meter. Additional water pH measurements were made using a double-junction combination electrode (Broadley-James) connected to a PHM93 pH meter (Radiometer). Body fluid P_{CO_2} measurements were made at 10 °C using a Cameron blood gas analyzer (Cameron Instrument Co., TX, USA). Body fluid P_{CO_2} measurements were accurate only if the samples were analyzed within 5 h of dissection. Although this seems like a long period, the animals still showed the expected distribution of internal chemicals (on the basis of values from other studies: Childress *et al.* 1991, 1993). In addition, the data from freshly collected worms agreed well with the data from experimental animals dissected immediately upon removal from the pressurized chambers (see Fig. 3A,B). We believe that this happens because the circulation and metabolism of the worms is greatly reduced when they are moved to water at 2 °C on collection and exposed to lower pressures on recovery; thus, internal parameters do not change over the '5 h' collection period. Within 5 h, P_{CO_2} drift was found to be negligible, but between 5 and 7 h after dissection the P_{CO_2} values increased by 25–30%. If the samples could not be analyzed within 5 h, blood P_{CO_2} was calculated instead (35 of 137 samples; see below). Each gas composition measurement took approximately 25 min, so all gas chromatograph, pH and P_{CO_2} samples were kept on ice in gas-tight syringes until analyses could be made. If an experimental worm had a trophosome with a pinkish color, which is indicative of trophosome degradation (Fisher *et al.* 1989), the data were excluded (36% of animals for 1992 and 9% for 1994). In addition, if any single time point experimental worm had a vascular blood or coelomic fluid pH below 6.9, indicative of poor circulation in *R. pachyptila*, probably due to damage during collection (Childress *et al.* 1991), the data were excluded (30% of animals for 1992 and 2% for 1994). Carbonic anhydrase activity was measured using the protocol described in Weis *et al.* (1989).

Water P_{CO_2} (mmHg; 1 mmHg=0.133 kPa) was calculated from pH and $\sum CO_2$ (mmol l^{-1}) measurements using the equation:

$$P_{CO_2} = [H^+] \times \sum CO_2 / \alpha \times (K_{app} + [H^+]),$$

where α is the solubility constant of CO_2 and K_{app} is the apparent equilibrium constant (Boutilier *et al.* 1984). Water solubility constants and equations for apparent pK (pK_{app}) were also taken from Boutilier *et al.* (1984); salinity was assumed to be 35‰. The solubility constant used for body fluid P_{CO_2} calculations was 0.48241 mmol l^{-1} kPa^{-1}, taken from Heisler (1990) for a body fluid at 8 °C with a molarity of 0.55 mol l^{-1} for dissolved species. The value for pK_{app} was calculated for a body fluid at 8 °C and pH 7.5 with a protein content of 33 g l^{-1} for coelomic fluid and 132 g l^{-1} for vascular blood (Terwilliger *et al.* 1980; Childress *et al.* 1984), an ionic strength of non-protein ions of 0.656 mol l^{-1} for coelomic fluid and 0.630 mol l^{-1} for vascular blood (Sanders and Childress, 1991) and a Na^+ concentration of 0.55 mol l^{-1} (Heisler, 1984, 1990). The pK_{app} values were 6.15 and 6.12 (which convert to K_{app} values of 7.08×10^{-7} and 7.59×10^{-7}, respectively), for coelomic fluid and vascular blood, respectively. Body fluid and water P_{CO_2} values were all measured and calculated for 101.3 kPa, ambient pressure. The increased hydrostatic pressure at depth would be expected to have small effects on P_{CO_2} through effects on both CO_2 solubility (Enns *et al.* 1965) and on pK (Issacs, 1981; Millero, 1983), but the relative relationships should be valid since all measurements were made at 101.3 kPa. Actual heme content was not taken into account for blood P_{CO_2} measurements because there is negligible buffering capacity and no CO_2 binding by the *R. pachyptila* hemoglobins (Childress *et al.* 1984, 1993; Toulmond *et al.* 1994).

Statistics

The Wilcoxon signed-rank test was used to test for differences between body compartments. The Kendall rank correlation was used to test for correlations. The Mann–Whitney *U*-test was used to test for differences in distribution between data sets. Simple regressions were used to show linear relationships, and multiple regressions were used to compare the influences of various parameters. Analysis of covariance (ANCOVA) was used to compare slopes and magnitudes of different data sets.

Values are presented in the text as means ± S.E.M., and values of *N* are given in the figure legends and the tables.

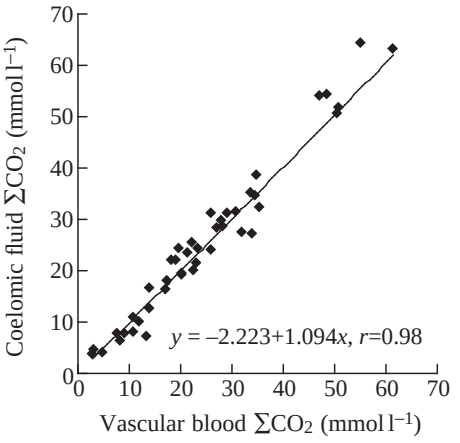

Fig. 1. Relationship between coelomic fluid and vascular blood total inorganic carbon, $\sum CO_2$, in the hydrothermal vent tubeworm *Riftia pachyptila* during single time point experiments. Worms were kept in high-pressure flowing-water aquaria at 8 °C and 21.5 MPa for 12–22 h. Water conditions were adjusted and ranged from 2 to 11 mmol l^{-1} for $\sum CO_2$, from 5.6 to 8.3 for pH and from 63 to 623 μmol l^{-1} for $\sum H_2S$. P<0.0001, Kendall rank correlation (N=43).

Results

Coelomic fluid versus *vascular blood*

Because it was difficult to collect enough vascular blood for all the measurements, vascular parameters were not measured on all animals. However, a direct relationship was observed between coelomic fluid and vascular blood parameters, so we present only the more complete coelomic fluid data here. For example, Fig. 1 shows the relationship between coelomic fluid and vascular blood total inorganic carbon, $\sum CO_2$ for animals in single time point experiments. As was found previously (Childress *et al.* 1984, 1991), coelomic fluid was shown to be representative of vascular blood for $\sum CO_2$ measurements, as they were not statistically different from each other (P=0.10 for freshly captured worms, P=0.77 for worms used in single time point experiments, Wilcoxon signed-rank test; Table 1). Coelomic fluid and vascular blood P_{CO_2} were, likewise, not significantly different (P=0.35 for freshly captured worms, P=0.09 for worms used in single time point experiments, Wilcoxon signed-rank test; Table 1). Coelomic fluid pH, however, appears to be slightly higher than vascular blood pH (P=0.0112 for freshly captured worms, P=0.0086 for worms used in single time point experiments, Wilcoxon signed-rank test; Table 1), as has been observed in previous studies (Childress *et al.* 1991).

Effects of site pH and $\sum CO_2$ on internal $\sum CO_2$

Table 2 shows both warm and hot water measurements for the 16 dives during which worms for this experiment were collected. 'Warm' water indicates water that was collected immediately surrounding the worm tubes (mean temperature 14 °C), while 'hot' water samples were collected directly from smokers (mean temperature 265 °C). X5, Riftia Field, Tubeworm Pillar, Rumour and Alvinellid Pillar are all at the 9° N site, while Genesis, Parigo, Elsa and Totem are located at the 13° N site. It was often not possible to collect both warm and hot water samples. For example, at the Riftia Field site, there is no smoker and, thus, no hot water to be sampled. We believe the P_{CO_2} values for the warm water around the worms at Riftia Field are actually higher than those measured during five of the collections because two other Riftia Field P_{CO_2} measurements were much higher, 8.7 and 12.6 kPa. Our justification for this is that a water sample cannot be biased to have a higher P_{CO_2} as a result of poor sampling. Rather, poor sampling (such as a bottle not completely filling) would result in a lowering of the $\sum CO_2$ and P_{CO_2}. Thus, the two high measurements probably reflect the environment and the others are probably too low. For this reason, in order to analyze the rest of the data, values for the Riftia Field water samples which we believed to be too low were given the average value of the two higher P_{CO_2} water samples (10.6 kPa). These data showed substantial variation among sites in $\sum CO_2$ and pH, resulting in different P_{CO_2} values as well. Temperature also seemed to be variable, but temperatures around the tubes were typically approximately 20 °C. The *in situ* pH analyzer confirmed the low pH values observed in water samples and suggested that the actual pH *in situ* around the worms may be well below 6.0 in some vent areas.

Table 1. *Comparison of acid–base parameters ($\sum CO_2$, pH and P_{CO_2}) for coelomic fluid and vascular blood from the hydrothermal vent tubeworm* Riftia pachyptila

	Freshly collected			Single time point		
Worms	Vascular blood	Coelomic fluid	*P* value	Vascular blood	Coelomic fluid	*P* value
$\sum CO_2$ (mmol l^{-1})	34.6±2.6	31.0±2.8	0.1049	25.9±2.9	26.4±3.4	0.7704
pH	7.37±0.04	7.40±0.03	0.0112	7.45±0.07	7.51±0.06	0.0086
P_{CO_2} (kPa)	6.10±0.81	5.78±0.68	0.3539	12.09±2.76	7.78±1.52	0.0912

Measurements were made on worms labelled as 'freshly collected' (N=36, mean ± S.E.M.) within 3–4 h of collection from the sea floor. Worms labelled as 'single time point' (N=43, mean ± S.E.M.) were kept in high-pressure flowing water aquaria at 8 °C and 21.5 MPa with water conditions ranging from 2 to 11 mmol l^{-1} for $\sum CO_2$, from 5.6 to 8.3 for pH and from 63 to 623 μmol l^{-1} for $\sum H_2S$.

The Wilcoxon signed-rank test was used to test for consistent differences between the coelomic and vascular compartments within individuals. All P values are for the Wilcoxon signed-rank test.

Table 2. *Physico-chemical characteristics of hydrothermal vent warm and hot water for dives from which* Riftia pachyptila *were collected for this study*

		Warm water					Hot water					ISAS readings of warm water					
Location	Dive number	*T* (°C)	$\sum CO_2$ (μmol l^{-1})	P_{CO_2} (kPa)	pH	$\sum H_2S$ (μmol l^{-1})	*T* (°C)	$\sum CO_2$ (μmol l^{-1})	P_{CO_2} (kPa)	pH	$\sum H_2S$ (μmol l^{-1})	*T* at plumes (°C)	*T* at top of tubes (°C)	*T* among tubes (°C)	pH at plumes	pH at top of tubes	pH among tubes
13° N																	
Genesis	2855	7.5	4114	4.24	6.19	2	263.3	18978	54.45	4.97	254						
	2874											5.1	12.6		7.7	6.8	
Parigo	2856	14.8	6534	5.00	6.38	6	280.0	24420	39.05	5.01	669	5.2	9.6	16.6			
Elsa	2857	21.5	5317	1.66	6.84	19	321.2	23575	39.68	4.43	2331						
Dying Genesis	2874	2.2	2877	1.05	7.21	0	259.0	27772	47.38	3.81	5815						
Totem	2875						345.6	25654	43.73	3.82	6092						
9° N																	
X5	2859	21.6	3600	NM	NM	0	NM	26462	44.25	4.57	1263			24.8			
Riftia Field	2861	16.5	2877	1.56	6.57	0						14.8	18.5	18.6			
Riftia Field	2863	19	7094	12.56	5.6	0						12.9	19.1	20.8			
Riftia Field	2865	18.6	3742	3.26	6.31	1						15.0	19.1	20.1			
Riftia Field	2868	17.5	2903	0.83	6.88	0											
Riftia Field	2869	14	3343	2.36	6.43	0						7.2	4.1	2.8	6.5	6.2	6.6
Riftia Field	2870	9.8	3315	1.67	6.61	1						15.0	14.6	22.9	5.7	5.2	5.3
Riftia Field	2873	19	6201	8.68	5.92	0						17.0	21.1	26.6	6.3	5.1	5.0
Tubeworm Pillar	2864	10	3496	2.01	6.54	63						5.4	7.9	21.7			
Rumour	2866	2	2680	0.34	7.23	0											
Alvinellid Pillar	2867						120.0	23952	40.00	4.59	3127						

Warm water samples were taken among the worms while hot water samples were taken around the smokers at the vent sites using titanium water samplers. It was not always possible to take both warm and hot samples at each site. ISAS samples used a thermistor sensor on a probe and an *in situ* pH electrode reading values for water drawn from near the probe tip. All dives were performed by the *Alvin* during a research cruise to the East Pacific Rise in 1994.

T, temperature; NM, not measured.

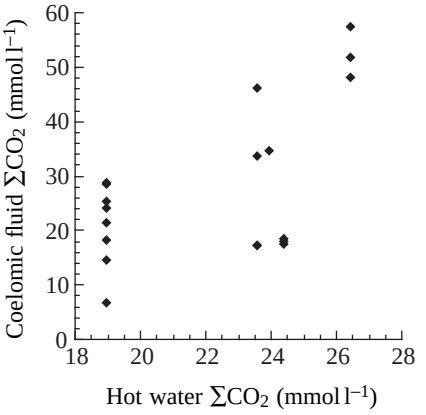

Fig. 2. Relationship between coelomic fluid total inorganic carbon, $\sum CO_2$, of freshly collected *Riftia pachyptila* and $\sum CO_2$ of the hot venting water at the respective vent sites (including the sites Genesis, Elsa, Alvinellid Pillar, Parigo and X5) (N=20; P=0.0334, regression significance y=–32.796+2.723x, r=0.58).

Worms that were examined immediately upon recovery showed coelomic fluid $\sum CO_2$ values that correlated well with the $\sum CO_2$ (P=0.033, significance of regression) of the hot venting water at that site, although the worms never come into contact with the hot water (Fig. 2). Worms collected from sites with low hot water $\sum CO_2$, for example the Genesis site with only 19 mmol l^{-1} $\sum CO_2$, had lower coelomic fluid $\sum CO_2$ values (7–29 mmol l^{-1}). Likewise, animals collected from sites with higher hot water $\sum CO_2$, such as the X5 site with 26.5 mmol l^{-1} $\sum CO_2$, had higher coelomic fluid $\sum CO_2$ values (48–57 mmol l^{-1}). This indicates that the properties of the hot water are significant factors in determining the characteristics of the diluted warm water around the worms.

Coelomic fluid $\sum CO_2$ values were also dependent upon the P_{CO_2} values of the surrounding warm water (Fig. 3A). Worms from sites with higher P_{CO_2}, such as Riftia Field, had higher coelomic fluid $\sum CO_2$ values. Figs 2 and 3A indicate that worms experiencing warm water and venting water with increased P_{CO_2} and elevated $\sum CO_2$ values also have elevated internal $\sum CO_2$ values. Worms also showed an increase in coelomic fluid $\sum CO_2$ as warm water pH decreased (P<0.0001, significance of regression; results not shown), indicating that CO_2 is probably the most influential factor affecting internal $\sum CO_2$ because CO_2 is more abundant than HCO_3^- at lower external pH values.

Single time point experiments

The same relationships between internal and external inorganic carbon were also seen during shipboard experiments. Fig. 3B shows that internal $\sum CO_2$ increases with increasing water P_{CO_2}. This increase in coelomic fluid $\sum CO_2$ for experimental worms is not significantly different in slope or magnitude (P=0.63 and P=0.32, respectively, ANCOVA) from the increase in internal $\sum CO_2$ seen for the freshly collected worms in Fig. 3A, indicating that the blood parameters measured on freshly captured worms are good indicators of the values of these same parameters in the worms *in situ*. A

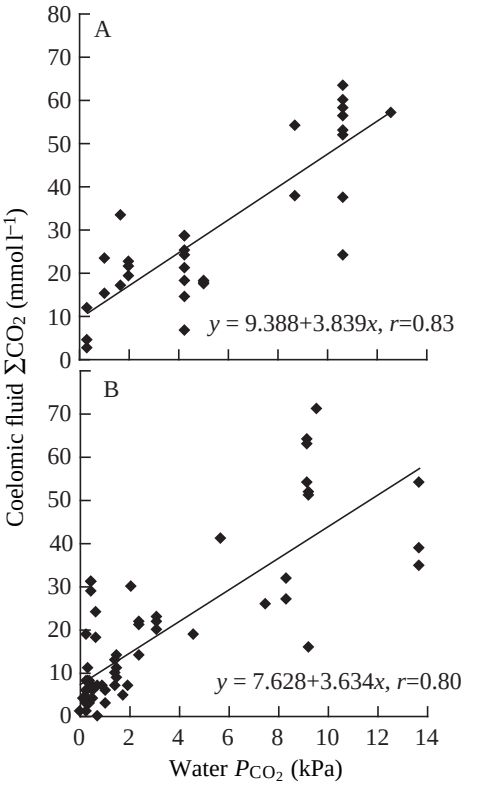

Fig. 3. (A) Relationship between coelomic fluid total inorganic carbon, $\sum CO_2$, of freshly collected *Riftia pachyptila* and P_{CO_2} of the warm water surrounding the worms (N=34; P<0.0001, regression significance). (B) Relationship between coelomic fluid total inorganic carbon, $\sum CO_2$, in the tubeworm *Riftia pachyptila* and the P_{CO_2} of surrounding water during 35 single time point shipboard experiments. Worms were kept in high-pressure flowing-water aquaria at 8 °C and 21.5 MPa for 12–22 h. Vessel conditions were controlled at fixed combinations of pH and $\sum CO_2$. $\sum CO_2$ values between 1.7 and 10.2 mmol l^{-1} and pH values between 5.6 and 7.6 were used (N=64; P<0.0001, regression significance).

multiple regression was used to determine whether P_{CO_2} or bicarbonate (HCO_3^-) levels in the external medium had the most influence on internal $\sum CO_2$ values. It was apparent from this test that P_{CO_2} plays a greater role (P=0.0002) in predicting internal $\sum CO_2$ than does HCO_3^- (P=0.16). This implies that increased internal $\sum CO_2$ is primarily due to diffusion of CO_2 and not to HCO_3^- uptake.

To test further the roles of external P_{CO_2} and [HCO_3^-] in influencing internal $\sum CO_2$, we carried out experiments in which external $\sum CO_2$ was kept fairly constant (between 6 and 10 mmol l^{-1}) while pH was varied over a wide range (from 5.6 to 6.6), thus changing the external carbon dioxide/bicarbonate ratios. Fig. 4A shows that external P_{CO_2} does indeed play an important role in determining coelomic fluid $\sum CO_2$ levels, whereas [HCO_3^-] does not (Fig. 4B). In fact, the two appear to be inversely related. Thus, blood $\sum CO_2$ in these worms depended upon the P_{CO_2} of the surrounding water. Additionally, Fig. 5 shows that internal P_{CO_2} increases with increasing external P_{CO_2} for the entire data set of single time point experiments.

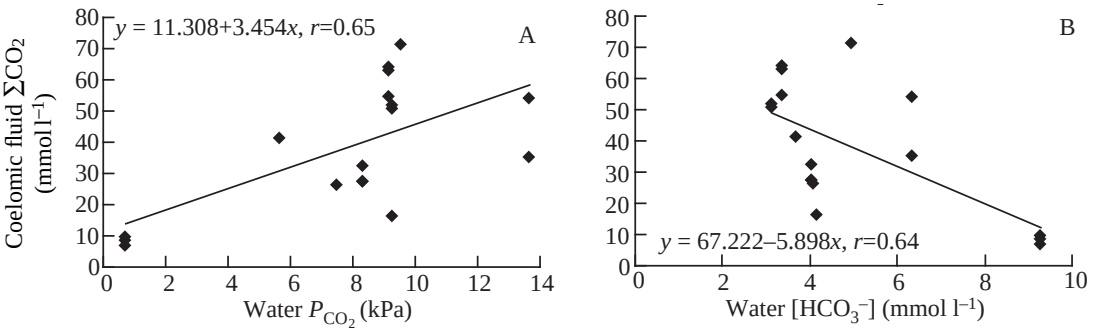

Fig. 4. Relationship between coelomic fluid total inorganic carbon, $\sum CO_2$, in the tubeworm *Riftia pachyptila* and the P_{CO_2} (A) and bicarbonate concentration [HCO_3^-] (B) of the surrounding water during nine single time point shipboard experiments. Worms were kept as stated in the legend to Fig. 3B. $\sum CO_2$ values between 5.6 and 10.2 mmol l^{-1} and pH values between 5.6 and 6.6 were used (N=16; for A and B; P<0.0001, regression significance for both plots).

Time course experiments

Control worms

Time course worms were first kept in maintenance vessels in surface sea water ($\sum CO_2$=2.1 mmol l^{-1}, P_{CO_2}=0.04 kPa, pH=8.2) until extracellular fluid $\sum CO_2$ was reduced to a low value (<5 mmol l^{-1}). Under these conditions, the worms showed a decrease in extracellular fluid $\sum CO_2$ over time (Fig. 6). During the first 25 h, coelomic fluid $\sum CO_2$ decreased from 50 to 19.6 mmol l^{-1} at a rate of 1.2 mmol l^{-1} h^{-1}.

When subsequently exposed to flowing water with an average $\sum CO_2$ of 4.8 mmol l^{-1} (range 4.2–5.5 mmol l^{-1}) and a mean P_{CO_2} of 6.2 kPa, the total extracellular fluid inorganic carbon of these worms increased (Fig. 7A). Between 0 and 7 h, the rate of increase in coelomic fluid $\sum CO_2$ concentration was 3.22 mmol l^{-1} h^{-1}, with $\sum CO_2$ leveling off after 7 h at a mean value of 27.8 mmol l^{-1} (Table 3). Vascular blood and coelomic fluid pH values, however, did not change over time of exposure (coelomic fluid shown in Fig. 7B).

To distinguish concentrating mechanisms from diffusion, worms were exposed to methane (a stable gas that is not metabolized by this symbiosis and does not dissociate to a charged form) at concentrations ranging from 0.1 to 0.3 mmol l^{-1}. Internal methane concentrations appeared to equilibrate with external concentrations between 4 and 7 h, after which coelomic fluid concentrations averaged 0.12 mmol l^{-1} and the mean water value was 0.13 mmol l^{-1} (Fig. 7C). Thus, no accumulation of methane above external levels was observed in either coelomic fluid or vascular blood, and the time to reach equilibrium was approximately the same as for CO_2.

One important question is the relationship between the vascular blood and coelomic fluid parameters over time, since the inorganic carbon is carried to the coelomic fluid in the vascular blood. As shown in Fig. 8, $\sum CO_2$ in the coelomic fluid closely tracks that in the vascular blood over time. For example, at 1 h, the coelomic fluid $\sum CO_2$ is 78 % of the vascular blood value and it rises to between 80 and 92 % of the vascular blood value between 2 and 5 h, reaching 100 % after 8 h. Thus, under conditions in which $\sum CO_2$ increased by approximately 600 %, coelomic fluid $\sum CO_2$ remained within approximately 20 % of the vascular blood $\sum CO_2$, even over shorter time periods. Coelomic fluid P_{CO_2} also tracks vascular blood P_{CO_2}, although with somewhat greater lag than is the

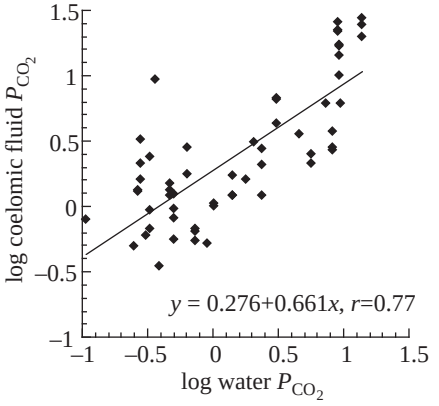

Fig. 5. Relationship between coelomic fluid P_{CO_2} (in kPa) in the tubeworm *Riftia pachyptila* and the P_{CO_2} (in kPa) of the surrounding water during 35 single time point shipboard experiments. Worms were kept under the conditions described in the legend to Fig. 3B (N=64; P<0.0001, regression significance).

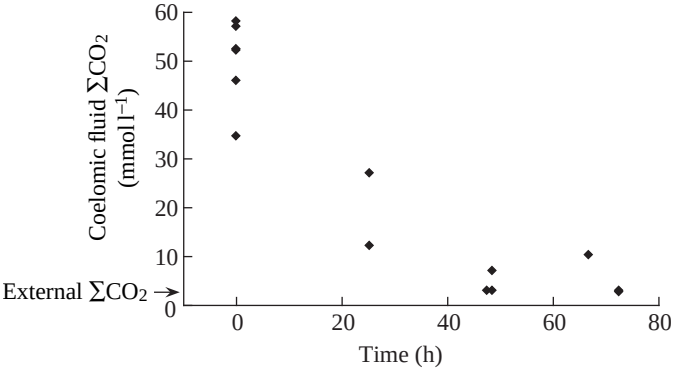

Fig. 6. Coelomic fluid total inorganic carbon, $\sum CO_2$, decreases over time for the vent tubeworm *Riftia pachyptila* kept in high-pressure flowing-water aquaria at 8 °C and 21.5 MPa and supplied with surface sea water (N=14). Time zero values are from freshly collected worms analyzed within 3–4 h of capture.

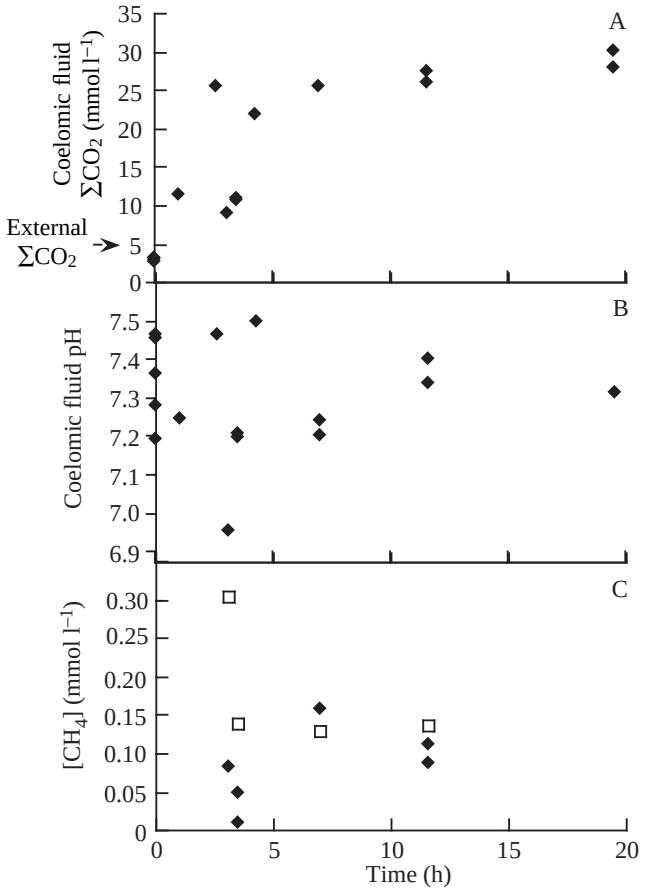

Fig. 7. Coelomic fluid total inorganic carbon, $\sum CO_2$ (A), pH (B) and $[CH_4]$ (C) over time for the vent tubeworm *Riftia pachyptila* exposed to external $\sum CO_2$ concentrations of approximately 5 mmol l^{-1}, with a P_{CO_2} of 6.2 kPa and a pH between 5.9 and 6.2 (N=15). In C, filled symbols represent coelomic fluid $[CH_4]$ while open symbols represent external medium $[CH_4]$.

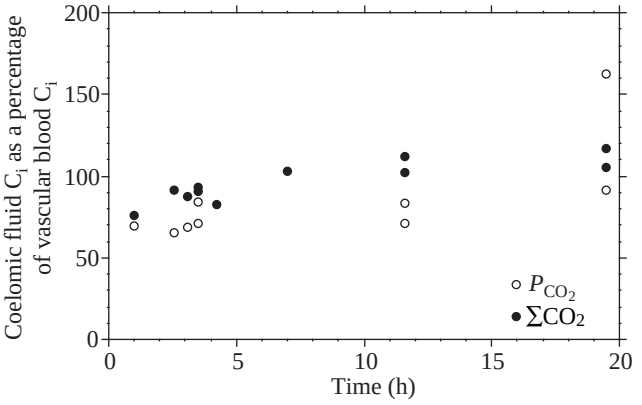

Fig. 8. Coelomic fluid inorganic carbon (C_i) as a percentage of vascular blood inorganic carbon over time for the vent tubeworm *Riftia pachyptila* exposed to external total inorganic carbon, $\sum CO_2$, concentrations of approximately 5 mmol l^{-1}, with a P_{CO_2} of 6.2 kPa and a pH between 5.9 and 6.2. Coelomic fluid and vascular blood inorganic carbon is shown as both P_{CO_2} (open symbols; N=9) and $\sum CO_2$ (filled symbols; N=11).

Table 3. *Effects of* N*-ethylmaleimide on acid–base parameters of the hydrothermal vent tubeworm* Riftia pachyptila

	Control	NEM-inhibited	*P* value
pH	7.27±0.07	6.90±0.06	0.0012
Rate of $\sum CO_2$ accumulation (mmol l^{-1} h^{-1})	3.22	2.20	N/A
$\sum CO_2$ plateau (mmol l^{-1})	27.80±0.70	17.10±1.80	0.0105
Coelomic fluid P_{CO_2} (kPa)	4.52±0.96	5.27±0.38	0.2482
Water P_{CO_2} (kPa)	6.42±1.17	6.58±0.02	0.9999
Final coelomic fluid P_{CO_2}/ water P_{CO_2}	0.71±0.08	0.80±0.06	0.3836

Mean ± S.E.M. coelomic fluid pH, rate of $\sum CO_2$ accumulation, $\sum CO_2$ plateau, coelomic fluid P_{CO_2} water P_{CO_2} and final coelomic fluid P_{CO_2}/water P_{CO_2} ratios for control (N=15) and NEM-inhibited (N=9) tubeworms *Riftia pachyptila*, kept in high-pressure flowing water aquaria and exposed to external $\sum CO_2$ concentrations between 4.6 and 5.5 mmol l^{-1} with a P_{CO_2} of 6.2 kPa and a pH between 5.9 and 6.2.

Inhibited worms were exposed to *N*-ethylmaleimide (NEM) at concentrations between 1.1 and 1.6 mmol l^{-1} for approximately 1–2 h.

P values are from the Mann–Whitney *U*-test.

NA, not applicable.

case for $\sum CO_2$, which primarily consists of HCO_3^-. The tendency for coelomic fluid P_{CO_2} to be somewhat lower than vascular blood P_{CO_2} is also found in the single time point data (Table 1). The rapid exchange of HCO_3^- between the vascular and coelomic compartments indicates that there is intimate contact between them and that the coelomic fluid is not a stagnant pool, but is effectively mixed.

Inhibited worms

Worms exposed to NEM were exposed to the same water conditions as the uninhibited (control) worms described in the previous section. However, these worms did not accumulate blood $\sum CO_2$ as rapidly when exposed to elevated $\sum CO_2$ (2.2 mmol l^{-1} h^{-1}; different from control worms in magnitude, P=0.0651, ANCOVA; Table 4) and $\sum CO_2$ did not reach the same level (17.1 mmol l^{-1}; Table 3). NEM-inhibited worms also had significantly lower coelomic fluid pH values than did control worms (P=0.0012, Mann–Whitney U-test; Table 3). This could reflect the action of NEM in preventing extracellular pH control by inhibiting ATP-dependent proton-equivalent ion transport. The coelomic fluid P_{CO_2} *versus* water P_{CO_2} ratios for control and NEM-inhibited worms did not differ significantly from each other over time (P=0.39, Mann–Whitney U-test; Table 3). Both groups approached coelomic fluid P_{CO_2}/water P_{CO_2} ratios slightly less than unity during the experiments, providing evidence that CO_2 diffusion is the primary mode of inorganic carbon uptake. Inhibition by NEM had no effect on internal methane levels (data not shown).

Table 4. *Rate of coelomic fluid $\sum CO_2$ increase for the tubeworm* Riftia pachyptila *during the first 4 h of time course experiments*

Group	*N*	Rate of $\sum CO_2$ increase (mmol l^{-1} h^{-1})	ANCOVA magnitude, *P* value	ANCOVA slope, *P* value
Control	7	3.2	–	–
NEM	5	2.2	0.0651	0.7964
Sulfanilamide	9	3.2	0.8876	0.9219
SITS	4	4.1	0.7206	0.3676

Worms were kept under the conditions stated in Table 3.

Worms were exposed to various inhibitors for approximately 1–2 h. Specific inhibitors included NEM, at concentrations between 1.1 and 1.6 mmol l^{-1}, sulfanilamide, an inhibitor of carbonic anhydrase, at concentrations between 1 and 1.4 mmol l^{-1}, and SITS, an anion exchange inhibitor, at a concentration of 0.2 mmol l^{-1}.

P values are from the ANCOVA test, to test for differences in slope from the slope for control worms.

Table 5. *Effect of various inhibitors on coelomic fluid pH of the tubeworm* Riftia pachyptila *at times between 5 and 20 h in time course experiments*

Group	*N*	Coelomic fluid pH	*P* value
Control	15	7.27±0.07	–
NEM	9	6.90±0.04	0.0012
Sulfanilamide	11	7.11±0.05	0.7598
DIDS	4	7.24±0.12	0.8578
DCCD	4	7.18 ±0.18	0.4202
SITS	4	7.32±0.19	0.4737

Worms were kept under the conditions stated in Table 3.

Worms were exposed to various inhibitors for approximately 1–2 h. Specific inhibitors included NEM, sulfanilamide, SITS, DIDS, another anion exchange inhibitor, at a concentration of 0.08 mmol l^{-1}, and DCCD, an inhibitor of H^+-ATPase, at a concentration of 1.2 mmol l^{-1}.

P values are from the Mann–Whitney *U*-test, to test for differences from control worms.

Sulfanilamide exposure resulted in no significant change in coelomic fluid $\sum CO_2$, pH or P_{CO_2} values. Sulfanilamide-exposed worms showed a rate of $\sum CO_2$ increase of 3.2 mmol l^{-1} h^{-1}, and this did not differ significantly in magnitude or slope from that of the control worms (P=0.89 and 0.92, respectively, ANCOVA; Table 4). They also had a coelomic fluid pH of 7.1, which was not significantly different from the pH of the controls (P=0.76, Mann–Whitney *U*-test; Table 5). Exposure to this inhibitor had no effect on coelomic fluid P_{CO_2}/water P_{CO_2} ratios over time (after leveling off, the control ratio was 0.71±0.08 and the ratio for worms in the presence of sulfanilamide was 0.71±0.03; P=1, Mann–Whitney *U*-test). Trophosome and plume samples analyzed for carbonic anhydrase activity showed no significant difference between tissues taken from control worms and tissue from worms exposed to sulfanilimide (P>1 and P=0.48 for trophosome and plume, respectively, Mann–Whitney *U*-test), indicating that the inhibitor was unable to reach the carbonic anhydrase in the tissues.

Exposure to SITS also failed to affect the coelomic fluid $\sum CO_2$ significantly or to increase pH (Tables 4, 5). While there were not enough short time period measurements taken for DIDS-exposed worms to determine differences in the coelomic fluid $\sum CO_2$ increase, no significant difference in coelomic fluid pH resulted from exposure to this inhibitor (P=0.86, Mann–Whitney *U*-test; Table 5). For DCCD-exposed worms, there was too much variation between individuals to calculate an accurate rate of coelomic fluid $\sum CO_2$ increase; however, the coelomic fluid pH of these worms did not differ significantly from that of the control worms (P=0.42, Mann–Whitney *U*-test; Table 5).

Discussion

CO_2 acquisition

The tubeworm *Riftia pachyptila* has an unusual mode of existence in that it relies entirely upon chemoautotrophic symbionts for its nutrition (Childress and Fisher, 1992). In exchange for organic carbon compounds produced by the bacteria, the worms must supply the symbionts with carbon dioxide. In previous research, vascular blood and coelomic fluid $\sum CO_2$ and P_{CO_2} of freshly collected worms have been shown to be high (maxima of 31 mmol l^{-1} $\sum CO_2$ and 4.1 kPa P_{CO_2}) and to reflect elevated P_{CO_2} and $\sum CO_2$ values around the worms. During the present study, the highest values measured for freshly collected worms were 21.1 kPa for coelomic fluid P_{CO_2} and 12.6 kPa for the P_{CO_2} in the water surrounding the worm tubes (these samples do not correspond exactly in location of sampling). Thus, P_{CO_2} values reported here are substantially higher than those found in our previous study for both the worms and surrounding water, supporting the contention that the worms are exposed to high P_{CO_2} *in situ*. The elevated P_{CO_2} *in situ* is partially due to somewhat elevated $\sum CO_2$ but is primarily a result of low pH values around the worms.

P_{CO_2} varies greatly from site to site because of variations in the pH and $\sum CO_2$ of the water surrounding the worms as a result of the mixing of hot, acidic, CO_2-rich vent water with cold, alkaline CO_2-poor deep sea water. The hot ‘source’ water characterizes the vent site as a whole, but the worms do not actually come into contact with it. There are large variations in the inorganic carbon conditions of the warm water, which is in direct contact with the worms. However, sites with higher hot water $\sum CO_2$ values typically have higher warm water values, and the freshly caught worms from these sites have higher internal $\sum CO_2$ values. Thus, the values of pH and $\sum CO_2$ in the hot vent water are apparently the strongest determinants of the exposure of a particular worm to elevated P_{CO_2}, although there is considerable variation in blood parameters, indicating microhabitat variation. Regardless of variation among vent sites, it is apparent that all sites investigated have substantially elevated P_{CO_2} and $\sum CO_2$ conditions.

It was proposed previously that elevated $\sum CO_2$ inside the worms could be the result of CO_2 diffusion, HCO_3^- uptake or both. We found that elevated external P_{CO_2} values resulted in increased internal $\sum CO_2$ and P_{CO_2} in the vascular blood and coelomic fluid of the experimental worms, but that elevated levels of external bicarbonate had no significant effect. These findings support a model in which CO_2 diffusion, rather than bicarbonate uptake, is the mechanism for inorganic carbon acquisition. They also demonstrate that the worms are able to concentrate $\sum CO_2$ internally as a result of their ability to maintain a more alkaline blood pH than that of the medium.

Worms exposed to appreciable levels of methane showed no internal accumulation of methane above external concentrations. It is clear that $\sum CO_2$ acquisition by *R. pachyptila* is different from that for methane, in that *R. pachyptila* can concentrate internal $\sum CO_2$ far above external values. The high P_{CO_2} at the vent sites provides a large external pool of CO_2, driving CO_2 diffusion across the plume, thus increasing the amount of CO_2 available to the symbionts. High concentrations of carbonic anhydrase in the plume and the alkaline pH inside the worm relative to outside apparently further enhance this diffusion by quickly converting CO_2 to bicarbonate (Childress *et al.* 1993; Kochevar *et al.* 1993). It was proposed that exposure to sulfanilamide would decrease internal $\sum CO_2$ because of a decrease in carbonic anhydrase activity. Because this enzyme is responsible for the hydration of carbon dioxide, speeding its conversion to bicarbonate and protons, and thus the enhancement of the CO_2 gradient into the worms, a decrease in carbonic anhydrase activity could have lowered the rate of diffusion of CO_2 into the worm and thus lowered the internal $\sum CO_2$ values. The lack of a significant decrease in internal $\sum CO_2$ in sulfanilamide-treated worms is apparently a result of the carbonic anhydrase associated with the plume being intracellular in location, and thus inaccessible to the inhibitor, rather than being on the surface, which is consistent with the results of Kochevar (1992). Similarly, the lack of effect of DIDS or SITS on internal $\sum CO_2$ and pH values suggests that no anion exchanger is present at the gill–water interface (apical membrane), but does not exclude their existence at the gill–blood interface (basolateral membrane).

Proton-equivalent ion transport

There are a number of biological processes that occur within the worms which release protons, such as the conversion of CO_2 into H^+ and HCO_3^-, and sulfide oxidation by the symbionts, resulting in the production of H^+ and SO_4^{2-}. Despite this, the worms maintain a stable extracellular pH at a value higher than that of the surrounding medium. This maintenance of an extracellular pH of approximately 7.5 must be accomplished by proton-equivalent ion export by the animal. Although the specific mechanism is still unknown, we did see dramatic effects on $\sum CO_2$ concentrations within the worms when they were unable to regulate their internal environment to the proper pH. Exposure to NEM resulted in a lowering of the coelomic fluid pH from 7.3 to 6.9 as CO_2 was taken up. On the basis of the pH values measured in NEM-exposed worms, we believe that NEM acted on H^+-ATPases. Although this is not direct evidence of an H^+-ATPase, we believe that the existence of one (or several) is very likely considering that NEM does inhibit H^+-ATPases (Lin and Randall, 1993), that we did see a dramatic decrease in extracellular pH in NEM-inhibited worms, and that high fluxes of proton equivalents are required to regulate pH in the face of large influxes of CO_2. Thus, NEM-inhibited worms were apparently unable to carry out proton-equivalent ion transport, indicating that regulation of body fluid pH across a wide range of internal $\sum CO_2$ levels occurs through proton-equivalent ion transport *via* an ATP-requiring process.

Although body fluid pH in the NEM-inhibited worms decreased, the relationship between coelomic fluid P_{CO_2} and water P_{CO_2} remained unaffected. In this case, a decrease in coelomic fluid pH resulted in a decrease in coelomic fluid $\sum CO_2$, leaving the P_{CO_2} slightly lower than the environmental P_{CO_2}. Thus, the worms maintained coelomic fluid P_{CO_2}/water P_{CO_2} ratios at slightly less than unity, which allowed for a CO_2 diffusion gradient favoring diffusion into the worm. This further indicates that diffusive CO_2 entry is the method of inorganic carbon acquisition in these worms, rather than some form of mediated HCO_3^- uptake. The lack of significant changes in blood gas parameters upon exposure to DCCD, DIDS and SITS is attributed to the lack of surface penetration by these chemicals (Brazy and Dennis, 1981; Cabantchik and Greger, 1992). In studies on other organisms, these inhibitors have apparently penetrated membranes (Al-Moghrabi *et al.* 1996); however, it is quite possible that *R. pachyptila* membranes are relatively impermeable to many substances as an adaptation to the poisonous nature of the environment in which they live.

While CO_2 transport at the water/epithelium interface is accomplished by diffusion of dissolved CO_2, as shown by the present results, an intracellular role for carbonic anhydrase is likely, given its high concentrations, and the involvement of bicarbonate transporter systems at the epithelium/blood boundary and/or at the bacteriocyte interface cannot be ruled out. The intracellular pH values within the plume cells and bacteriocytes, although yet unmeasured, are also critical aspects of the mechanism of inorganic carbon uptake and transport in *R. pachyptila*.

Comparison with other animals

In comparison to other animals, including worms, animal hosts of algal symbionts and animals exposed to hypercapnic conditions, it is apparent that the pH regulation seen in *R. pachyptila* is unprecedented. A study investigating the hydrogen ion activity in various polychaete species reported that body fluid pH ranged from 6.84 to 7.44 in 25 species (Wells, 1974). Considering that *R. pachyptila* lives at an environmental pH of approximately 6.0, it is notable that it maintains such a large pH gradient between itself and its environment. Compared with other host–symbiont

Table 6. *Comparison of internal pH changes in various animals exposed to high levels of CO_2*

Animal	Initial P_{CO_2} (kPa)	Elevated P_{CO_2} (kPa)	pH decrease	Time for pH compensation (h)	% pH compensation	Reference
Callinectes sapidus	0.53	1.33	0.3–0.4	24	65	Cameron (1989)
(blue crab)	0.53	6.00	0.4	ND	ND	Cameron and Iwama (1987)
Squalus acanthias	0.04*	1.06–1.33	0.5	24–36	Within 0.1 pH units	Claiborne and Evans (1992)
(spiny dogfish)						
Conger conger	0.08	1.09	0.4	10	90	Toews *et al.* (1983)
(a marine teleost)						
Riftia pachyptila	0.04	6.20	No change	NA	100	Present study
(a vent tubeworm)						

Initial P_{CO_2} is the P_{CO_2} at which the animals started, and elevated P_{CO_2} is the P_{CO_2} to which they were exposed for the duration of the experiment.

In all animals, the initial internal pH decrease was eventually partially compensated for by an increase in internal [HCO_3^-], resulting in some internal pH compensation.

ND, no data; NA, not applicable (for *Riftia pachyptila* there was no initial pH decrease and, thus, no time for pH compensation).

*Assumed value.

relationships, it appears that *R. pachyptila* is also unusual in its pH-regulating abilities. For example, the giant clam *Tridacna gigas*, which plays host to algal symbionts, has a hemolymph pH that varies by ±0.8 pH units throughout the day. When the inorganic carbon concentrations change in the host as a result of photosynthesis by the algae, the clam cannot regulate its pH accordingly, with hemolymph pH reaching 8.1 at mid-day and falling to 7.2 at night (Fitt *et al.* 1995). In contrast, *R. pachyptila*, despite numerous bacterial and chemical processes affecting the internal carbon concentrations, maintains its extracellular pH constant and alkaline with respect to the external environment.

Another useful comparison can be made by surveying the responses of marine animals to hypercapnia. Most studies that have dealt with the effects of elevated external P_{CO_2} on the extracellular acid–base balance in marine animals concern crustaceans or fishes (Truchot, 1987). In these cases, CO_2 diffuses into the animals and causes a decrease in extracellular fluid pH, which is later partially compensated for by an increase in internal HCO_3^- levels, presumably *via* Cl^-/HCO_3^- exchange or H^+ export (Table 6) (Toews *et al.* 1983; Cameron and Iwama, 1987; Cameron, 1989; Claiborne and Evans, 1992). For many animals, this initial pH decrease is immediate (within 1 h) and the time required for compensation is 10–36 h. In *R. pachyptila*, however, even a 160-fold increase in external P_{CO_2} results in no change in coelomic fluid (or vascular blood) pH (Fig. 7B; Table 6). Thus, although the response of *R. pachyptila* to elevated P_{CO_2} may be similar to the response of non-symbiotic animals to hypercapnia, the rate of pH regulation is extraordinarily high and the compensation is unusually complete.

We propose a model for inorganic carbon uptake in *R. pachyptila* in which CO_2 diffuses across the outer epithelium of the plume as a result of high external P_{CO_2} caused by the low environmental pH and elevated environmental ΣCO_2. Once in the plume cell, CO_2 concentration is brought to steady state with HCO_3^- through catalysis by carbonic anhydrase. The excess protons are then eliminated *via* a proton pumping mechanism to maintain a relatively alkaline extracellular pH. This mechanism functions at extremely high rates, which concentrates inorganic carbon at the alkaline pH inside the worms and appears to be a further specialization of *R. pachyptila* for supporting autotrophic endosymbionts. It is possible that the acquisition of inorganic sulfide also occurs through diffusion of the undissociated species, H_2S, which could be concentrated *via* the same mechanisms.

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