

Hypotaurine and thiotaurine as indicators of sulfide exposure in bivalves and vestimentiferans  
from hydrothermal vents and cold seeps

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Running head: Hypotaurine and thiotaurine in sulfide exposure

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## Abstract

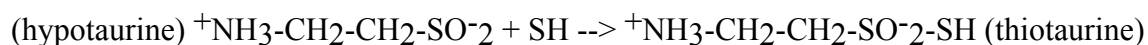
Vesicomid clams, vestimentiferans, and some bathymodiolin mussels from hydrothermal vents and cold seeps possess thiotrophic endosymbionts, high levels of hypotaurine and, in tissues with symbionts, thiotaurine. The latter, a product of hypotaurine and sulfide, may store and/or transport sulfide non-toxically, and the ratio to hypotaurine plus thiotaurine ( $Th/[H+Th]$ ) may reflect an animal's sulfide exposure. To test this, we analyzed seep and vent animals with in situ sulfide measurements. *Calyptogena kilmeri* clams occur at high-sulfide seeps in Monterey Canyon, while *C. (Vesicomya) pacifica* clams occur at seeps with lower levels but take up and metabolize sulfide more effectively. From one seep where they co-occur, both had gill thiotaurine

contents at 22-25 mmol/kg wet mass, and while *C. (V.) pacifica* had a higher blood sulfide level, it had a lower Th/[H+Th] (0.39) than *C. kilmeri* (0.63). However, these same species from different seeps with lower sulfide exposures had lower ratios. *Bathymodiolus thermophilus* (East Pacific Rise [EPR 9°50'N]) from high- (84  $\mu$ M) and a low- (7  $\mu$ M) sulfide vents had gill ratios of 0.40 and 0.12, respectively. Trophosomes of *Riftia pachyptila* (EPR 9°50'N) from medium- (33  $\mu$ M) and low- (4  $\mu$ M) sulfide vents had ratios of 0.23 and 0.20, respectively (not significantly different). *Ridgeia piscesae* vestimentiferans (Juan de Fuca Ridge) have very different phenotypes at high- and low-sulfide sites, and their trophosomes had the greatest differences: 0.81 and 0.04 ratios from high- and low-sulfide sites, respectively. Thus Th/[H+Th] may indicate sulfide exposure levels within species, but not in interspecies comparisons, possibly due to phylogenetic and metabolic differences. Total H+Th was constant within each species (except in *R. piscesae*); the sum may indicate the maximum potential sulfide load that a species faces.

## Problem

Certain animals around marine hydrothermal vents and cold seeps have formed a symbiotic relationship with chemosynthetic microbes. In particular, vesicomid clams, vestimentiferans, and some bathymodiolin mussels take up hydrogen sulfide from vent or seep emissions for thiotrophic endosymbionts. These endosymbionts oxidize the sulfide ultimately to drive organic carbon fixation (Van Dover & Fry 1994). However, hydrogen sulfide is toxic to animals because it can bind to iron and disrupt mitochondrial function (Fisher 1990). Some of these animals have special sulfide binding proteins (e.g., modified hemoglobins) in their blood or hemolymph for transporting sulfide non-toxically from the environment to the symbionts (Arp *et al.* 1987; Childress *et al.* 1993; Kraus 1995; Zal *et al.* 2000). However, these proteins do not transport sulfide within cells (with the exception of intracellular hemoglobins reported in *Solemya* clams: Kraus *et al.* 1996), and other mechanisms of intracellular defense against sulfide have been proposed. For example, there are specialized sulfide-oxidizing organelles in epidermal tissue in some polychaetes including vestimentiferans (Menon *et al.* 2003). In some species such as the vent mussel *Bathymodiolus thermophilus*, sulfide is rapidly converted to thiosulfate as a means of detoxification (Powell & Somero 1986). Elemental sulfur has also been found in many of these animals; it has been proposed to be a non-toxic energy reserve produced by the bacteria (Vetter 1985; Arndt *et al.* 2001).

In recent years, another mechanism involving thiotaurine and hypotaurine has been proposed to serve in protection from and/or transport of sulfide (Alberic & Boulegue 1990; Pranal *et al.* 1995; Pruski *et al.* 2000b). These unusual amino acids can be found at high levels in many vestimentiferans and bivalves from vents and seeps (Alberic 1986; Pranal *et al.* 1995; Pruski *et al.* 2000a). They are generally found only at low levels in hemolymph or blood, and thus are assumed to be primarily intracellular (Pruski *et al.* 2000a, Yin *et al.* 2000). In tissues with endosymbionts, hypotaurine appears to react with sulfide to produce thiotaurine (Pruski *et al.* 2000b):



The source of SH could be a free radical, a ligand donated by a carrier protein R as R-SSH, or a group donated by glutathione-SSH or other thiols. Synthesis of thiotaurine appears to be enzymatic (Pruski & Fiala-Medioni 2003). The reaction is reversible, and might serve to store sulfide non-toxically within cells, then release it as the endosymbionts deplete free sulfide. This

would be advantageous as sulfide concentration surrounding the animals can vary (Barry *et al.* 1996; Shank *et al.* 1998).

As evidence for the linkage between the thiotaurine reaction and symbiosis, hypotaurine is high in all tissues in these animals but thiotaurine occurs at high levels only in thiotrophic symbiont-bearing tissues, namely gills in some bivalves and trophosomes in vestimentiferans (Pruski *et al.* 2000a; Fiess *et al.* 2002). As a contrasting example, thiotaurine is very low in gills of methanotrophic mussels from cold seeps (Pruski *et al.* 2000b). The ratio of thiotaurine to total hypotaurine plus thiotaurine ( $\text{Th}/[\text{H}+\text{Th}]$ ) has been proposed to be an indicator of the level of sulfide exposure in these animals (Pranal *et al.* 1995) and thiotaurine has been proposed to be a general marker of thiotrophic endosymbiosis (Pruski *et al.* 2000b). Indeed, the ratio tends to be higher in animals with higher environmental exposures to sulfide (Pruski *et al.* 2000a). Also, laboratory studies show that thiotaurine contents increase during sulfide exposure in symbiont-bearing tissues of vesicomyids, bathymodiolins, vestimentiferans (Pruski & Fiala-Médioni 2003) and shallow-living solemyid clams (Joyner *et al.* 2003).

The role of thiotaurine has recently been expanded by the discovery that two species of gastropods from hydrothermal vents contain substantial levels of thiotaurine (Rosenberg *et al.* 2006), even though they lack endosymbionts. Nevertheless, the  $\text{Th}/[\text{H}+\text{Th}]$  ratio decreased in gastropods held in the lab with low or no sulfide while it stayed high in individuals held with high sulfide. These gastropods may be exposed to sulfide as a result of grazing on vent bacteria, and the thiotaurine finding supports the idea that this solute is used for sulfide detoxification and not necessarily for symbiosis.

The question addressed here is whether thiotaurine is made by vent and seep animals in situ in proportion to sulfide exposure, and thus whether an estimate of in situ sulfide exposure can be made on field-collected samples by analyzing thiotaurine and hypotaurine contents (which can be easier to measure than sulfide). To date, the best correlations between sulfide exposure and thiotaurine have come from laboratory studies; correlations between amino acids and sulfide levels in situ have only been made with specimens not from the same time or site as the sulfide measurements. Here we have analyzed these amino acids in a variety of vent and seep animals (originally collected for other studies) most of which had associated ambient sulfide values (and blood/hemolymph measurements for some).

First, the vesicomid seep clams *Calypptogena/Vesicomya pacifica*, *C. kilmeri*, and *Ectengena extenta* were analyzed. *C. (V.) pacifica* and *C. kilmeri* are the dominant vesicomids at many cold seeps in Monterey Canyon off California. The two can be found living together, though one species dominates at certain locations; *C. kilmeri* prefers areas of high sulfide in the sediment, generally 4-18 mM, whereas *C. (V.) pacifica* is found in areas with lower sulfide levels, from trace levels to 4 mM (Barry *et al.* 1996, 1997). These differences are attributed to physiological and biochemical adaptations that give *C. (V.) pacifica* a much higher efficiency in both the uptake of sulfide through the foot and the oxidation of sulfide in the gills (Goffredi *et al.* 2002). *C. kilmeri* in contrast has lower uptake and oxidation activity, presumably reflecting its need for higher sulfide environments. Specimens of both species were obtained from the same seep and from different seeps, along with measurements of sulfide levels in the sediment and/or the hemolymph (blood) (Goffredi *et al.* 2002).

*Ectengena extenta*, closely related to *C. kilmeri* according to molecular analysis (Peek *et al.* 1998), was collected at deep seeps (3200 m) in Monterey Canyon. Its sulfide exposure is unknown, but its gills have much higher levels of elemental sulfur (S<sup>0</sup>) than the other vesicomids (Goffredi *et al.* 2004). Elemental sulfur has been proposed to be an energy reserve (as noted earlier), produced aerobically and then possibly used during anoxia (Vetter 1985; Arndt *et al.* 2001); it may be particularly important in animals most frequently experiencing the anoxia associated with sulfide-laden waters.

The second type of animal analyzed was the bathymodiolin mussel *Bathymodiolus thermophilus*, collected from two different locations within a hydrothermal vent site on the East Pacific Rise. Precise sulfide measurements in the water were also made near the animals. Bathymodiolins can have one or both of two different symbionts that specialize on either sulfide or methane for chemosynthesis (Distel *et al.* 1995), although in previous work only the thiotrophic symbiont was found in *B. thermophilus* (Fisher *et al.* 1987). The mussels can also obtain nutrients through filter feeding (Page *et al.* 1991); thus they may have lower overall sulfide usage, which could explain their relatively low hypotaurine and thiotaurine contents (Pruski *et al.* 2000b).

Vestimentiferans were the final type of symbiotic animal analyzed. Specimens of the giant tubeworm *Riftia pachyptila* were collected from two very different vent sites on the East Pacific Rise (Govenar *et al.* 2005); sulfide measurements were made in the water near their plumes and

at the base of their tubes. *Ridgeia piscesae* vestimentiferans were taken from two very different habitats on the Juan de Fuca Ridge. This tubeworm can exhibit very distinct phenotypes, depending on the local habitat and possibly sulfide-acquisition strategies. Although sulfide levels were not available for the *Ridgeia* specimens used here, the two collection sites are known to have very different sulfide levels (Urcuyo *et al.* 2003). Animals at diffuse-flow basalt sites are exposed to low sulfide and generally have a characteristic "long-skinny" (LS) morphology, while animals from a high-sulfide chimney have a "short-fat" (SF) body (Robigou *et al.* 1993; Sarrazin *et al.* 1997; Urcuyo *et al.* 1998; 2003). The morphologies are so different that they were once considered to be separate species, but genomic analyses have revealed them to be the same (Southward *et al.* 1995, 1996; Black *et al.* 1997, 1998; Carney *et al.* 2002). Both LS and SF types were used in this study.

Thiourine and hypourine are closely related to taurine, an amino acid often found as a major organic osmolyte in shallow-water invertebrates. Like all osmoconforming marine invertebrates, cells of polychaetes and mollusks accumulate free amino acids, methylamines and sometimes polyols to equalize their cellular osmotic pressures with that of the extracellular fluid and seawater. These organic osmolytes often have other cytoprotective functions beyond providing osmotic balance, and vary in composition in correlation with metabolic adaptations, temperature, and ocean depth (Yancey *et al.* 2002; Yancey 2005). Taurine contents, for example, decline with ocean depth (at least in bivalves, gastropods and anthozoans; Pruski *et al.* 2000a; Fiess *et al.* 2002; Yancey *et al.* 2002, 2004; Rosenberg *et al.* 2006). In general taurine levels have not been found to shift with changing sulfide exposure, but Joyner *et al.* (2003) recently found changes in taurine as well as hypourine and thiourine in shallow symbiotic clams. Therefore, contents of taurine, as well as other major sulfur-containing organic solutes, were also measured in all specimens to determine whether there were any trends.

## **Study Areas**

Animals were collected from, and sulfide measurements were made at 1) Monterey Canyon (California) cold seeps, including Clam Field (36° 44.0' N, 122° 2.0' W; 950 m depth) on January 5, 1999, Invert Cliff (1000 m) on September 21, 1999, and Crescent Slump (3200 m) on November 3, 2000, 2) Eel River cold seeps off Eureka, California (510-520 m depth), on April 29, 2001, 3) hydrothermal vents (2500-2550 m depth) at the Mussel Bed (December 10 to 12,

2002), Tica (December 1 to 14, 2002) and Riftia Fields (December 1 to 11, 2002) sites in the 9°50' N area of the East-Pacific Rise (EPR), and 4) the Main Endeavour Field of the Juan de Fuca Ridge on May 12, 2001, at the Clam Bed site (47° 57'N, 129° 05'W; 2194 m depth) and on May 10, 2001, at the Smoke and Mirrors sulfide edifice called Strawberry Fields (47° 56'N, 128° 05'W; 2184 m depth).

## **Materials and Methods**

### *Specimen Collection*

Specimens were collected using various remotely operated vehicles (ROVs) from several locations, most originally for other studies. *Calyptogenia (Vesicomya) pacifica* were collected at Clam Field (Monterey Canyon) and Eel River seeps, and *C. kilmeri* from Clam Field and Invert Cliff (Monterey). *Ectengena extenta* were from the Crescent Slump (Monterey) seeps. *Riftia pachytila* were collected at Tica and Riftia Field vents (9°50' N EPR). *Bathymodiolus thermophilus* were collected at two different locations in the Mussel Bed vents (9°50' N EPR). *Ridgeia piscesae* were collected from Main Endeavour Field of the Juan de Fuca Ridge. Those from the low-sulfide Clam Bed site had the LS morphology, while those from the high-sulfide Strawberry Fields edifice had the SF morphology (see Problem section).

Some animals were immediately frozen on the ship, while others were kept alive in chilled (4° C) seawater on the ship, then later were either frozen or dissected for tissues that were immediately frozen. Freezing was at -70° C or on dry ice on the ship, followed by storage at -70 to -80° C in the laboratory.

### *Sulfide analysis*

For *C. (V.) pacifica* and *C. kilmeri* from Monterey Canyon, sulfide levels ( $\Sigma\text{H}_2\text{S}$ ) in the sediment pore water and in their hemolymphs were measured in a previous study by gas chromatography (for the same specimens used here) (Goffredi *et al.* 2002). For *pacifica* specimens from Eel River seeps, sulfide levels in sediment pore water at the collection site were determined by W. Ziebis using an amperometric sensor (published in Levin *et al.* 2003). Total concentrations of acid volatile sulfide in the environment of *R. pachytila* and *B. thermophilus* were determined in situ as described in Le Bris *et al.* (2006), using the submersible flow analyzer ALCHIMIST installed on the DSRV *Alvin*. Large spatial gradients were observed at the scale of tubeworms (Le Bris *et*

*al.* 2006). Only the measurements obtained at the level of plumes were considered for this study, as the most relevant of sulfide exposure.

### *Amino Acid Analysis*

Samples were shipped on dry ice to Whitman College where they were stored at -70°C until analysis. Frozen samples were weighed then rapidly homogenized in a glass dounce on ice in 1 ml of 70% cold ethanol, and kept on ice for at least 3 hours before centrifugation at 15000 x g for 20 minutes to remove proteins. Ethanol precipitation of unwanted proteins was used rather than the more common acid precipitation because acid degrades thiotaurine (Pruski *et al.* 2000b). Supernatants were dried overnight in a vacuum centrifuge then resuspended in 500 to 1000 µl of purified water. All samples were passed through C-18 delipidizing cartridges (Varian, Inc.) and 0.45 micron filters (Millipore, Inc.) as described by Wolff *et al.* (1989). Samples were then analyzed for amino acids using high performance liquid chromatography (HPLC) as previously described (Wolff *et al.* 1989; Yin *et al.* 2000). Thiotaurine standard was synthesized by the method of Cavalini *et al.* (1963).

### *Statistical Analysis*

Data are presented as means ± S.D. Statistical significance ( $P < 0.5$ ) was determined using Student's t-tests or ANOVA with Student-Newmann-Keuls post-tests. Arcsin conversions were used for statistics of ratios.

## **Results**

### *Vesicomysids: Thiotaurine and Hypotaurine*

Thiotaurine was found at substantial levels in vesicomysid gills, with values ranging from about 6.9 to 70 mmol/kg wet mass (Table 1, top rows). For *C. (V.) pacifica*, specimens from the site (Clam Field) with 4 mM ambient sulfide were compared to ones from the site (Eel River) with ≤ 2 mM ambient sulfide (Table 1). The former had higher thiotaurine (22.3 vs. 6.9) and lower hypotaurine (36.6 vs. 56.6), and thus a higher Th/[H+Th] ratio (0.39 vs 0.11). For *C. kilmeri*, specimens from the site (Clam Field) with 4 mM ambient sulfide and average of 1.1 mM blood (hemolymph) levels were compared to ones from the site (Invert Cliff) with higher (7-9 mM) ambient sulfide but lower blood levels (about 0.6 mM) (Table 1). The former had higher

Table 1 here
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thiourine (25.2 vs 13.1) and lower hypourine (14.5 vs 21.8), and thus a higher Th/[H+Th] ratio (0.63 vs 0.39) (Table 1 and Fig. 1).

Interspecies comparisons yielded a different pattern for *C. (V.) pacifica* and *C. kilmeri* from the same site (Clam Field seep). *C. (V.) pacifica* had significantly higher blood levels of sulfide than *C. kilmeri* (2.7 vs 1.1 mM), yet the two species had statistically the same thiourine levels at 22.3 and 25.1 mmol/kg, respectively (Table 1). Moreover, *C. (V.) pacifica* had a lower Th/[H+Th] ratio than *C. kilmeri* (0.39 vs. 0.63) (Table 1 and Fig. 1).

*Ectengena extenta* clams (not shown in Table 1) had the highest content of gill thiourine ( $70.5 \pm 5.9$  mmol/kg), lowest content of hypourine ( $7.2 \pm 0.7$ ) and highest Th/[H+Th] ratio, at  $0.91 \pm 0.004$ .

For the two species from Clam Field, foot tissue was also available for analysis (Table 2). Thiourine was extremely low in both, while hypourine was found at moderate levels that were lower than in gills (Table 1),

Fig 1 here

#### *Bathymodiolins: Thiourine and Hypourine*

In *Bathymodiolus thermophilus* mussels from the EPR Mussel Bed site, both thiourine and hypourine were found at substantial levels in gill tissue (Table 1, middle rows). Mussels from the two collection sites were exposed to ambient waters with 84  $\mu$ M and 7  $\mu$ M sulfide, respectively. The former had higher thiourine (7.8 vs 3.1 mmol/kg wet mass.), lower hypourine (12.2 vs 21.0), and a higher ratio (0.40 vs 0.12) (Table 1 and Fig. 1).

For foot tissue (Table 2; available for the low-sulfide group). thiourine was not statistically different than zero, but hypourine contents were the same as in gills (Table 1).

Table 2 here

#### *Vestimentiferans: Thiourine and Hypourine*

In the *Riftia pachyptila* tubeworms from the EPR sites, thiourine levels were high in the trophosome (Table 1, lower middle rows). Worms from the two collections sites, Tica and Riftia Field, were exposed to different levels of sulfide: at the tube bases, the maximum ambient values were 283 and 81  $\mu$ M, respectively, however at plume level the averages were 33  $\mu$ M and 4  $\mu$ M sulfide, respectively. The two groups showed no statistical differences: the averages for the Tica and Riftia Field groups were, respectively, 16.5 and 14.8 mmol/kg wet mass. for thiourine, 55.1 and 59.4 for hypourine, and 0.23 and 0.20 for the Th/[H+Th] ratio (Table 1 and Fig. 1).

In the *Ridgeia piscesae* tubeworms from Juan de Fuca vents, trophosomes varied greatly between the SF form, living on high-sulfide chimneys, and the LS form, living at a diffuse-flow basalt site (Table 1, lower rows). The SF trophosomes were greener than the LS ones, and the SF ethanol extracts were much darker in greenish-yellow color. In comparison to the LS group, the SF group had much higher thiotaurine content (49 vs 0.77 mmol/kg wet mass.), lower hypotaurine content (11 vs 17), and a much higher Th/[H+Th] ratio (0.81 vs 0.04) (Table 1 and Fig. 1).

Vestimentum and plume tissue from *Riftia pachyptila* were also analyzed (Table 2); these contained levels of thiotaurine considerably lower than in trophosomes (Table 1), but had high levels of hypotaurine.

#### *All Species: Total Thiotaurine plus Hypotaurine*

Totals of thiotaurine and hypotaurine contents of all specimens were calculated for symbiont-bearing tissues, and the results are shown Table 1 (third to last column) and Fig. 2. Values did not differ within most species, even in those in which hypotaurine and thiotaurine differed considerably between test groups. The only exception to this was *Ridgeia piscesae*, in which the total was 60 mmol/kg wet mass in the high-sulfide SF groups and 18 mmol/kg wet mass in the low-sulfide LS group.

Fig 2 here

#### *All Species: Taurine*

Taurine contents of all specimens are shown in the second-to-last column of Tables 1 and 2. For symbiont-bearing tissues (Table 1), values did not differ within species, even in those in which hypotaurine and thiotaurine differed considerably between test groups. Contents correlated inversely with depth of collection, consistent with previous studies. The vesicomid clams from Eel River seeps at 0.5 km had 49 mmol/kg wet mass; the two vesicomid species from Monterey Canyon from about 1 km depth had taurine contents of about 37-41 mmol/kg wet mass. (Table 1). *Ridgeia piscesae* were from ~2.1 km and had contents of about 29-33 mmol/kg wt wt. Both *Riftia pachyptila* and *Bathymodiolus thermophilus* were from 2.5 to 2.55 km, and had contents ranging from about 11 to 15 mmol/kg wet mass. Finally, the deepest species, *Ectengena extenta* from 3.2 km, had contents of 10 mmol/kg wet mass (not shown in Table 1)

### *All Species: Other Osmotically Significant Organic Solutes*

The HPLC method revealed a number of other low-molecular-weight organic solutes at medium to high concentrations in all tissues. These solutes are presumed to serve as organic osmolytes, though they may have other functions. Some of these contained sulfur in vestimentiferans, as follows. In *Riftia pachyptila* trophosomes, an unknown tentatively identified as N-methylhypotaurine (Yin *et al.* 2000) was found at estimated levels of  $15.0 \pm 3.3$  and  $13.8 \pm 1.5$  mmol/kg wet mass in medium- and low-sulfide specimens, respectively (not significantly different). In *Ridgeia piscesae* trophosomes, for SF and LS phenotypes, respectively, N-methyltaurine (Yin *et al.* 2000) was found at  $14.0 \pm 6.5$  and  $11.6 \pm 3.1$  mmol/kg wet mass (not significantly different) and N-methylhypotaurine at  $14.4 \pm 4.4$  and  $28.1 \pm 1.1$  mmol/kg ( $P < 0.05$ ). All tissues in all species also contained cysteine and methionine but at low levels ( $< 1$  mmol/kg) that did not vary between test groups within species. Similar results were found for other tissues without symbionts (gill, foot, plume).

The other major organic solutes in all tissues analyzed were glycine betaine (GB) and glycine in most species, and (in decreasing order) alanine, proline and GB in vestimentiferans. The sum total of these solutes plus the methylated taurine derivatives are shown in the final columns of Tables 1 and 2. *B. thermophilus* tissues also contained substantial amounts of a solute that did not differ in HPLC peak area between groups. It reacted positively with ninhydrin, indicating an amine, but its elution time did not match any amino acid standard. Thus it could not be quantified.

### **Discussion**

As found in previous studies, thiotaurine was considerably higher in tissues with symbionts (Table 1) than tissues without (Table 2). Our data for the symbiont-bearing tissues are generally consistent with the hypothesis that Th/[H+Th] ratios give an indication of sulfide exposure (Pranal *et al.* 1995; Pruski *et al.* 2000b) within species. However the ratios may not be useful for interspecies comparisons. We propose that a different parameter, the sum of hypotaurine and thiotaurine (H+Th), is a consistent and useful indicator of maximum sulfide exposure across species.

### *Th/[H+Th] Ratio as an Indicator of Current Exposure*

As predicted by the hypothesis, the Th/[H+Th] ratio was lower in symbiont-bearing tissues within most species in each group having the lower sulfide exposure (Table 1, Fig. 1). In *Calyptogena (Vesicomya) pacifica*, the correlation of the ratio to sulfide exposure was found for both sediment and blood/hemolymph sulfide levels. For *C. kilmeri*, the correlation was with the internal (blood/hemolymph) sulfide level only, a result suggests that internal sulfide may be a better indicator of in situ exposure than ambient sulfide. Thus, in future studies, measurements of internal sulfide will be needed. For the other species, blood/hemolymph values were not available, but the differences in ambient sulfide were mostly large enough that it seems reasonable to assume that these values reflect internal exposure. Indeed, earlier work on *Riftia pachyptila* found that internal sulfide levels were linked to total ambient sulfide concentrations (Goffredi *et al.* 1997). For *Ridgeia piscesae*, precise sulfide exposures were not available for these specimens, but the large sulfide differences between the habitats for the LF and SF phenotypes are well known (Robigou *et al.* 1993; Sarrazin *et al.* 1997; Urcuyo *et al.* 1998, 2003). For example, in one study an aggregate of LF forms was exposed (at plume level) to a mean sulfide concentration of 0.1  $\mu\text{M}$  (Urcuyo *et al.* 2003). Moreover, the different intensities of greenish color we noted for the SF and LF trophosomes may be a marker of different sulfide exposures, because greenish color is highly correlated with tissue contents of elemental sulfur (Pflugfelder *et al.* 2005).

The exception was *Riftia pachyptila*, in which the difference between ratios was not significant. While the two sites, Tica and Riftia Field, differ considerably in their overall chemistry including maximum ambient sulfide levels (Govenar *et al.* 2005), the magnitude and absolute difference in sulfide exposures between the two groups at plume level were considerably lower than for the other animals (Table 1). It is possible that the tubeworms were collected during a period of relatively low sulfide exposure; for example in another study, sulfide levels at *Riftia* base level ranged from 190 to 980  $\mu\text{M}$  (Shank *et al.* 1998), compared to 81-283  $\mu\text{M}$  in this study. Furthermore, in previous studies, these animals typically had higher Th/[H+Th] ratios (e.g., 0.55; Pruski *et al.* 2000a). Thus our data for *Riftia* do not contradict the hypothesis. However, our data suggest that the ratio may only be a useful indicator between groups subjected to large differences in exposure.

While Th/[H+Th] ratio may thus be useful as a rough indicator of exposure for intraspecies studies in some cases, it cannot be readily used for interspecies comparisons. This is most clearly shown in the data for *C. (V.) pacifica* vs *C. kilmeri*. For specimens from the same seep (Clam Field), the former had much higher blood sulfide levels (2.7 vs 1.1 mM), but had a much lower Th/[H+Th] ratio (0.39 vs 0.63), and both had about the same content of thiotaurine (Table 1). Possibly the ratio also reflects the metabolic turnover rate of sulfide, which is much higher in *C. (V.) pacifica* than in *C. kilmeri* (Goffredi *et al.* 2002).

#### *Total Hypotaurine plus Thiotaurine as an Indicator of Maximum Exposure*

This study revealed for the first time that a different parameter, total H+Th, may be consistent within most species, regardless of exposure and individual thiotaurine and hypotaurine levels (Fig. 2). We propose that H+Th is an indicator of *potential* sulfide load. That is, a species which may sometimes experience high internal sulfide loads may need to have a high reserve of hypotaurine for converting the sulfide to thiotaurine, regardless of sulfide exposure in any given period. This hypothesis is supported not only by the totals themselves but by these species features and differences:

1) *Calyptogena (Vesicomya) pacifica* vs *C. kilmeri*. The former had much higher H+Th totals (Fig. 2) despite having lower Th/[H+Th] ratios (Fig. 1). This is consistent with their very different metabolic adaptations. *C. (V.) pacifica* routinely has much higher blood sulfide levels than *C. kilmeri*, as in the specimens used here at a seep where both were exposed to sulfide up to 4 mM (Table 1). The difference is due to a variety of *C. (V.) pacifica* adaptations including a larger foot (which takes up sulfide from the environment), larger plasma volume, and higher concentration of zinc (which binds sulfide in transport proteins) (Goffredi *et al.* 2002). Moreover, other specimens of *C. kilmeri* from high-sulfide seeps (9-18 mM) had blood/hemolymph sulfide levels much lower than those of *C. (V.) pacifica* at the Clam Field (4 mM) seep (Goffredi *et al.* 2002). Thus, the high levels of hypotaurine in *C. (V.) pacifica* may be produced so that it can be converted to thiotaurine in certain conditions. For example, if internal sulfide levels rise during some conditions, thiotaurine production could increase temporarily to prevent sulfide toxicity and/or to provide an energy reserve for periods of lower sulfide availability.

2) *Bathymodiolus thermophilus*: while one group of these mussels had a fairly high T/[H+Th] ratio of 0.40, both groups had some of the lowest total H+Th of all species in this

study, along with the LS-type *Ridgeia piscesae* (Figs 1, 2). The low H+Th is consistent with this species' relatively low energy input from its thiotrophic symbionts, as indicated by its lack of elemental sulfur in its gills and low activity of symbiont enzymes (Fisher *et al.* 1987; Felbeck *et al.* 1981).

3) *Riftia pachyptila*: the specimens here had fairly low T/[H+Th] ratios, consistent with their relatively low sulfide exposures (4 - 33  $\mu$ M). However, their H+Th values were among the highest (Figs 1, 2). This could be a general species adaptation that allows the worms to tolerate much higher sulfide exposures than the specimens used here (e.g., Shank *et al.* 1998). Indeed, blood levels of sulfide have been found in *Riftia* at 3.3 mM (Childress *et al.* 1984), higher than those of *C. (V.) pacifica* (Table 1), which had somewhat lower H+Th values (Fig. 2). Overall, the low T/[H+Th] ratio combined with a high total H+Th in these *Riftia* specimens is consistent with the hypotheses that the ratio is indicative of recent exposure and that the total correlates with maximum exposure.

4) *Ridgeia piscesae*: the pattern in this species for H+Th was unlike that of the other species, since the values were dramatically different between the SF and LS phenotypes (Fig. 2). However, this is not necessarily inconsistent with our hypothesis that H+Th relates to maximum exposure. These two phenotypes are so different that they were once thought to be separate species, and expression of some of their genes has been shown to be quite different between the two forms, and even within a form depending upon collection site (Carney *et al.* submitted). Thus it is possible that, in addition to altered gene expression for very different body forms, gene regulation related to hypotaurine-thiotaurine levels is also adjusted to reflect the maximum exposures in the very different habitats. Additionally, the *Ridgeia piscesae* forms have very different sulfide acquisition strategies. The LS growth form likely takes in sulfide from thin, sulfide-permeable posterior extensions (roots) of its tube (Urcuyo *et al.* 2003) while the SF form, without posterior roots, takes in sulfide through the plume as *Riftia* does. While sulfide levels within the substrate may be in the range of 100  $\mu$ M (Urcuyo *et al.* 2003) for the LS animals, these animals' exposure to sulfide through their roots may be limited by a surface area that is smaller than that of the plumes of the SF form.

5) In addition to the species for which we had sulfide data, *Ectengena extenta* had high levels of elemental sulfur in their gills, suggesting a very high level of sulfide exposure (as noted earlier). Their gills also had both the highest T/[H+Th] ratio and H+Th total. The idea that high

total H+Th is a marker of maximum exposure is consistent with this, although measurements of the animals' ambient and internal sulfide exposures will be necessary to fully test this.

Another possible adaptive reason for having high hypotaurine (and a high H+Th) is oxidative stress (Pruski *et al.* 2000a). Oxygen-centered (as well as sulfur-centered) radicals are generated in the presence of HS<sup>-</sup>, trace metal catalysts, and oxygen (Tapley *et al.* 1999). Hypotaurine is a strong antioxidant and can react with oxygen radicals (Aruoma *et al.* 1988). Whether the animals in this study with the highest hypotaurine contents are exposed to greater oxidative stress is uncertain, though the higher metabolic rate of *C. (V.) pacifica* compared to *C. kilmeri* (Barry *et al.* 1997) may generate more oxygen radicals.

#### *Taurine and Other Taurine Derivatives*

Taurine contains correlated roughly with depth of collection. This is a trend seen in several previous studies (Pruski *et al.* 2000a; Fiess *et al.* 2000) including animals without endosymbionts (Yancey *et al.* 2004; Rosenberg *et al.* 2006). The trend has been attributed to a reduction in dietary source of taurine with depth (Pruski *et al.* 2000a). However, shallow solemyid clams (which have symbionts) interconvert taurine, hypotaurine and thiotaurine (Joyner *et al.* 2003), and larvae of at least one bivalve species can synthesize taurine *de novo* (Welborn & Manahan 1995). Thus a dietary source of taurine itself is not necessarily required in all mollusks. Other reasons for reduction in taurine with depth (e.g., (e.g., high pressure inhibition of reactions involved in taurine synthesis) need to be considered.

The other major taurine derivatives, N-methyltaurine in *Ridgeia*, and possibly N-methylhypotaurine in *Riftia*, are unusual in that they have not been reported at osmotically significant levels in other marine invertebrates except for *Lamellibrachia vestimentiferans* (Yin *et al.* 2000). Their functions are unknown, but they may be an adaptation to pressure. Other methylated amines have been found to increase with depth in other organisms, and those solutes can stabilize proteins against inhibition by high pressure (Yancey *et al.* 2004; Yancey 2005).

#### **Conclusions**

Hypotaurine (H) and thiotaurine (Th) together are among the most abundant of all organic compounds in some vent and seep animals, but not in other marine invertebrates. Thus it is important to understand their function. This seems to be their ability to detoxify sulfide for basic

cytoprotection and/or storage for thiotrophic symbionts (Alberic & Boulegue 1990; Pranal *et al.* 1995; Pruski *et al.* 2000b). The ratio of Th/[H+Th] increases with sulfide exposure in most species, and may serve as a rough indicator of such exposure in intraspecies studies. However, for interspecies comparisons, the ratio should be interpreted within the context of the total H+Th, which may be an indicator of the maximum sulfide exposure for each species or significantly different phenotype within a species.

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## Figure Legends

Fig. 1. Ratios of thiotaurine to sum of hypotaurine and thiotaurine ( $\text{Th}/[\text{H}+\text{Th}]$ ) for symbiont-bearing tissues, from Table 1. "1" and "2" designate specimens with higher and lower sulfide exposures, respectively. Values are means  $\pm$  1 SD; \*different from the same species at the other seep or vent site; †different from the other species at the same seep ( $p < 0.05$ ).

Fig. 2. Sum of hypotaurine and thiotaurine ( $\text{H}+\text{Th}$ ) for symbiont-bearing tissues, from Table 1. Values are means  $\pm$  1 SD; \*different from the same species at the other seep or vent site; †different from the other species at the same seep ( $p < 0.05$ ).

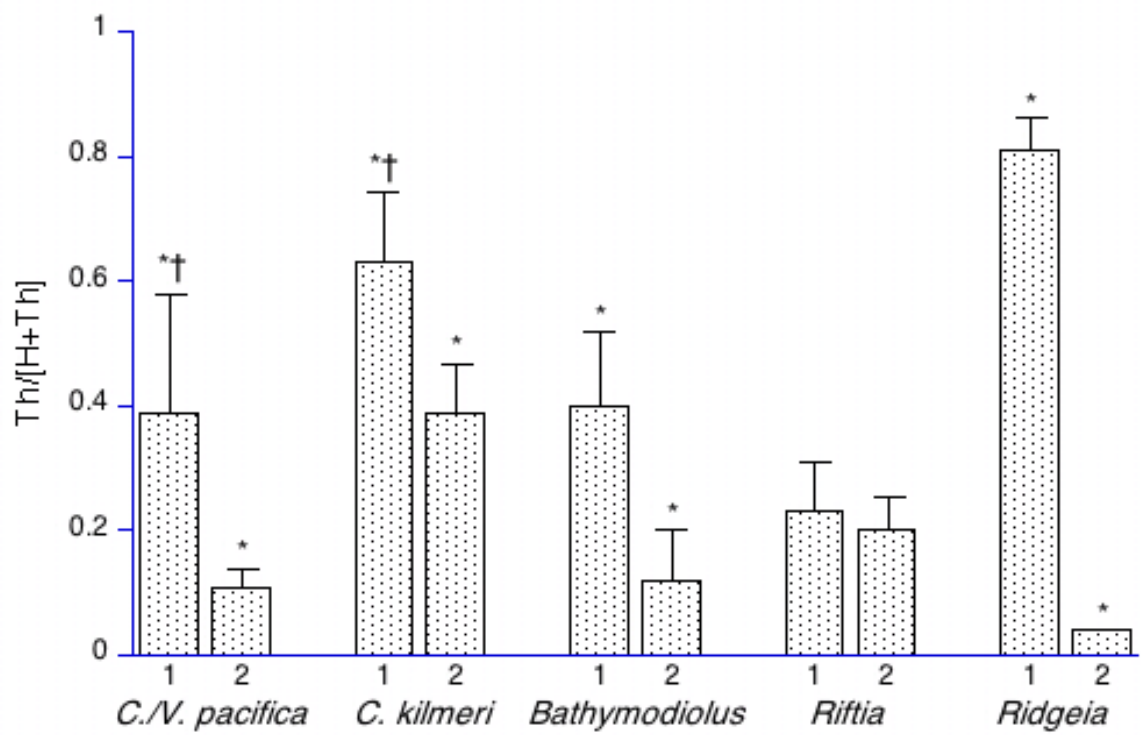


Fig. 1

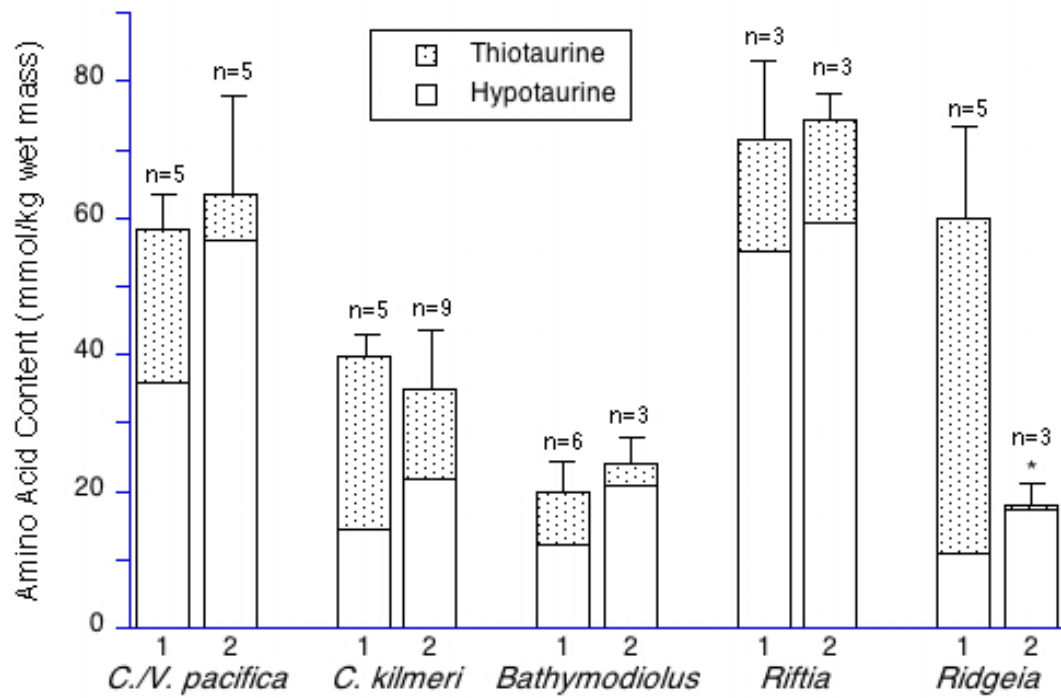


Fig. 2