

# Adaptations to hypoxia in hydrothermal-vent and cold-seep invertebrates

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**Abstract** The deep sea harbors very unusual environments, such as hydrothermal vents and cold seeps, that illustrate an apparent paradox: the environmental conditions are very challenging and yet they display a high biomass when compared to the surrounding environment at similar depth. Hypoxia is one of the challenges that these species face to live there. Here, we review specific adaptations of their respiratory system that the species have developed to cope with hypoxia, at the morphological, physiological, and biochemical levels. Most studies to date deal with annelids and crustaceans, and trends can be drawn: development of ventilation and branchial surfaces to help with oxygen extraction, and an increase in finely tuned oxygen binding proteins to help with oxygen storage and transport. Beside these respiratory adaptations most animals have developed enhanced anaerobic capacities and specific ways to deal with sulfide.

**Keywords** Hypoxia · Invertebrates · Annelids · Crustacea · Mollusks · Hemocyanin · Hemoglobin · Respiration · Oxygen · Sulfide

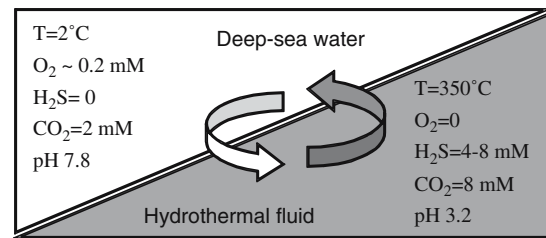
## 1 Introduction

The discovery of hydrothermal vents on the Galápagos Ridge in 1976 (Corliss and Ballard 1977) revolutionized our vision of the deep-sea, which was previously notorious for its very low biomass and high species diversity (Hessler and Jumars 1974). Numerous animals from the vicinity of fluid emissions associated with plate tectonics were brought back to the surface (Corliss and Ballard 1977; Ballard and Grassle 1979; Corliss et al. 1979). Subsequent exploration at other sites on mid-oceanic ridges and back-arc basins has extended our knowledge of the fauna associated with hydrothermal vents. When compared to the surrounding areas, these communities exhibit very high biomass, and a relatively low species diversity. The abundance of these organisms, contrasting with the harsh conditions of the environment, has stimulated research on their physiological and biochemical adaptations. Stable isotope values of their tissues indicate that the high biomass is supported by local chemosynthetic primary production rather than by photosynthesis-based organic matter sinking from the surface (Fisher 1995). Chemosynthesis is carried out by bacteria, free-living or in symbiotic association with invertebrates. These bacteria oxidize the reduced chemicals contained in the hydrothermal fluid (Fe, CH<sub>4</sub>, H<sub>2</sub>S), using oxygen or nitrate as a final electron acceptor, to produce

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energy and fix carbon dioxide into organic matter (Fisher 1996). This hydrothermal fluid is formed by the interaction of the sea-water seeping through the oceanic crust with the hot rocks. The fluid is changed markedly and usually contains high levels of heavy metals,  $\text{CO}_2$  and sulfide, is completely devoid of  $\text{O}_2$ , and has a low pH (Table 1 and references therein). The hydrothermal vent communities completely rely on the emission of hydrothermal fluid. However, its mixing with the deep-sea water strongly affects the surrounding environment and the animal communities it harbors. Individual species occupy different niches whose characteristics vary with the proportion of hydrothermal fluid, and even specific locations are exposed to highly variable, often challenging conditions (Fig. 1) (Johnson et al. 1988; Chevalloné et al. 1991, 1992; Chevalloné 1986; Le Bris et al. 2003). Due to the highly chaotic conditions and to technical difficulties, very few in-situ measurements of oxygen have been published to date. A study of microhabitat variations in mussel beds at the Rose Garden site yielded typical oxygen concen-



**Fig. 1** Schematic representation of water mixing around hydrothermal vents. Bottom water (clear) mixes with hydrothermal fluid (grey shade). Typical water characteristics are given for each. Animals are exposed to varying proportions of each fluid according to their proximity of the vent. In addition, the mixing is chaotic and at a fixed position, the proportion of hydrothermal vent fluid can vary quickly (arrows)

tration values between 0 and 110  $\mu\text{M}$ , this latter value being that of ambient sea-water in the area (Fisher et al. 1988). The harsh conditions (low oxygen, high levels of sulfide, heavy metals, radioactivity, etc.) encountered in these food-rich environments require specific adaptations for survival that may explain the very high proportion of endemism (95%) observed in the

**Table 1** Physical and chemical parameters of hydrothermal fluid from black smokers of different origin compared to typical deep-sea water (after Von Damm 1990, modified by Magenheimer and Gieskes 1992)

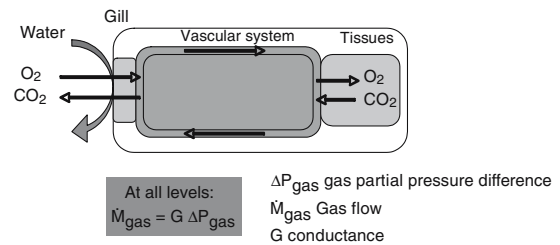
Physical–chemical parameters	South Juan de Fuca Ridge	East Pacific Rise 21°N	East Pacific Rise 13°N	Deep-sea water
Temp. (°C)	224–285	273–355	317–380	2
pH	3.2	3.3–3.8	3.2	7.8
Alkalinity	0	– 0.50 à – 0.19	– 0.74 à – 0.40	2.3
Li (mM)	110–1,810	891–1,322	688	26
Na (mM)	700–800	432–510	560	464
K (mM)	37.3–51.6	23.2–25.8	29.6	9.8
Rb (mM)	28–37	27–33	14.1	1.3
Be (nM)	95–150	10–37	–	0
Mg (mM)	0	0	0	52.7
Ca (mM)	77.3–96.4	11.7–20.8	55	10.2
Sr (mM)	230–312	65–97	175	87
Al (mM)	1.9	4.0–5.2	–	0.020
Cl (mM)	896–1,090	489–579	740	541
$\text{SiO}_2$ (mM)	22.7–23.3	15.6–19.5	22	0.16
$\text{SO}_4$ (mM)	0	0	0	27.9
$\text{H}_2\text{S}$ (mM)	3–4.4	6.6–8.4	4.0	0
Mn (mM)	2.61–4.48	0.7–1.0	0.8–1.2	<0.001
Fe (mM)	10.3–18.7	0.75–2.42	1.05–1.85	<0.001
Cu (mM)	<2	<2–44	–	0.007
Zn (mM)	<900	40–106	–	0.01
Se (mM)	<1	<1–73	–	2.5
As (nM)	0	<30–452	–	27
$\text{NH}_3$ (mM)	0	0	–	<0.01

collections of fauna at hydrothermal vents (Tunnicliffe 1991).

The first—and to date best studied—cold-seeps were discovered in the Gulf of Mexico in the 1980s (Paull et al. 1984; Kennicutt et al. 1985). Cold seeps inhabited by dense communities are now known from more than 30 locations in the world (Sibuet and Olu 1998; Tyler et al. 2003). Conditions at cold seeps are often hypoxic, as a result of the removal of free  $O_2$  in the deep-sea water by spontaneous reaction with sulfides contained in the fluid diffusing from the sediment. In contrast to hydrothermal vents, these sulfides are biogenic in nature: anaerobic bacteria in the sediment utilize sea-water sulfate as the final electron acceptor for the degradation of hydrocarbons seeping up (Cordes et al. 2005). A study at the Brine Pool site in the Gulf of Mexico showed that oxygen in water samples taken between the mussels averaged  $39 \mu\text{mol l}^{-1}$  and was often undetectable by gas chromatography ( $<5 \mu\text{mol l}^{-1}$ , Smith et al. 2000).

To date, no physiological work has been published on invertebrates from whale-fall communities. These animals are also potentially exposed to lower levels of oxygen. Some studies report the adaptations of pelagic organisms to low oxygen levels in oxygen minimum zones (for example, see Childress 1975; Childress and Seibel 1998), characterized by stable hypoxic conditions. Benthic invertebrates from oxygen minimum layer areas are usually small in size (Levin 2003). This may be due to the fact that these areas are not associated with strong emissions of reduced fluids that are necessary for local primary production that is at the base of the very high biomass that characterizes hydrothermal vents and cold seeps.

Interestingly the  $O_2$  consumption rates of hydrothermal-vent and cold-seep animals at low environmental  $O_2$  tensions are similar to those of related, shallow-water species measured at higher environmental  $O_2$  tensions (Childress and Mickel 1985; Fisher et al. 2000; Hourdez et al. 2002), indicating specific adaptations for  $O_2$  uptake in the former group, such as higher blood  $O_2$  affinities and more efficient respiratory organs. Here, we review morphological, physiological, and biochemical adaptations of the respiratory



**Fig. 2** Conceptual representation of gas flows in a metazoan with a circulatory system. See text for details

system. To date, these adaptations have mainly been studied in polychaetes and crustacea but data on other groups will be discussed when available.

Gas uptake (and elimination) by an organism is limited by several factors at different levels (Fig. 2). At all levels, the gas flow is driven by convective or diffusive processes, and is proportional to the difference of partial pressure of the considered gas between two locations, and to a conductance factor  $G$ . Details of parameters affecting  $G$  will be discussed for the different levels of adaptation considered. At the interface with the environment, the respiratory exchange organs (gills, branchiae) are essential for oxygen uptake. The oxygen that is taken up is then transported by the circulatory system to the tissues that need it. The mitochondria, where oxidative respiration takes place, are the actual site of oxygen consumption.

## 2 Adaptations for oxygen extraction

### 2.1 Ventilation

The first way to improve oxygen extraction is to renew the diffusion layer at the surface of the gas exchange organs. The water in the diffusion layer is indeed quickly depleted in oxygen and its extraction becomes more difficult. Convective circulation of water and blood at the gill level contributes to increase oxygen extraction in an integrative process. This maintains an optimal difference of partial pressure between the two sides of the diffusion barrier.

The gills of annelids are external (i.e., not contained in branchial chambers) and these animals have little control over the ventilation

of the gill surface by water, except through movements of cilia which are abundant in vent species (Jouin-Toulmond et al. 1996; Hourdez and Jouin-Toulmond 1998; Hourdez et al. 2001). Perfusion of the gills by body fluids may also increase but this parameter is difficult to measure in polychaetes, especially in species like scaleworms whose gills are perfused by the coelomic fluid, circulated by cilia that line the coelomic cavity (Hourdez and Jouin-Toulmond 1998).

Decapod crustacea possess a branchial chamber in which water may be circulated by specialized appendages, the scaphognathite and associated epipodites. Although regulation of ventilation flow rate is well documented for shallow-water species (McMahon 2001) and some deep-sea species (Belman and Childress 1976), no study addresses this behavioral adaptation in vent or seep species. Generally, acute hypoxia induces a rapid increase in ventilation flow but it slows down under chronic exposure, probably due to the high energetic cost of increasing water pumping (McMahon 2001).

Mollusca circulate water through their mantle cavity by ciliary movement at the surface of the gills. This is a normal process for these filter-feeding animals. However, in clams of the genus *Calyplogena*, the digestive system is reduced and the bulk of the nutrition is obtained through symbiotic bacteria contained in the gill filaments rather than through water filtration (Kennish and Lutz 1992). The respiratory function may well be the only one remaining in these clams, in addition to harboring the symbiotic bacteria.

## 2.2 Gill surfaces

The conductance  $G$  is a function of the ratio of surface area over diffusion distance. Extraction of oxygen across gills can thereby be increased by increasing the total surface area or by decreasing the diffusion distance through the epithelium, or both.

An increased specific gill surface area has been reported in numerous cold-seep and hydrothermal vent polychaetes (Table 2). This is especially meaningful when compared to close relatives from well-oxygenated environments. In the family Polynoidae (scaleworms), littoral species are

usually devoid of gills whereas about half of the hydrothermal vent and cold-seep species possess well developed gills (Hourdez and Jouin-Toulmond 1998; Hourdez unpub. data). This increase in area for gas exchange has also been reported in benthic invertebrates from oxygen minimum zones all around the world (Levin 2003).

Besides an increase in gill surface area, a reduction of the diffusion distance can also help gas exchange across the gills. Most of the vent species studied to date show very short diffusion distances (Table 2). These short distances are possible through the use of intraepidermal blood vessels found in the gills of the polychaetes from hypoxic environments (Jouin and Gaill 1990; Hourdez et al. 2001). These intraepidermal extensions of blood vessels are absent in the gills of the shallow-water trichobranchid polychaete *Terebellides stroemii*, a species closely related to alvinellids (Jouin-Toulmond and Hourdez 2006). The scaleworm *Branchipolynoe* spp. is an exception to this reduced diffusion distance. However, their gills are mere finger-like extension of their body-wall that are perfused by the coelomic fluid and not by blood vessels. The bodywall is very

**Table 2** Gill surface areas and diffusion distances for annelids from hydrothermal vent (HTV), littoral (Lit.), and cold-seep (CS) environments

Species	Environment	Specific gill surface area (cm <sup>2</sup> /g)	Diffusion distance (μm)
<i>Alvinella pompejana</i> <sup>a</sup>	HTV	12	1–3
<i>Paralvinella grasslei</i> <sup>a</sup>	HTV	47	4
<i>Riftia pachyptila</i> <sup>b</sup>	HTV	12	1–3
<i>Branchipolynoe symmytilida</i> <sup>c</sup>	HTV	14.2	10
<i>Branchipolynoe seepensis</i> <sup>c</sup>	HTV	10.3	9
<i>Branchipolynoe pettiboneae</i> <sup>c</sup>	HTV	7.7	10
<i>Glycera convoluta</i> <sup>c</sup>	Lit.	1.5–2	?
<i>Terebellides stroemi</i> <sup>d</sup>	Lit.	6	5–8
<i>Arenicola marina</i> <sup>e</sup>	Lit.	4	8–14
<i>Methanoaricia dendrobranchiata</i> <sup>f</sup>	CS	8	4

<sup>a</sup> Jouin and Gaill (1990); <sup>b</sup> Andersen et al. (2002);

<sup>c</sup> Hourdez and Jouin-Toulmond (1998); <sup>d</sup> Jouin-Toulmond and Hourdez (2006); <sup>e</sup> Jouin and Toulmond (1989);

<sup>f</sup> Hourdez et al. (2001)

thin there (typically 10  $\mu\text{m}$ ) whereas it is 150  $\mu\text{m}$  thick anywhere else on the body (Hourdez and Jouin-Toulmond 1998).

No specific data are available for other taxonomic groups. However, it is well known that mollusks that are symbiotic with sulfide-oxidizing or methanotrophic bacteria possess much larger gills (that contain the symbionts) than species that are not symbiotic (Childress and Fischer 1992). No data are available on the diffusion distances and gill surface areas for crustacea from cold seeps and hydrothermal vents. However, Williams (1980), in his original description of the vent crab *Bythograea thermydron*, reports that they have large gills, apparently to facilitate oxygen uptake from the hypoxic environment. Conversely in *Rimicaris exoculata*, the peculiar morphology of the gill chamber and hypertrophy of the scaphognathite and first maxillipeds' epipodites leaves only a reduced space for gills (Segonzac et al. 1993). A comparative study of specific gill surface area, and scaphognathite and perfusion performance among bythograeid crabs and alvinocarid shrimps is clearly lacking at present. This is likely to be a level at which adaptations can be observed as Johnson and Rees (1988) showed that there were variations in gill surface areas depending on habitat and lifestyle in four shallow-water crab species.

### 3 Adaptations for oxygen transport

Once in the circulatory system, the conductance for oxygen will depend on the product of the capacitance ( $\beta b$ ) of the blood (i.e., how much oxygen can be carried per unit volume of blood) and of the blood flow ( $V_b$ ). In order to improve oxygen delivery to the organs, one can either increase  $\beta b$  or  $V_b$ . The presence of oxygen binding proteins (respiratory pigments) increases  $\beta b$ , and increased heart rate or stroke volume can perfuse the tissues with more blood.

#### 3.1 Modulation of heart rate

A measure of the regulation of heart rate in annelids from hypoxic environments has never been attempted. However, Mickel and Childress (1982a) measured variations in heart rate of the crab *Bythograea thermydron* as a function of

pressure and temperature. An increase of temperature from 5°C to 20°C triggered an increase of heart rate, probably in response to an increased metabolic demand.

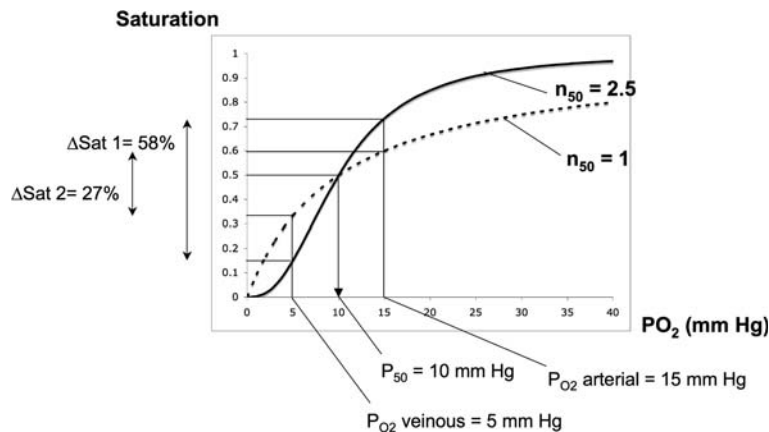
#### 3.2 Oxygen binding proteins (OBPs)

Most hydrothermal vent organisms possess respiratory pigments. These were studied in some mollusks, polychaetes, and crustaceans.

The main properties of OBPs are affinity - measured by  $P_{50}$ , the oxygen partial pressure at half saturation -, cooperativity - measured by  $n_{50}$  the slope of the Hill plot at  $P_{50}$ , and the Bohr effect - measured by  $\phi = \Delta \log P_{50} / \Delta \text{pH}$  (Fig. 3). Depending on their oxygen binding properties, respiratory pigments may serve to store oxygen, to facilitate oxygen diffusion, or to actually transport oxygen from the gas exchange organs to the tissues. Oxygen storage OBPs usually exhibit a high affinity (allowing uptake of oxygen from the circulating OBP), low cooperativity and are insensitive to pH changes. Conversely, OBPs that serve as transporters usually exhibit a lower affinity, some cooperativity and are affected by changes in pH (Bohr effect). Their cooperativity allows them to release more oxygen for a given variation of partial pressure between arterial and venous blood (Fig. 3). These blood parameters, however, are not known for the majority of deep-sea species, and we have to rely on indirect interpretation derived from affinity, cooperativity and Bohr effect measurements.

##### 3.2.1 OBP as an oxygen storage system

Respiratory pigments can be used as oxygen storage. It has been calculated, based on an oxygen consumption rate independent of the partial pressure and starting with respiratory pigments fully saturated, that the bound oxygen could represent an autonomy of up to 1 h 30 min in the commensal hydrothermal vent polychaete *Branchiopolynoe seepensis* (Hourdez and Weber 2005). This value only reaches 30 min in the cold-seep polychaete *Methanoaricia dendrobranchiata* (Hourdez et al. 2002). Very little data are available in other species. However, in the hydrothermal vent copepod *Benthoxynus spiculifer*, the



**Fig. 3** Saturation curve for a hypothetical oxygen binding protein (OBP) with a  $P_{50}$  of 10 mm Hg. Dashed curve for an OBP with a cooperativity coefficient  $n_{50} = 1$  and solid line for an OBP with a cooperativity coefficient  $n_{50} = 2.5$ .

For each OBP, the difference of saturation ( $\Delta\text{Sat}$ ) is given for a difference of partial pressure of  $\text{O}_2$  of 10 mmHg between arterial blood and venous blood

amount of hemoglobin in the body can represent no more than 2 min of autonomy at 15°C and less than 30 s at 25°C (Hourdez et al. 2000b). This hemoglobin pool would only allow very short forays into anoxic areas to look for food before the animals have to rely on anaerobic metabolism for their energetic needs. In such animals, the hemoglobin's primary function is more likely in  $\text{O}_2$  acquisition from the environment (Hourdez et al. 2000b), maintaining a free-oxygen (i.e., not bound to Hb) gradient from outside of the animal to the internal milieu while the respiratory surfaces are ventilated.

### 3.2.2 OBP as a transport system

There is a general trend for a high oxygen affinity, which would facilitate uptake at the level of the gills, and a strong Bohr effect (decrease of affinity when pH decreases) that would allow the release of this oxygen at the level of metabolically active tissues. The high affinity for oxygen would leave little of it free in the body fluids, thereby maintaining a gradient between the inside and the outside of the body, even when the environmental levels of oxygen are very low.

**3.2.2.1 Mollusks** Bivalves, along with vestimentiferans, are the main organisms with symbiotic sulfide oxidizing and/or methanotrophic bacteria at hydrothermal vents and cold-seeps (Tunnicliffe

1991). Vesicomysid clams are the only vent and seep bivalves known to possess circulating respiratory pigments to date. These Hbs are intracellular (erythrocytic) and low-molecular weight, consisting of tetramers made of three different chains of 13–14 kDa (Terwilliger et al. 1983; Arp et al. 1984; Zal et al. 2000b). *Calyptogena magnifica* erythrocytes suspended in sea-water after washing show a moderate  $\text{O}_2$  affinity ( $P_{50} = 8.5$  mm Hg, pH 7.15, 8°C (Arp et al. 1984)) that is lowered when the erythrocytes were suspended in the original serum ( $P_{50} = 14.0$  mm Hg, pH 6.75, 9°C). Dissolved in distilled water the Hb showed a much higher  $\text{O}_2$  affinity ( $P_{50} = 3.8$  mm Hg, pH 6.99, 8°C), suggesting the presence of intracellular effectors that decrease the affinity in the erythrocytes (like ATP or 2,3-DPG for vertebrate Hb). The functional properties of the Hb were affected by sulfide that oxidizes Hb in vitro (Terwilliger et al. 1983; Arp et al. 1984) and impedes measurement of the sulfide sensitivity of  $\text{O}_2$  binding (Arp et al. 1984).

The symbiont-harboring gastropod species *Alviniconcha hessleri* forms dense populations at hydrothermal vents in the Western Pacific (Mariana, North Fiji, and Lau back-arc basins) where it dominates the fauna. Its modified gills contain chemoautotrophic sulfide-oxidizing bacteria that provide the snail with carbon compounds. These gills also contain a tissue globin at a concentration of 65  $\mu\text{mol/kg}$  wet

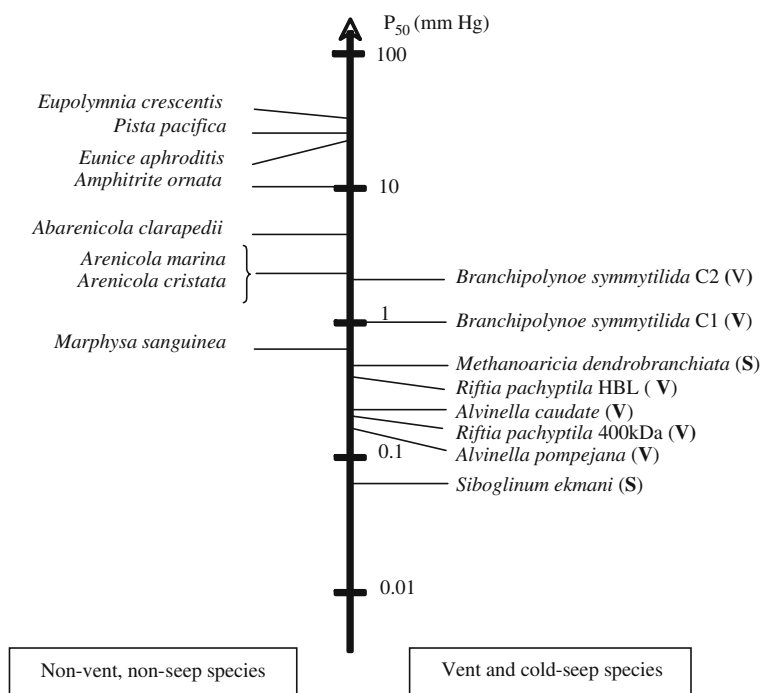


weight gill (Wittenberg and Stein 1995). Wittenberg (1985) also reports the presence of an intracellular globin in the gills of the mussel *Bathymodiolus heckerae* from the Florida Escarpment. Gastropods also possess circulating hemocyanins but there is no published data on their properties and characteristics for vent species. The same is true for the hemocyanin of the vent octopus *Vulcanoctopus hydrothermalis*.

**3.2.2.2 Polychaetes** Polychaetes represent an important proportion of the biomass and diversity at hydrothermal vents and cold seeps (Tunnicliffe 1991). They also occupy most of the ecological niches at hydrothermal vents where metazoans can be found, from the coldest (and least constraining) to the warmest. All the species studied to date possess hemoglobin(s) in high concentrations, with a high affinity for oxygen

when compared to shallow-water relatives, and a strong Bohr effect (Fig. 4, Hourdez and Weber 2005).

Vestimentiferan tubeworms (siboglinid polychaetes) are often the most commonly encountered metazoan animals in hydrothermal vent and cold-seep communities. They lack a mouth, digestive tract and anus (Jones 1981, 1988), and their nutritional needs are entirely provided for by symbiotic sulfide-oxidizing bacteria harbored in the ‘trophosome’, an internal, well-vascularized organ (Cavanaugh et al. 1981; Felbeck 1981; Felbeck et al. 1981; Jones 1981). The first thoroughly studied vestimentiferan was the giant tubeworm *Riftia pachyptila*. This worm possesses two Hbs in its vascular blood and another in its coelomic fluid (Arp et al. 1990; Zal et al. 1996b). The blood Hbs are a hexagonal bilayer (HBL) Hb of ~3.6 MDa (as typically encountered in vascular blood of other annelids) and a 400 kDa Hb that is



**Fig. 4**  $P_{50}$  values (log scale) for extracellular Hbs from vent and seep marine polychaetes compared to non-vent and non-cold seep species. Experimental conditions and references: *Arenicola marina*, pH 7.6, 20°C; *Siboglinum ekmani*, pH 6.5, 20°C; *Alvinella pompejana*, pH 7.6, 20°C; *Alvinella caudate*, pH 7.6, 20°C; *Pista pacifica*, pH 7.0, 20°C; *Marphysa sanguinea*, pH 7.3, 20°C; *Eunice aphrod-*

*itis*, pH 7.0, 20°C; *Arenicola cristata*, pH 7.7, 20°C; *Abarenicola clarapedii*, pH 7.43, 20°C; *Eupolymnia crescentis*, pH 5–7, 10°C; *Branchiopolynoe symmytilida* Hbs C1 and C2, pH 7.5, 20°C; *Methanoaricia dendrobranchiata*, pH 7.5, 20°C; *Riftia pachyptila* HBL and 400 kDa Hbs, pH 7.0, 30°C. (V) Vent species, (S) Seep species. After Hourdez and Weber (2005)

specific of siboglinid tubeworms. The coelomic fluid contains another 400 kDa Hb, differing from the vascular one in its subunit composition (Zal et al. 1996a). The O<sub>2</sub> binding properties of vestimentiferan Hbs are similar to those of other annelids from hydrothermal vents and cold seeps. In *Riftia*, both Hb types have very high O<sub>2</sub> affinities, with P<sub>50</sub> values of 0.47 and 0.27 mm Hg for the HBL-Hb and the 400 kDa, respectively, at pH 6.97 and 30°C. The smaller Hb consistently showed a higher affinity, except at pH values above 7.6 (Arp et al. 1990), which correspond to in vivo pH values (Arp and Childress 1981). This suggests that the O<sub>2</sub> affinity difference between the coelomic (400 kDa) Hb and the vascular (HBL and 400 kDa) Hbs may easily reverse, allowing a bi-directional transfer between the coelomic and vascular compartments. This would buffer the effects of changes in environmental O<sub>2</sub> tension. The two types of Hbs also differ markedly in their pH sensitivity ( $\phi = -0.35$  and  $-0.04$ , for the HBL and 400 kDa Hbs, respectively). The cooperativity is also lower in the 400 kDa Hb than in the HBL Hb ( $n_{50} = 1.6$  and  $2.4$ , respectively at pH  $\approx 7$ ), which correlates with the number of subunits (ca. 24 vs. 144 globin chains). Hbs from three species of *Siboglinum* that occur in hypoxic fjords comprise only 400 kDa molecules (Terwilliger et al. 1987), all showing very high O<sub>2</sub> affinities and a slight reverse Bohr effect between pH 7.0 and 7.9 ( $\phi = +0.18$  at 15°C and  $+0.25$  at 20°C; (Terwilliger et al. 1987)). According to our definition, these properties would favor a storage role for the 400 kDa Hb and a transport role for the HBL Hb. A key and unique characteristic of vestimentiferan Hbs is their ability to bind sulfide reversibly and with a high affinity (Arp and Childress 1983; Arp et al. 1984; Childress and Fischer 1992; Zal 1998; Zal et al. 1998). This will be dealt with in a later section.

Species of Alvinellidae (a vent-endemic family) and the cold-seep orbinid *Methanoaricia dendrobranchiata* possess a typical extracellular annelid hexagonal bilayer (HBL) hemoglobin in their vascular system (Terwilliger and Terwilliger 1984; Toulmond et al. 1990; Zal et al. 1997, 2000a; Hourdez et al. 2000a). Although the structure of the Hb is very similar to that of other annelid

hemoglobins, the functional properties show a high affinity for oxygen and a strong Bohr effect that allows the release of the bound oxygen near metabolically active (i.e., acidic) tissues (Terwilliger and Terwilliger 1984; Toulmond et al. 1990; Hourdez et al. 2000a).

In addition to their HBL hemoglobin, alvinellids also possess a circulating intracellular hemoglobin contained in coelomocytes (Jouin-Toulmond et al. 1996; Hourdez et al. 2000a), as it is common in the closely-related terebellids (Weber 1978). However, in contrast to terebellids where the oxygen transfer is unidirectional (usually from extra- to intracellular Hbs), driven by differences in affinities for oxygen between the extra- and the intracellular Hbs, oxygen transfer can be bi-directional in *Alvinella pompejana*, the only alvinellid species for which functional properties were studied for both hemoglobins (Hourdez et al. 2000a). This property most likely allows the buffering of environmental oxygen variations using the coelomic compartment as an oxygen store. This is especially meaningful in the anterior part of the body where a dense network of capillaries is surrounded by numerous erythrocytes (see Sect. 3.3). There, a very important transfer can occur between the vascular and the coelomic compartments.

The discovery of large amounts of hemoglobin in the hydrothermal vent scaleworms *Branchiopolynoe symmytilida* and *B. seepensis* (Polychaeta; Polynoidae) came as a surprise as littoral species of the same family are completely devoid of circulating respiratory pigments (Hourdez et al. 1999a). There are two Hbs, one is a dimer (HbC2) and the other a trimer (HbC1) of tetradomain subunits. Multidomain (i.e., several globin domains in the same polypeptide) subunits are unique not only in the Polynoidae family but in all polychaetes, indicating that it most likely evolved in the lineage to which *Branchiopolynoe* spp. belong. These Hbs are contained in the coelomic cavity, exhibit a high affinity for oxygen and a strong Bohr effect (Hourdez et al. 1999b). Their cooperativity is low (cooperativity coefficient typically between 1 and 1.9), suggesting a function of oxygen storage rather than oxygen transporter. However, the coelomic fluid circulates inside the gills of the branchiate species and



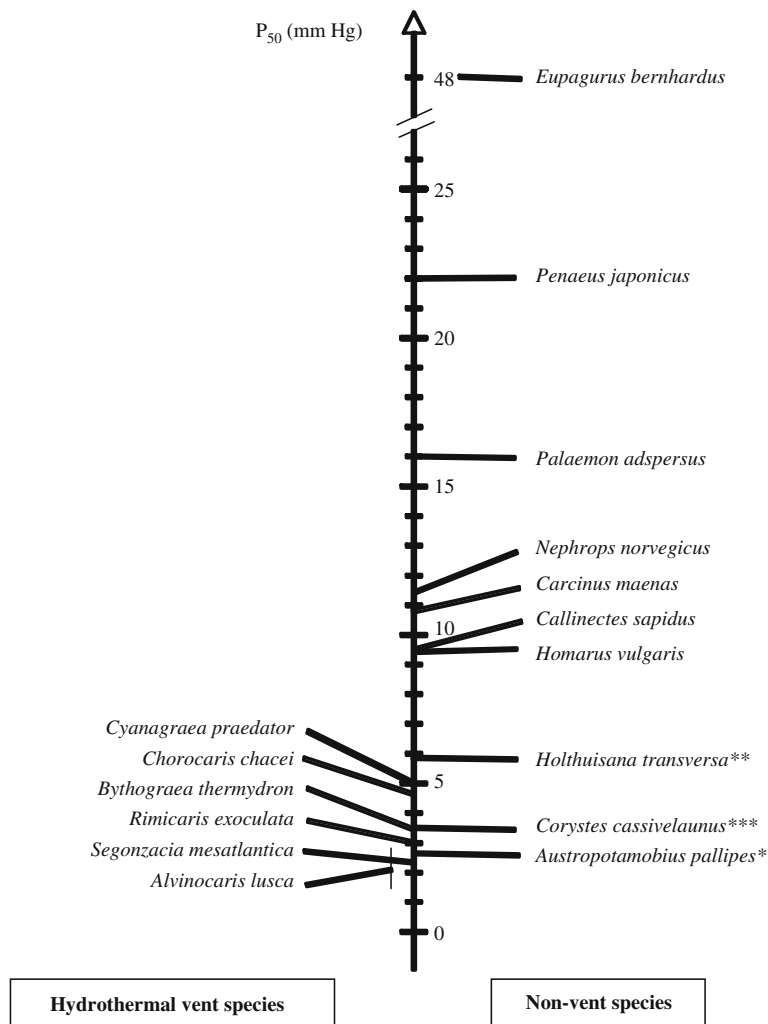
the hemoglobin probably helps maintain a concentration gradient between the hypoxic environment and the inside of the body. The strong Bohr effect is also indicative of a role in oxygen transport but we would have to know the variation of oxygenation level and pH within the coelomic fluid to confirm this. In addition to the Bohr effect, there is a specific CO<sub>2</sub> effect in HbC2 (but not in HbC1), independent of pH, which decreases the affinity when PCO<sub>2</sub> increases. This further increases the unloading of oxygen near metabolically active tissues (Hourdez et al. 1999b). Hemoglobin has been observed in numerous other hydrothermal vent and cold seep species of scaleworms (SH pers. obs.). All these species belong to vent- and seep-endemic subfamilies of polynoids (Branchiplicatinae, Branchipolynoinae, Branchinotogluminae, Lepidontopodinae, and some Macellicephalinae). The two other subfamilies encountered near hydrothermal vents (Harmothoinae and Iphioninae) are not endemic and are only found at the periphery of the sites, where conditions are less challenging. Species belonging to these two latter subfamilies do not possess circulating Hbs. Given the high metabolic cost of producing such large amounts of proteins, their presence is most likely an adaptation to the chronic hypoxia these worms are exposed to.

**3.2.2.3 Crustacea** Crustacea are also common at hydrothermal vents and cold-seeps but all published data come from hydrothermal vent species so far. Hemocyanin is the most common respiratory pigment but hemoglobin has been found in two species of copepods. The more mobile nature of large decapod crustacea (shrimps and crabs) allows them to avoid areas where conditions are too harsh. Nonetheless, the conditions they encounter are often hypoxic and one can expect specific adaptations.

Mickel and Childress (1982b) reported that the hydrothermal vent crab *Bythograea thermydron* can tolerate periods of anoxia. In addition, this crab can maintain its oxygen consumption rate constant until a critical P<sub>O<sub>2</sub></sub> value of 12 mm Hg, i.e., about 7.5% of saturation with air. This value is 3–4 times the P<sub>50</sub> of the hemocyanin contained

in the hemolymph of *B. thermydron* (Sanders et al. 1988). This P<sub>50</sub> value is small compared to those found in non-vent species and falls within the range reported for other hydrothermal vent species of crabs and shrimps (Fig. 5 and references therein). No data are available for cold-seep endemic species to date. As in polychaetes, this high affinity helps extract oxygen from the chronically hypoxic surrounding water. It is compensated by a strong Bohr effect that allows the release of oxygen near metabolically active tissues (Sanders et al. 1988; Lallier and Truchot 1997; Lallier et al. 1998; Chausson et al. 2001). The affinity is increased by the presence of lactate (the final product of anaerobic metabolism in crustacea) that will thereby favor the loading of oxygen at the level of the gills if the tissues produce lactic acid (Sanders et al. 1988). It was also shown that the affinity is increased by the presence of thiosulfate, the product of sulfide detoxification in *B. thermydron* (Vetter et al. 1987). An effect of lactate has also been reported for two species of vent shrimps, *Chorocaris chacei* (Lallier et al. 1998), and *Rimicaris exoculata* (Lallier and Truchot 1997). The vent-chimney crab *Cyanagraea praedator* also possesses hemocyanin with a high affinity for oxygen and a strong Bohr effect (Sanders 1989; Chausson et al. 2001). Surprisingly, lactate does not have any effect on the affinity nor the Bohr factor in this species (Chausson et al. 2001).

In arthropod hemocyanins, there usually is a decrease of oxygen affinity when the temperature increases, as a consequence of the exothermic nature of this reaction (Truchot 1992). Interestingly, all the hemocyanins from hydrothermal vent species show either no effect or a slightly reverse effect of temperature on oxygen affinity (Sanders et al. 1988; Lallier and Truchot 1997; Lallier et al. 1998; Chausson et al. 2001, 2004). More detailed studies will determine whether the temperature insensitivity is due to specific adaptations in the hemocyanin molecule itself or the result of side effects of the overall oxygenation reactions. Whatever the cause of this temperature insensitivity, this may well be an adaptation to the highly variable conditions (for which temperature is an indicator) encountered at deep-sea hydrothermal vents: despite



**Fig. 5** P<sub>50</sub> values for decapod hemocyanins from vent-endemic species compared to some representative non-vent species. Experimental conditions and references: *Eupagurus bernhardus* 15°C, pH 7.83; *Penaeus japonicus* 25°C, pH 7.6; *Palaemon adspersus* 15°C, pH 7.85; *Nephrops norvegicus* 10°C, pH 7.9; *Carcinus maenas* 15°C, pH 7.84; *Callinectes sapidus* 20°C, pH 7.6; *Homarus vulgaris* 15°C, pH 7.9; *Holthuisana transversa* 25°C, pH 7.35; *Corystes cassivelaunus* 10°C, pH 7.9; *Austropotamobius pallipes* 15°C, pH 7.9 (all these species, see review

by Truchot (1992)); *Cyanagraea praedator* 15°C, pH 7.5 (Chausson et al. 2001); *Chorocaris chacei* 15°C, pH 7.5 (Lallier et al. 1998); *Bythograea thermydron* 15°C, pH 7.5 (Sanders et al. 1988); *Rimicaris exoculata* 15°C, pH 7.5 (Lallier and Truchot 1997); *Segonzacia mesatlantica* 15°C, pH 7.5 (Chausson et al. 2004); *Alvinocaris lusca* 15°C, pH 7.5 (Sanders et al. 1988). \* fresh-water species. \*\* species with mixed land/water way of life. \*\*\* species living buried in the sand

the changes in temperature, the affinity of hemocyanin for oxygen will remain high. This characteristic explains at least in part why the critical PO<sub>2</sub> varies little between 2°C and 20°C (Mickel and Childress 1982b). The lack of temperature sensitivity of oxygen binding has also been observed for shallow water crustacea that are exposed to highly variable temperatures

such as the hermit crab *Pagurus bernhardus* (Jokumsen and Weber 1982) and the shrimp *Palaemon elegans* (Morris et al. 1985). In this context, the study of cold-seep relatives of the vent species would be most interesting to determine whether these species that are used to cold water (with little or no variations of temperature) have temperature-sensitive hemocyanins.

In the crab *Cyanagraea praedator* and in the shrimp *Rimicaris exoculata*, there is another factor—whose identity is still unknown—that decreases the oxygen affinity of hemocyanin and that can be eliminated by dialysis (Lallier and Truchot 1997; Chausson et al. 2001). This phenomenon has also been observed for terrestrial crabs of the family Ocypodidae (Morris and Bridges 1985; Bridges et al. 1997), and in the coconut-tree crab *Birgus latro* (Morris et al. 1988). In *Ocypodes serratan*, this small molecule (<5,000 Da) is capable of binding large amounts of CO<sub>2</sub> (Bridges et al. 1997). The factor remains to be identified and its effect on the hemocyanin studied.

In a study on Mid-Atlantic Ridge hydrothermal vent crab *Segonzacia mesatlantica*, Chausson et al. (2004) investigated the effect of severe hypoxia (6.25  $\mu$ M O<sub>2</sub>, i.e., 3.7 times less than the critical PO<sub>2</sub> below which *Bythograea thermydron* oxygen consumption drops and the animal goes anaerobic) on short term—6 h—changes in hemocyanin composition in the hemolymph. Comparing two batches of crabs, one maintained in severe hypoxia, and the other in saturation with air (referred to as “hyperoxia”), the authors report an increase of the hexameric (from 68% to 72%), the dodecameric (16% to 18%), and a decrease of the monomeric (from 13% to 7%) fractions. However, the overall chain composition did not change significantly. Unfortunately, the data were obtained from pools of 4 crabs for each condition, which does not give access to inter-individual variability. This is especially important because the dodecameric-to-hexameric fractions ratio may be variable in natural populations, as has been shown in the blue crab *Callinectes sapidus* (Greaves et al. 1992).

Some species of hydrothermal vent crustacea have been reported to possess hemoglobin: the copepods *Benthoxynus spiculifer* (Hourdez et al. 2000b) and *Scotoecetes introrsus* (Sell 2000). Although Hb has been reported in some bottom-dwelling littoral and lake species (Fox 1957), little is known about Hbs of these animals probably because of their small size. Interestingly, these Hb-containing copepods identified by Fox (1957) were collected from muddy and reduced environments with low O<sub>2</sub> and high sulfide levels.

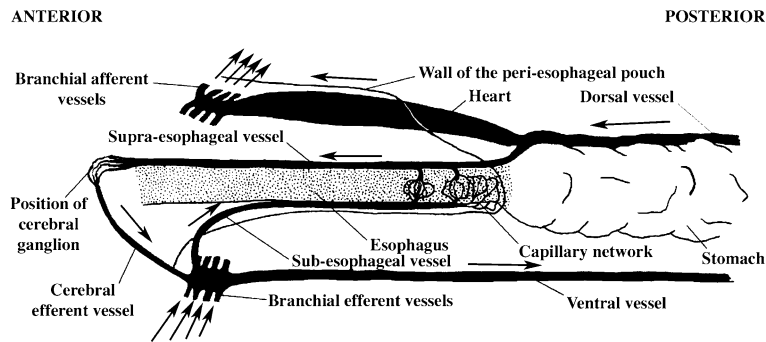
In the Branchiopod *Daphnia magna*, Hb synthesis is induced by ambient hypoxia (Fox et al. 1951; Kobayashi and Hoshi 1982) and animals with high Hb concentrations are able to maintain normal oxygen consumption rates even at low O<sub>2</sub> tensions that are lethal for individuals with low Hb (Kobayashi and Hoshi 1984). The low hemoglobin concentrations reported for the two vent copepod species make it very unlikely that the hemoglobin could be used as a significant oxygen store (Hourdez et al. 2000b; Sell 2000). The very high affinity of *B. spiculifer* hemoglobin also indicates that its main function is to maintain a concentration gradient from the outside to the inside of the animal, even when environmental PO<sub>2</sub> are very low (Hourdez et al. 2000b). Hemoglobin thus extends the inhabitable environment of small crustaceans like copepods and allows them to inhabit the base of tubeworm bushes.

### 3.3 Circulatory system adaptations

In alvinellid polychaetes, the gular membrane extends posteriorly, enveloping the esophagus and a very developed capillary network (Fig. 6). In addition, the peri-esophageal pouch thus formed contains very numerous erythrocytes (coelomocytes with high concentrations of hemoglobin). This setup, with a high density of capillaries and coelomocytes, forms an internal gas exchange system (Jouin-Toulmond et al. 1996). A study of the functional properties of the Hbs (the coelomic, intracellular Hb and the vascular, extracellular Hb) showed that they possess very similar functional properties under the same conditions, thereby allowing bidirectional transfer in the gas exchange system (Hourdez et al. 2000a). This most likely buffers the variations of oxygen levels inside the body, in particular for the brain area, directly irrigated by the blood after it goes through the capillary network (Fig. 6).

## 4 Adaptations of oxygen cellular utilization

Even though the species studied possess specific adaptations that allow them to extract oxygen despite its low levels in the environment, they most likely often have to rely on anaerobic metabolism. In addition, sulfide can inhibit the



**Fig. 6** Anterior circulatory system in *Alvinella pompejana* showing the periesophageal pouch containing the capillary network. Coelomocytes (not represented) are very dense in the pouch and in close contact with the capillaries. Oxygenated blood coming from the gills (branchial

efferent vessels) merge ventrally and the sub-esophageal vessel can carry this blood to the capillary network where it exchanges gases with the coelomocytes. These capillaries then fuse with the supra-esophageal vessel and oxygen can be sent directly to the cerebral ganglion

electron transport chain (Powell and Somero 1986) and anaerobic metabolism may be happening even when oxygen is not completely absent. Hand and Somero (1983) measured enzymatic activities in order to determine which metabolic pathways were present in *Calyptogenia magnifica*, *Riftia pachyptila*, *Bythograea thermydron*, and *Alvinella pompejana*. All the species studied exhibit cytochrome c oxidase and citrate synthase activities, both indicative of aerobic metabolism. The use of aerobic metabolism was also supported by the high activity of two glycolytic enzymes, phosphofructokinase and pyruvate kinase.

Several hydrothermal vent and cold-seep species have been studied for their ability to survive periods of anoxia. The crab *Bythograea thermydron* can survive at least 12 h (Mickel and Childress 1982b), the clam *Calyptogenia magnifica* at least 16 h at 14°C (Arp et al. 1984), and the siboglinid polychaete *Riftia pachyptila* at least 36 h at 8°C (Childress et al. 1984), and up to 60 h at 15°C (Arndt et al. 1998). In the cold-seep polychaete *Methanoraria dendrobranchiata*, a  $TL_{50}$  of 133.5 h in anoxia without sulfide was calculated (Hourdez et al. 2002). The ice-worm *Hesiocaeca methanicola*, a hesionid polychaete that lives on methane ice, has a  $TL_{50}$  of 99 h in complete anoxia (Fisher et al. 2000). All these data indicate a good capacity of these species to survive under anoxia for extended durations. This also indicates a good capability for anaerobic metabolism.

In *B. thermydron*, Hand and Somero (1983) detected high levels of lactate dehydrogenase (a typical enzyme for anaerobic metabolism in crustacea). Both the siboglinid *R. pachyptila* and the clam *C. magnifica* exhibited high level of malate dehydrogenase, a typical enzyme for anaerobic metabolism in invertebrates other than crustacea. In *Alvinella pompejana*, phosphoenolpyruvate carboxykinase, alanopine dehydrogenase, strombine dehydrogenase, and lactate dehydrogenase activities, markers of anaerobic metabolism, were also detected (Desbruyères et al. 1998). This observation indicates either that the worms were exposed to hypoxia prior to collection or that they are pre-adapted to hypoxia and continuously express these enzymes.

Arndt et al. (1998) studied the effect of prolonged anoxia on *Riftia pachyptila*. These authors followed the levels of malate, glycogen, aspartate, and succinate in the tissues for 60 h. From the high levels (up to 26 mM in the blood) encountered under normoxia, malate quickly drops and levels of succinate rise to very high values (up to 17 mM in the blood). Succinate is a well known final product of anaerobic metabolism in annelids, along with short-chain volatile fatty acids (Grieshaber et al. 1994). However, none of these fatty acids were excreted by the worm. Glycogen, mainly found in the trophosome, was only used after 48 h of anoxia. Succinate formed during anoxia cannot account for the decrease of malate and glycogen, suggesting that there might

be other end products for *Riftia*'s anaerobic metabolism. Arndt et al. (1998) also report the absence of alanopine dehydrogenase, octopine dehydrogenase, and strombine dehydrogenase activities. Lactate dehydrogenase activity was found to be moderate in the vestimentum and in the body-wall, and absent in the other tissues tested. However, only traces of L-Lactate could be detected in the worms after anoxia exposure, suggesting a limited importance of this pathway in anaerobic metabolism. Other opine dehydrogenases activities (using lysine, taurine, etc...) were not tested.

## 5 Dealing with sulfide

At hydrothermal vents, oxygen and sulfide concentrations vary inversely (Johnson et al. 1988). Sulfide has a specific effect that affects the survival of animals. The cold-seep polychaete *Methanoaricia dendrobranchiata* has a  $TL_{50}$  of 99.1 h in anoxia +1 mmol  $l^{-1}$  sulfide, 119.6 h in anoxia +60  $\mu$ mol  $l^{-1}$  sulfide, and 133.5 h in anoxia without sulfide (Hourdez et al. 2002).

Sulfide can poison the mitochondrial electron transfer chain and disrupt the aerobic metabolism (Grieshaber and Völkel 1998). This may explain at least in part the high levels of enzymes involved in anaerobic metabolic pathways as the organisms may have to rely on it even if oxygen is present as a result of sulfide poisoning. In addition, sulfide can bind to hemoglobins and poison oxygen transport.

Organisms that are symbiotic with sulfide oxidizing bacteria may be protected from sulfide toxicity by the sink the bacteria represent. However, when the bacteria are not located in tissues directly in contact with the sulfide-containing water, the organism must transport sulfide without being poisoned by it. Sulfide-binding molecules are then necessary. Although other annelid hemoglobins may bind sulfide none can bind such large amounts as *Riftia pachyptila* HBL hemoglobin, that transports both sulfide and  $O_2$  from the environment to the symbiotic bacteria in the trophosome, avoiding both poisoning by free sulfide and spontaneous reaction between the two molecules. The site of sulfide binding is still currently debated (Zal et al. 1998; Flores et al.

2005; Numoto et al. 2005; Flores and Hourdez in press) but it appears clear that it is not bound to the heme group. Based on crystal structure data, Flores et al. (2005) challenged the idea that sulfide was bound to free cysteines in the 400 kDa Hb from *Riftia* (Zal et al. 1998). The crystal data showed that the free cysteines were located in highly hydrophobic pockets that should keep the sulfide ions from binding. Alternatively, the Hb possesses zinc ions at specific locations (bound to histidine residues) that could be sites for sulfide binding. Flores et al. (2005) and Flores and Hourdez (in press) also reported that specific chelation of the zinc ions by TPEN inhibited the binding of sulfide while this binding was unchanged after capping of the free cysteines by NEM. A similar crystallographic study on the 400 kDa Hb from *Oligobranchia mashikoi* (another siboglinid polychaete) showed that the free cysteines were accessible to ethyl-mercury ions and that there were no zinc ions (Numoto et al. 2005). The specific amino acids involved in the binding of zinc in *Riftia* 400 kDa Hb are not conserved in the Hb from *O. mashikoi*. Interestingly, this latter species has been shown to possess methanotrophic (instead of sulfide-oxidizing) symbionts (Kimura et al. 2003), thereby relieving the selective pressure to bind sulfide to transport it to the symbionts. Numoto et al. (2005) also report that the amount of free sulfide in solution decreased in presence of that Hb, suggesting that it did bind sulfide. Some uncertainty remains though as they did not try to release that sulfide to show it was bound and not oxidized (Flores and Hourdez in press). Although the clam *Calyptogena* body fluid does bind sulfide (and transports it from its foot that extends in deep cracks in the basalt to its symbionts in its gills) the carrier is not Hb but a large (several million Dalton) zinc-rich molecule (reviewed in Childress and Fischer 1992) that is a heme-free, glycosylated protein and could be a lipoprotein (Zal et al. 2000b).

Species that do not have body fluids capable of binding sulfide may oxidize sulfide in their mitochondria and even get some ATP out of the process such as in the polychaetes *Arenicola marina* (Völkel and Grieshaber 1997), and the bivalves *Geukensia demissa* (Parrino et al. 2000;



Doeller et al. 2001; Kraus and Doeller 2004) and *Solemya reidi* (O'Brien and Vetter 1990).

## 6 Conclusion

From the limited studies conducted so far on deep-sea hydrothermal vents or cold seep endemic species, anatomical and biochemical approaches have identified general trends regarding their respiratory adaptations to their hypoxic environment. Large branchial surface areas and abundant oxygen binding proteins seem to be shared by crustaceans and annelids inhabiting these extreme environments with an inclination to develop oxygen storage mechanisms to buffer the variations of oxygen supply in the water. Adaptations to low oxygen levels in invertebrates from hydrothermal vents and cold seeps seem similar. It seems however that the seep species are able to withstand longer exposure to anoxia, which may be an adaptation to the more stable conditions encountered at seeps. In this aspect, benthic species from oxygen minimum zones (OMZ) may show capabilities similar to those of the seep species. Very few studies are available to date but we know that some OMZ species exhibit enlarged gas exchange surfaces, and high activities of enzymes involved in anaerobic metabolism (for review see Levin 2003).

However, in vivo physiological experiments, although difficult to conduct on these hard-to-reach animals, are definitely needed in order to show that the anatomical or biochemical adaptations identified so far are really at work in these animals and allow them to maintain an aerobic metabolism comparable to littoral relative species despite the potential toxicity of their surroundings (sulfide, heavy metals).

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