Can the hemoglobin characteristics of vesicomyid clam species influence their distribution in deep-sea sulfide-rich sediments? A case study in the Angola Basin

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Abstract

Vesicomyids live in endosymbiosis with sulfur-oxidizing bacteria and therefore need hydrogen sulfide to survive. They can nevertheless live in a wide range of sulfide and oxygen levels and depths, which may explain the exceptional diversity of this clam family in deep-sea habitats. In the Gulf of Guinea, nine species of vesicomyid clams are known to live in cold-seep areas with pockmarks from 600 to 3200m deep, as well as in the organic-rich sediments of the Congo deep-sea fan at 5000 m deep. Our previous study showed that two species living in a giant pockmark have different oxygen carriers, suggesting different adaptations to hypoxia. Here, we studied the hemoglobin structure and oxygen affinity in three other species, *Calyptogena valdiviae*, *Elenaconcha guiness* and *Abyssogena southwardae* to determine whether the characteristics of their oxygen carriers contribute to their distribution in sulfide-rich sediments at a regional scale. Documenting pairwise species associations

in various proportions, we give a semi-quantitative account of their local distribution and oxygen and sulfide measurements at seven sites. Mass spectrometry showed that each vesicomyid species has four intracellular monomeric hemoglobin molecules of 15-16 kDa, all differing in their molecular mass. As expected, the monomers showed no cooperativity in oxygen binding. Their oxygen affinities were very high (below 1 Torr), but differed significantly. *C. valdiviae* had the highest affinity and was dominant in the Harp pockmark, the site with the lowest oxygen content (half the value of fully oxygenated water). *A. southwardae* dominated in the Congo Lobe area, the site with the deepest sulfides. We discuss how hemoglobin may favor an active, vertical distribution of vesicomyids in sulfide-rich sediments.

Keywords:

hemoglobin, mass spectrometry, oxygen affinity, symbiont-bearing bivalve, blood-clams, cold seeps, sulfide-rich sediments.

Introduction

Vesicomyid clams are heterodont bivalve mollusks that live worldwide from 100 to 9500 m depth and commonly colonize hydrothermal vents, cold seeps and whale falls (Krylova and Sahling, 2010; Sibuet and Olu, 1998). To date, about 120 different species have been identified from morphological diagnosis, COI sequencing or both (Audzijonyte et al., 2012; Krylova and Sahling, 2010). In the vesicomyid family, the Pliocardiinae subfamily has a broad bathymetric distribution at the oceanic scale, but only one genus is eurybathic (the small-sized *Isorropodon*); all the other large-sized genera inhabit either the bathyal or abyssal zones (Krylova and Sahling, 2010). Several authors have argued in favor of a depth-segregation hypothesis (Goffredi et al., 2003; Kojima and Ohta, 1997; Olu et al., 1996), mainly driven by species-specific differences in egg buoyancy, larval development and

dispersal, but possibly also due to other pressure-related physiological or biochemical adaptations among juveniles and adults (Goffredi et al., 2003).

At the species level, several studies have demonstrated a trans-oceanic distribution for genetically similar species (Audzijonyte et al., 2012; Decker et al., 2012b; Kojima et al., 2004; Teixeira et al., 2013; van der Heijden et al., 2012). Audzijonyte et al. (2012) identified seven species with a trans-Pacific distribution, and two species with an Indo-Pacific distribution. *Abyssogena southwardae* shows a trans-Atlantic distribution along the equatorial belt, colonizing seeps on the western and eastern continental margins, as well as vents on the Mid-Atlantic Ridge (Teixeira et al., 2013). This ocean-wide species distribution indicates either long-distance larval dispersal and/or unknown stepping-stone habitats that enable gene flow to and from either side of the ocean on a generational time-scale (Audzijonyte et al., 2012; Teixeira et al., 2013).

At a regional scale, studies have demonstrated that the environmental characteristics of the biotope likely account for the patchy distribution of species (Barry et al., 1997; Goffredi and Barry, 2002; von Cosel and Olu, 2009). More locally, the distribution of two species co-occurring in unequal proportions in a cold-seep site may arise from their different biological or physiological characteristics. For example in the Monterey Bay, *Calyptogena pacifica* (Dall, 1891) and *Phreagena kilmeri* (Bernard, 1974) seem to have different reproductive strategies (Lisin et al., 1997), different physiological metabolisms with regard to sulfide binding in the hemolymph (i.e. “blood”) and different sulfide uptake by symbionts (Barry et al., 1997; Goffredi and Barry, 2002). In the Gulf of Guinea, *Laubiericoncha chuni* and *Christineconcha regab* differ by the presence or absence of an oxygen carrier in their hemolymph, an adaptation to hypoxic events (Decker et al., 2014).

Sulfide and oxygen are two main factors in vesicomyid biology, because these clams live in symbiosis with sulfide-oxidizing bacteria that are maternally acquired during embryogenesis (Cary and Giovannoni, 1993; Endow and Ohta, 1990; Szafranski et al., 2014). The symbionts are housed within bacteriocytes in the gills, and provide the bivalve with carbohydrates (Cavanaugh, 1983; Fiala-Médioni and Le Pennec, 1987). To fuel its symbionts, the clam takes up sulfides via its foot buried in

the sulfide-rich sediments, whereas oxygen and carbon dioxide are supplied by the seawater, above the sediments, flowing through the siphons (Arp et al., 1984). These elements are then distributed to all tissues via the hemolymph. To date, the hemolymph of seven vesicomyid species has been analyzed and all, except one, show hemoglobin in their red blood cells (Kawano et al., 2003; Suzuki et al., 2000; Suzuki and Ohta, 2000; Suzuki et al., 1989a; Suzuki et al., 1989b; Terwilliger et al., 1983; Zal et al., 2000). In vesicomyids, the structure and number of hemoglobins vary among species, from monomers in *L. chuni* (Decker et al., 2014) to tetramers in "*Calyptogena" magnifica* Boss and Turner, 1980 (Terwilliger et al., 1983; Zal et al., 2000). Variations in hemoglobin structure affect oxygen affinity. Circulating hemoglobin greatly enhances oxygen transport, but may also promote oxygen storage during hypoxic periods (Weber and Vinogradov, 2001). Thus, the preliminary hypothesis of our study was that the most "efficient" hemoglobin (i.e. able to bind oxygen with the greatest affinity) facilitates survival in the most extreme habitat (i.e. subject to hypoxia with reduced or sulfide-rich sediments). To test this hypothesis, we analyzed the distribution of the various vesicomyid species in the Gulf of Guinea.

The present study investigated whether the characteristics of the vesicomyid hemoglobins drive the observed distribution of vesicomyid species in various sulfide-rich environments on the Gabon and Congo margins. The clam habitats are found either in cold seeps in pockmarks or in areas of organic matter accumulation in the Congo deep-sea fan, both of which are more or less hypoxic and reduced habitats. We analyzed the hemoglobins of three vesicomyid species (*Calyptogena valdiviae, Elenaconcha guiness,* and *A. southwardae*) to (1) detail their molecular structure and (2) measure their oxygen affinity. We compiled our data with those from a previous globin study on *L. chuni* and *C. regab* at the Regab pockmark (Decker et al., 2014). We compared our results with the clam species distribution along the bathymetric gradient, taking into account the chemical characteristics of their biotopes, especially the oxygen and sulfide levels around the clams.

Study Area

The analysis was performed on three vesicomyid species from the Gulf of Guinea. In this gulf, four clam species have been known since the Valdiviae expedition in the 1930s (Thiele and Jaeckel, 1931). Recent explorations using a remotely operated vehicle (ROV) increased the number of known vesicomyid species to nine (Rodrigues et al., 2012; Teixeira et al., 2013; von Cosel and Olu, 2009), each associated with a different geological setting (Table 1). Six of them have been extensively studied for their molecular taxonomy, biology, or symbiosis (Decker and Olu, 2012; Decker et al., 2013; Decker et al., 2014; Duperron et al., 2012; Rodrigues et al., 2012; Teixeira et al., 2013). The various chemosynthesis-based ecosystems known so far in the Gulf of Guinea have a broad bathymetric range: from 500 m depth for the Guiness pockmarks along the Gabon margin, to 5000 m in the terminal lobes of the Congo deep-sea fan (Table 1, Figure 1) (Olu-Le Roy et al., 2007; Ondréas et al., 2005; Sahling et al., 2008; Sibuet and Vangriesheim, 2009). Several pockmarks with sulfide-rich sediments and carbonate concretions (Sibuet and Vangriesheim, 2009) have been found in the Guiness pockmark area (Figure 1). Despite their great patchiness and apparently low methane fluxes, (Sibuet and Vangriesheim, 2009), seeps support a large diversity of chemosynthetic symbiont-bearing taxa. Among the vesicomyids, three species have been recorded: *C. valdiviae* (Thiele and Jaeckel, 1931), *E. guiness,* Cosel and Olu 2009 and *Isorropodon bigoti,* Cosel and Salas 2006. The two former species colonize two different pockmarks (Harp and Kilkenny), where variable fluid emissions may explain their distribution (von Cosel and Olu, 2009). The small *I. bigoti* clam is rarely observed and is buried in the sediment at Kilkenny (Rodrigues et al., 2012).

About 500 km further south, Worm Hole and Regab are two large pockmarks or pockmark clusters at a similar depth (about 3000-3200 m), about 100 km apart (Figure 1). Regab is on the northern slope of the Congo canyon, and its faunal assemblages have been described in detail (Menot et al., 2010; Olu-Le Roy et al., 2007). Worm Hole and Regab share several species, such as the mussel *Bathymodiolus boomerang* and the siboglinid tubeworm *Escarpia southwardae* (Olu-Le Roy et al., 2007; Sahling et al., 2008). However, the vesicomyid species differ: *C. regab* and *L. chuni* both live in

Regab but are not found at Worm Hole, which seems to be colonized only by a few clustered individuals of *A. southwardae* (Krylova et al. 2010;(Teixeira et al., 2013).

West of Regab, the terminal lobes of the Congo deep-sea fan (Figure 1) are located 750 km away off the coast at 5000 m depth. They are fed by turbidites containing labile organic matter delivered by the Congo River (Rabouille et al., 2009; Savoye et al., 2009). This material is mostly of terrigenous origin, with an estimated terrestrial contribution as high as 79±3% in sites on or near the active channel (Stetten et al., 2015). The lobe area is characterized by high to very high sedimentary rates for this depth (0.6 to 22 cm yr^{-1}, i.e. 2-3 orders of magnitude higher than values usually recorded at these depths) (Rabouille et al., in press this volume). This sedimentation results in sediments with very high organic carbon content (3 to 5% in wet weight), compared with what is usually found in abyssal sediments (generally <0.3% and rarely 1%) (Stetten et al., 2015). These lobes are characterized by very patchy colonization of chemosynthesis-based ecosystems, including microbial mats and vesicomyid beds (Sen et al., submitted). Two different species of vesicomyids have been sampled there: *A. southwardae* and *C. regab* (Decker et al., 2014). Both species are associated with sulfide-oxidizing symbionts (Decker et al., in prep.), which are sustained by sulfides produced locally from anaerobic methane oxidation and oxidation of organic matter (Pastor et al., Submitted).

Methods

Sampling strategy

Vesicomyid bivalves were collected during two scientific cruises aboard the R/V *Pourquoi Pas?* using the ROV *Victor 6000*: the WACS cruise (in January-March 2011, chief scientist Karine Olu-Le Roy) and the CONGOLOBE cruise (from December 2011 to January 2012, chief scientist: Christophe Rabouille). *C. valdiviae* and *E. guiness* specimens were collected in the Guiness area on the Harp and Kilkenny pockmarks (Table 2, Figures 1 and 2). *A. southwardae* was collected at the Worm Hole pockmark and in the terminal lobes of the Congo deep-sea fans (CoL02 (Site A), CoL03 (Site F), CoL09 (Site C) and

CoL11 (Site B)) (Table 2, Figure 2). *C. regab* was also collected in the terminal lobes of the Congo deep-sea fan together with *A. southwardae*. Specimens were collected using nets and blade cores manipulated by the ROV. Total clam densities per square meter were estimated from blade core-samples (0.0303 m^2) (1 to 4 replicates). The proportion of each species was estimated by totaling? the number of individuals of each species counted in nets and blade cores at each site. The authors (KO and CD) sorted the collected species, and Dr. Elena Krylova, a specialist in vesicomyid taxonomy, checked their identifications on the basis of morphological criteria. Molecular identifications were also done to ensure the identification of the species from the Congo deep-sea fans (Teixeira et al., 2013; S. Arnaud-Haond, personal communication).

Chemical measurements in the habitats

Tube-core samples (6 cm diameter, 30 cm length) were taken in the clam aggregates (beds) to characterize the oxygen and sulfide profiles of the studied sites (Pastor et al., Submitted). The CALMAR benthic chamber (Caprais et al., 2010) was also deployed on vesicomyid beds to measure gas exchange (oxygen and methane) at the sediment-water interface (Khripounoff et al., 2015; Khripounoff et al., in press this volume).

Oxygen microsensors (Unisense) were used to measure oxygen concentration profiles in the sediment cores sampled by the ROV, following the method described in Rabouille et al. (2009). For the other tube cores, pore water was extracted at a resolution of 1-2 cm using Rhizon moisture samplers (Seeberg-Elverfeldt et al., 2005) inserted into the holes of pre-drilled push-core liners. $ZnCl_2$ was added immediately after pore water extraction for sulfide analysis. Oxygen concentration in the water column above vesicomyids was obtained by chemical analysis of 10 ml of water sampled *in situ* with the CALMAR using the modified Winkler titration method (Carritt and Carpenter, 1966). Methane concentration was determined using static headspace gas chromatography (HSS-GC) (Sarradin and Caprais, 1996). At the laboratory, pore water sulfide concentrations were analyzed using standard photometric procedures (Cline, 1969; Fonselius, 1983).

Hemoglobin analyses

After retrieval, bivalves were quickly transferred to a cold room at 4°C. Three specimens of *C. valdiviae* and *E. guiness* and five of *A. southwardae* (3 from the Lobe area and 2 from Worm Hole) were dissected for the hemoglobin analysis. The others were identified, counted, measured and stored in 4% buffered formalin. Hemolymph was sampled using a 1 mL syringe, inserting the needle between the valves at the anterior end of the clam, or after opening it (Table 2). All dissected samples were frozen in liquid nitrogen and stored at -80°C in the laboratory until further use. For each of the three vesicomyid species, three individuals (exceptionally two) were successively analyzed by high-performance liquid chromatography (HPLC), mass spectrometry (MS) and in the diffusion chamber (Table 2). We purified the hemolymph by HPLC (size-exclusion chromatography, i.e. SEC) on a Superose-12 10/300 GL Tricorn column (GE Healthcare), with a fractionation range of 1 to 300 kDa. We performed two types of SEC electrospray-ionization mass spectrometry (ESI-MS) analyses: one in native conditions and one in denaturing conditions. In the native conditions, we used 50 mM ammonium acetate (pH 6.2) for SEC elution and for ESI-MS. Ammonium acetate preserves the non-covalent interactions between the molecules in their original state and allows identification of the various hemoglobin molecules in the exact multimeric state they have in the hemolymph. The second analysis in denaturing conditions aims to separate the multimers, but not break the disulfide bridges. For this analysis, we purified the hemolymph samples by SEC in a saline HEPES buffer, and then performed the ESI-MS analyses in a mix of water/acetonitrile/formic acid to expose the inner structure of the molecule for better ionization. Further technical conditions about ESI-MS are detailed in (Decker et al., 2014).

Oxygen-binding affinities of hemoglobins were obtained in a gas-diffusion chamber (Sick and Gersonde 1969) as described in (Decker et al., 2014). The pH was measured with a microelectrode. Oxygen-binding properties and pH measurements were carried out at 5°C. Technically, 5°C is the lowest possible temperature to measure the oxygen partial pressure (PO_2) at which hemoglobin is

half saturated with oxygen (P_{50}) and avoid water condensation in the sample. Therefore, this is the closest possible temperature to the deep-seawater temperature (2.6°C at Regab pockmark (Decker et al., 2012a), 5.3°C at Guiness and 2.4°C in the Lobe area).

Values of P_{50} and n_{50} (Hill's cooperativity coefficient at P_{50}) were derived from the linear regression of the Hill plots ($\log(S/1-S)=f(\log(PO_2))$) for saturation values in the linear portion of the curve, i.e. at saturation values between 20-80%.

Statistical methods

We used a Kruskal-Wallis test followed by a Behrens-Fisher (BF) post-hoc test for non-parametric multiple comparisons of the P_{50} values between species. All analyses were performed using the free open source R Environment (R Development Core team, 2010). The NPMC library was used for non-parametric multiple comparisons (Helms and Munzel, 2008)

Results

Habitat characterizations and clam densities

Seven sites were sampled along the Congo-Angola margin in three areas: Guiness, Worm Hole and the Congo deep-sea fan (Table 3, Figure 2).

Guiness area

In the Guiness area (Fig. 1), vesicomyids were observed in the two pockmarks Harp and Kilkenny, but the other pockmarks only revealed bivalve shells and concretions. Harp and Kilkenny are relatively small, about 300 m diameter and are located 2 km apart, at 580 to 680 m depth, respectively. The density of vesicomyids was lower at Harp than at Kilkenny (165 *vs* 561 ind.m^{-2} respectively) (Table 3) At Harp, we observed a few small beds (about 50 cm diameter) of *C. valdiviae* (Fig. 2A). In contrast, Kilkenny was colonized by numerous small patches of *E. guiness* and *C. valdiviae*, which appeared to be segregated among the microbial mats, which covered an area of about 15 m in diameter (Figure 2B). Both pockmarks were characterized by low oxygen concentrations in the water column (about

140 µM, Table 3), but had the deepest measured oxygen penetration depths in the sediment (0.65 cm, Table 3). At Kilkenny, high sulfide concentrations and fluxes were measured in the vesicomyid bed dominated by *E. guiness* (Table 3, Figure 2B), whereas there were no sulfide fluxes at the Harp pockmark, where only *C. valdiviae* was sampled (Table 3, Figure 2A).

Worm Hole area

The Worm Hole pockmark (Fig. 1) is found at 3090 m depth. The sulfide concentration was high in the sediment with a maximal value at 14 cm among the few isolated individuals of *A. southwardae* that colonized the area (Table 3, Figure 2C).

The Congo deep-sea fan (Lobe area sites)

In the canyon of the Congo deep-sea fan, *C. regab* and *A. southwardae* co-occurred in mixed patches, but in various densities and proportions (Table 3, Figure 2D-G). At site B, which was located in a lobe not currently in the axis of the Congo channel, we sampled a patch of about 130 ind.m^{-2} with both species, where *A. southwardae* dominated (Figure 2D). In contrast, at sites A, F and C located in the axis of the Congo channel, *C. regab* was always the dominant species (Table 3). At site A, vesicomyids formed dense aggregates of about 750 ind.m^{-2} (Table 3) along the southern slope of the canyon in the "Vesico Bay" area (Figure 2E). At site F, vesicomyids were found in low density (about 120 ind.m^{-2}) surrounding microbial mats on the top of a small hill (Figure 2F). At site C, vesicomyids were sampled in a very dense bed (about 1390 ind.m^{-2}), in a heart-shaped patch (Table 3, Fig. 2G).

In all four study sites in the Lobe area, oxygen concentrations in the water above the vesicomyids ranged from 215 to 240 µM (Table 3). Oxygen penetration in the sediment was deeper at sites B and F than at the other sites (Table 3). The sulfide concentration in the sediment was highest at site A (8 mM) where it peaked at 10 cm below surface, and the sulfide peak (maximum concentration within the upper layer of the sediments) varied from 2.5 cm at site F to 17 cm at site B.

Structural analysis of the hemoglobins

The three study species had hemoglobin in their blood cells. The volume of blood collected from a single clam ranged from 600 µl to 1.5 ml in *C. valdiviae and E. guiness,* and from 2 to 10 ml in A. *southwardae.* The SEC chromatograms of the hemolymph of all three species are shown in Figure 3. On all spectra, the first minor fraction close to the exclusion volume of the column absorbed mainly at 280 nm, corresponding to protein aggregates. The heme protein profiles of the hemolymph from *C. valdiviae* and *E. guiness* showed two very close peaks eluting in the narrow time range of 25-27 min (Figure 3A, B), but *A. southwardae* from both sites showed only a single peak (Figure 3C, D). This single peak and each fraction of the double peaks were purified and analyzed separately using MS. For all vesicomyid species, the MS analysis showed that each peak contains monomers of slightly varying masses within the range of 15-16 kDa. Regarding the double peaks of *C. valdiviae* and *E. guiness*, the peak that eluted first contained a mixture of two monomers: the heaviest and the lightest monomers characterized for each vesicomyid species. The peak that eluted last contained a mixture of all four monomers identified in each respective species (Table 4). Moreover, measurements on the native hemolymph of *C. valdiviae* and *E. guiness* showed the presence of only monomeric units in the natural state of the hemolymph, and no dimerization (Figure 4A, B). The single peak in *A. southwardae* also corresponded to four different monomers of about 15-16 kDa that showed no dimerization either.

The expected error on a mass result is about 0.005% according to the ESI-MS instrument specifications. On each sample, several mass measurements were taken and the results were averaged to estimate mass precision. A comparison of measurements showed that these percentages of error and the observed precision can identify similar masses as being identical molecules with an uncertainty of about 1 Da. We thus sorted the monomers? by increasing molecular mass and called them globin G1, G2, etc. The masses of the globins of *C. valdiviae* and *E. guiness* were very similar, but those of *E. guiness* were always 6 Da lighter than those of *C. valdiviae* (with the exception of chain 4 that was only 4 Da lighter). *A. southwardae* also had four different

monomeric globin chains (Figure 4C), with masses that were identical (at the precision of 1 Da) among individuals from the same site, but only two globin masses were common to all specimens sampled at Worm Hole and Lobe B (Table 4).

Oxygen-binding properties

Oxygen-binding properties were measured on the purified hemoglobins (Figure 5) at 5°C and pH 6.5. The value of 6.5 was the pH measured in the hemolymph just after collection on board at 5°C. The oxygen-binding affinities were very high and lower than 1 Torr for all three species (Figure 5). *C. valdiviae* showed the greatest affinity with a P_{50} of 0.13 ± 0.01, which was significantly lower than that of the other species, with values of 0.24 ± 0.04 for *E. guiness* and 0.21 ± 0.06 and 0.32 ± 0.03 for *A. southwardae* at Worm Hole and site xx, respectively (Kruskal-Wallis test, $p<0.05$ and non-parametric pairwise multiple comparisons, $p<0.001$). All measurements were significantly different, except the values of *A. southwardae* from the Lobe area and *E. guiness* from the Guiness area (non-parametric multiple comparisons, $p = 0.9$). There was no cooperativity (n_{50}) in any of the molecules because n_{50} was always close to 1.

Discussion

In the Gulf of Guinea, four out of five analyzed vesicomyid species have intracellular circulating hemoglobin (this work and Decker et al, 2014). They live at 600 to 5000 m depth. Other studied vesicomyids include four species from cold seeps along Japan (1000-3600 m) and the vent species *C. magnifica* from the East-Pacific Rise (2600 m depth) (See Table 4 for details). The extensive depth range of clams with hemoglobin (and even the one without), suggests that circulating hemoglobins in vesicomyid clams are not the factor that determines the distribution of the vesicomyids species between the bathyal and the abyssal zones. Nonetheless, in the Gulf of Guinea, we recorded *C. valdiviae* and *E. guiness* only at Guiness pockmarks (580-680 m depth), whereas *A. southwardae, L.*

chuni and *C. regab* (the latter with no hemoglobin) were found in deeper pockmarks (3000 m depth) or lobe areas (5000 m depth), confirming the previously observed depth segregation (Cosel and Olu 2009). Taken together with what is recorded worldwide, *C. valdiviae* and *E. guiness* appear to be "bathyal" species, whereas *A. southwardae, L. chuni and C. regab* appear to be "abyssal" species (Table 1). This species segregation according to depth may be attributable to the distance of larval dispersal and larval tolerance to the constraints of the pelagic environment for abyssal species, or pressure tolerance for bathyal species. However, segregation according to larval dispersal cannot involve hemoglobin because veliger larva do not have circulating hemoglobin. It not known when, after settlement, hemoglobin is translated by the vesicomyid genome nor the clam size (probably very small) at which hemoglobin acts as functional oxygen carrier and storer. Although this study cannot answer this question, it is known that hemoglobin can store oxygen and persist in hypoxic conditions during vesicomyid benthic life stages. Their molecular masses can distinguish their hemoglobin structures, indicating their properties as oxygen carriers, and thus their potential resistance to hypoxic events in the habitats in which the larvae settle.

Monomeric hemoglobin structures differ between each species

C. valdiviae, E. guiness and *A. southwardae* from the Gulf of Guinea have four different monomeric globin chains of 15-16 kDa in their hemolymph, and each had a different, distinct mass (Table 4). In contrast, *L. chuni* from the Regab pockmark has only three types of monomers within the same mass range of 15 kDa (Decker et al., 2014). A monomeric structure is also typical of the hemolymph of *P. nautilei* from the Japan Trench, but its molecular mass is greater: about 18 kDa (Kawano et al, 2003). Three other cold-seep vesicomyids from Japan *(A. kaikoi, P. soyoae* and *P. tsubasa)* likely have globins of about 15-16 kDa, which are associated as homodimers (Kawano et al., 2003; Suzuki and Ohta, 2000; Suzuki et al., 1989a). In contrast, the vent clam *C. magnifica* possesses a tetrameric hemoglobin of about 68 kDa, made up of various covalently bound homotetramers, homodimers or heterodimers (Zal et al., 2000) (Table 4). When the blood becomes concentrated (in experimental or

in natural conditions) monomers tend to dimerize, and dimers tend to become tetramers (Kraus and Wittenberg, 1990; Suzuki and Ohta, 2000; Suzuki et al., 1989a; Zal et al., 2000). However, we observed only monomers in the native hemolymph of our vesicomyid species. A multimeric state enables intra-molecular cooperativity (i.e. enhances oxygen binding affinity, once the first oxygen molecule is bound). A monomeric state does not have this advantage, as confirmed by the value of their Hill coefficients, which were all close to unity.

The molecular masses of monomers differed among the species, which may reflect differences in their primary structures due to their various phylogenetic origins. Although all vesicomyids originate from a common ancestor that diverged between vents and seeps in the Cenozoic era (40 millions years ago) (Amano and Kiel, 2007; Peek and Gustafson, 1997), none of the analyzed vesicomyid species in the Gulf of Guinea are closely related, even the species that co-occur (Decker et al., 2012b). They are not related to the above-cited vesicomyids species from Japan either. The multiplicity of globins is frequent in mollusks, because most bivalves possess two hemoglobins with different affinities (Nagel, 1985; Weber and Vinogradov, 2001), and two slightly different molecular structures may lead to slightly different tridimensional shapes of heme pockets, thereby influencing oxygen binding affinity. The globin diversity among vesicomyid clams may thus enable the modulation of the oxygen affinity of monomers to match to the oxygen levels of the surrounding environment. This diversity may either result from structural genetic diversity at the population level, or arise from modulations in the transcription of the globin genes according to individual metabolic variation and/or due to environmental changes, as shown in the vent tubeworm *Ridgeia piscesae* living on chimneys or on basalts (Carney et al., 2007). Our results in *A. southwardae* show that globin masses can also vary within the same species between individuals sampled at two different sites (e.g. Worm Hole and the Lobe area), whereas within a given site, the masses were identical among individuals (with the precision of 1 Da). Only two of the four globin chains (G''3 and G''4) were similar between the individuals from different sites. The G''3 and G''4 chains differ by 16 Da, indicating that either G"4 is an oxidized form of chain G''3, or that their primary structures are different. The two

other pairs of chains (G"1a, G"2a; G"1b, G"2b) differed between the two sites. However, the study of the mitochondrial cytochrome oxidase subunit I gene of *A. southwardae* from Worm Hole and Lobe confirmed that the specimens of both areas belong to the same taxon. However, because only a few individuals were sampled at Worm Hole, it is difficult to conclude as to the genetic divergence between the two populations (Teixeira et al., 2013). Genetic divergence may explain some of the inter-site variation between globin masses, but this hypothesis requires confirmation with an analysis on a larger number of specimens.

Hemoglobins with a very high range of oxygen-binding affinities

The hemoglobins from the three vesicomyids species have very high affinity, all within a range below 1 Torr (Figure 5). These values are among the highest levels found in bivalves (Figure 6), but within the range of the values observed for cytosolic gill hemoglobin of symbiont-bearing bivalves living in the sulfide-rich sediments of shallow waters, such as solemyids and lucinids (Dando et al., 1985; Kraus et al., 1996; Weber and Vinogradov, 2001). Mollusk cytosolic hemoglobins usually have higher oxygen affinities than circulating hemoglobins (Weber and Vinogradov, 2001). For example, the solemyid *Myrtea spinnifera* lives at 33 m depth in the silty sand of Ypsesund (close to Bergen, Norway), where free sulfides in the upper 10-15 cm of sediments can reach concentrations of up to 11 µM in the interstitial water, but bound sulfides (i.e. acid-labile) are as high as 625 µM dm^{-3} at 11 cm depth (Dando et al., 1985). However, these values are about 10-100 times lower than the sulfides measured around the vesicomyids in the seeps, further emphasizing the very high oxygen affinities found in cold-seep clams.

When comparing circulating hemoglobin, all the measured P_{50} values were much lower than those found for the vent clam *C. magnifica* (P_{50}= 7.6 ± 0.23 Torr at pH 6.78 and 10°C) (Terwilliger et al., 1983), showing that cold-seep clams have greater oxygen affinity than this vent clam. However, the oxygen concentrations at hydrothermal vents in the environment of *C. magnifica* (about 110 µM in

the water (Arp et al., 1984)) are comparable to what has been measured in the Guiness area, which is about half the standard concentration in coastal water (Aminot and Kérouel, 2004). The greater affinity of seep clams may confer allow them to burrow deep inside the sediments and remain separated from the oxygenated water source for some time, whereas burrowing in the vent basalt is not possible for *C. magnifica*. Sulfide concentrations are usually high at vents (Luther et al., 2012) because sulfide is a component of the hydrothermal fluids, whereas sulfide content is nil or very low in the bottom water at seeps, being produced by anaerobic methane oxidation in the 15 cm below the seafloor (Cambon-Bonavita et al., 2009; Cruaud et al., 2015; Elvert et al., 2005; Pop Ristova et al., 2012) where vesicomyids take up sulfides via their foot (Arp et al., 1984). Although all found within a narrow range, the affinities of vesicomyid hemoglobin from the Gulf of Guinea species were clearly distinct. We hypothesize that their specificities are related to the chemical conditions of their habitats (see below).

High affinity of hemoglobins in bathyal species is an adaptation to low oxygen levels in ambient waters

Among all species studied in the Gulf of Guinea, *C. valdiviae* colonizing the Guiness and Harp pockmarks had the hemoglobin with the highest affinity (0.13 ± 0.01) for oxygen of all vesicomyid species, including *L. chuni* previously studied in Decker et al. (2014) (0.45 ± 0.18). An interesting observation is that *C. valdiviae* dominated where the oxygen concentrations in the water column were the lowest, i.e. at the Harp pockmark (Table 3 and Figures 7, 8). The oxygen concentration in the water at Harp is about 100 µM lower than in the Lobe area and in the deeper pockmarks, and is only about half of that in fully oxygenated coastal sea water (at 20°C and salinity of 34) (Aminot and Kérouel, 2004). The other species colonizing the Guiness pockmarks, *E. guiness,* also shows very high affinity, slightly lower than *C. valdiviae*. It was only sampled at the Kilkenny pockmark, where it was dominant (Table 2, 3). The water column above this pockmark also showed relatively low oxygen values that were only slightly greater than those at Harp. Moreover, high sulfide concentrations were

measured in vesicomyid beds (the highest of all vesicomyid biotopes studied in this paper, both below the surface of the sediment and in the top first 15 cm of the sediment). Moreover, the Guiness site showed the only positive sulfide flux through the sediment-water interface measured in this study (Table 3). The sulfide concentration at Kilkenny was one of the highest observed in the vesicomyid beds at cold seeps, with a value similar to those measured at the Hydrate Ridge associated with *C. pacifica* and *P. kilmeri* (Sahling et al., 2002). The upward sulfide flux from the sediment into the water may result in anoxic periods when local currents bring these fluid flows around the clam siphons. Thus, among all the sites we studied in the Gulf of Guinea, the two pockmarks at Guiness appeared to be the most extreme habitats with high sulfide and low oxygen levels, and this may explain why the two species with the highest hemoglobin affinity live in these pockmarks.

Hemoglobin in* Abyssogena southwardae *as an advantage to dig for deep sulfides

The blood clam *A. southwardae* was abundant in the lobes of the Congo deep-sea fan, where it was the dominant species at site B. It was the only species sampled at the Worm Hole pockmark where only three individuals were sampled. The structure of its globin chain differed between both areas, suggesting a relationship with hemoglobin affinity. For example, hemoglobin affinity was significantly higher for individuals living in the Lobe area than for those living in the Worm Hole pockmark (Figure 5). Oxygen levels in the water column (measured in the Lobe clam beds) were only slightly lower than the oxygen levels in surface waters, and sulfide levels close to the interface (1 cm depth) were very low in patches of the Lobe area as well as at Worm Hole. Therefore, oxygen and sulfide concentrations in the water around the abyssal vesicomyids may not be very limiting in comparison with other sites. The maximum sulfide concentrations measured within the sediments in *A. southwardae* patches were relatively high such as at Worm Hole, but were within the range of those associated with other vesicomyid species of this study (Table 3). In contrast, *A. southwardae* dominated where the sulfide peak was the deepest, from 14 cm depth in the Worm Hole pockmark

(Pastor et al., Submitted), to 17 cm depth at site B in the Lobe area (Table 3, Figure 7). To obtain sulfides for its symbionts, *A. southwardae* may need to dig deeper within the sediments, temporarily separating the clam from the oxygenated surface (oxygen penetration is limited to the first cm at the most). We hypothesized that its high oxygen affinity gives *A. southwardae* an advantage over *C. regab*, which, without hemoglobin, dominates at all other sites of the Lobe area (A, C and F) where the sulfide levels were more similar to that of the sediment-water interface. In addition, the very long shell length of *A. southwardae* and its elongated shell shape, clearly appear to be an adaptive advantage to burrow more easily and deeper (Figures 7, 8). The same hypothesis was proposed to explain the relative distribution of *L. chuni* and *C. regab* in the Regab pockmark, with the former with high oxygen affinity and long siphons, being assumed to be favored at low flux areas in the pockmark, where sulfides are produced slightly deeper than in the higher flux areas (Decker et al., 2014; Krylova and Cosel, 2011) (Figures 7, 8).

The paradox of the success of *C. regab* with no hemoglobin

Although *C. regab* does not possess hemoglobin, it dominated the hemoglobin-containing species *A. southwardae* in three Lobe sites except site B (Table 3), and *L. chuni* in the Regab pockmark (Decker et al, 2012). Despites the lack of hemoglobin for oxygen transport and storage, *C. regab* can settle in cold seeps and the reduced sediments of the Gulf of Guinea, where it is the only species and has densities as high as 1400 ind.m^{-2} in the Lobe area, and from 500 to 1000 ind.m^{-2} in the Regab pockmark (Decker et al., 2012a). This species was associated with the highest methane fluxes in the Lobe area (Table 3) as well as in the Regab pockmark (Decker et al., 2012a; Khripounoff et al., 2015), and seemed associated with sulfide-rich sediments closer to the surface than the hemoglobin species (*A. southwardae* and *L. chuni*) occurring in the same areas (Decker et al., 2014; Pop Ristova et al., 2012) (Figure 8). This species may be able to bury into the sediment using its foot containing myoglobin (Decker et al, 2012), but *C. regab* shells generally appear high above the surface, where the siphons can reach the oxygen in the surrounding seawater. This behavior may partially result in

an over-estimation of its dominance, making it easier to see and count *C. regab*, than other buried vesicomyid species, such as *L. chuni,* which often barely shows its siphons at the surface, or *A. southwardae* in the Lobe area. The fact that hemoglobin-species generally remain more buried may contribute to the underestimation of their abundance at these sites. Moreover, the vascularized foot of *C. regab* also allows lateral movements and help withstand highly dynamic environment with transient fluxes at the Regab pockmark (Marcon et al., 2014) and high sedimentation rates at Lobe C (Rabouille et al., in press this volume).

However, *C. regab* is a very successful species and enjoy high reproductive success. During the evolution of the vesicomyid species, *C. regab* appears to have lost the ability to synthesize hemoglobin through a loss of function or the implementation of another strategy (i.e. a temporary anaerobic metabolism?). Furthermore, hemoglobin synthesis may have a metabolic cost , and *C. regab* may benefit from this economy to invest in higher reproduction or growth rates (Guillon et al., subm). Moreover, other biological traits may contribute similarly, such as more efficient symbiont strains enabling a faster growth in *C. regab* compared to *L. chuni* (Decker et al. in prep).

In conclusion, in addition to depth segregation factors which likely influence the vesicomyid clam distribution in the Gulf of Guinea, *C. regab* without hemoglobin appears as to be the dominant clam species when high methane fluxes provide high sulfide levels close to the sediment surface. This species is dominant at the most active part of the Regab pockmark, and in the terminal lobe area of the Congo deep-sea fan currently receiving the high organic matter inputs. However, hemoglobin with high oxygen affinity is likely to be advantageous for other vesicomyid species to cope with low oxygen levels either in the bottom layers, such as in bathyal cold seeps of the Gabon margin, or in the upper sediment layers when clams need to burrow to obtain sulfides located at around 15 cm below the sediment-water interface, as in low methane flux areas, or older turbidite depositional areas (Figure 8).

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Figure 1: Location of the study sites and the sampled vesicomyid species. Scale bar, 1 cm.

Figure 2: Views showing the three vesicomyid species in their habitats along the Congo-Angola margin. Guiness area: Harp pockmark colonized by *Calyptogena valdiviae* (A); Kilkenny pockmark with *Elenaconcha guiness* and *C. valdiviae* (B); Worm Hole pockmark with a few *Abyssogena southwardae* specimens (C); Lobes of the Congo deep-sea fan: a clam bed at site B dominated by *A. southwardae* (D); a dense vesicomyid bed at site A, dominated by *Christineconcha regab* (E); some *C. regab* and *A. southwardae* around microbial mats at site F (F); a heart-shaped patch of vesicomyid clams at site C dominated by *C. regab* (G).

Figure 3: Size-exclusion chromatography of hemoglobins on a Superose 12 column: *C. valdiviae* hemoglobin (A); *E. guiness* hemoglobin (B); *A. southwardae* from Worm Hole pockmark hemoglobin (C); *A. southwardae* from the Lobes site B (D). The column was equilibrated with vesicomyid buffer (pH 6.2) described in Childress et al. (1991).

Figure 4: Electrospray ionization-mass spectrometry (ESI-MS) spectra of *Calyptogena valdiviae* hemoglobin (A): Comparison of native conditions (i) with denaturant conditions (ii) (MaxEnt deconvolution spectra). Masses calculated from multiply charged spectra were: G1 : 15774 ± 1 Da; G2 : 16023 ± 1 Da; G3 : 16080 ± 1 Da; G4 : 16541 ± 1 Da (i); G1 : 15158 ± 1 Da; G2 : 15407 ± 1 Da; G3 : 15464 ± 1 Da; G4 : 15925 ± 1 Da (ii). ESI-MS spectra of *Elenaconcha guiness* hemoglobin (B): native conditions at cone voltage of 130 V (i) native conditions at cone voltage of 210 V (heme dissociation) (ii) and denaturant conditions (iii) (all MaxEnt deconvolution spectra). Masses calculated from multiply charged spectra were: G'1 : 15768 ± 1 Da; G'2 : 16016 ± 1 Da; G'3 : 16074 ± 1 Da; G'4 : 16537 ± 1 Da (i); G'1 : 15152 ± 1 Da; G'2 : 15400 ± 1 Da; G'3 : 15458 ± 1 Da; G'4 : 15921 ± 1 Da (ii and iii). Native ESI-MS spectra of *Abyssogena southwardae* hemoglobin (Worm Hole) (C): cone voltage set

to 150 V (i); cone voltage set to 220 V: dissociation of the heme complex (ii), mass range from 15900-16800 Da; range from 15000-15900 Da (MaxEnt deconvolution spectra). Masses calculated from multiply charged spectra were: G''1a : 15169 ± 1 Da; G ''2a : 15183 ± 1 Da; G''3 : 16004± 1 Da; G''4 : 16020 ± 1 Da (i and ii?).

Figure 5: Oxygen affinity of the hemoglobin of vesicomyid bivalves from the Gulf of Guinea measured at 5°C and at pH 6.5. WH: Individuals from the Worm Hole pockmark; L: Individuals from the Lobe site B.

Figure 6: Oxygen affinity of the hemoglobin of vesicomyid bivalves expressed as P_{50}, i.e. the partial pressure of oxygen necessary to saturate the hemoglobin at 50% of its capacity. The symbiotic species in the boxed area all have P_{50} values in the range of 0.1-1 Torr (mm Hg), indicating very high affinity.

Figure 7: Correlation between mean individual shell length of the dominant species and the maximum sulfide concentration depth at each sampled site. See Table 3 for data. Blue: *C. regab*, Green: *E. guiness,* Purple: *A. southwardae.* Circle represents *C. regab* from the Regab pockmark (Marker W03, unpublished data).

Figure 8: Diagram illustrating the vertical distribution of the vesicomyid species in their habitats. Dominant species are represented in dark brown.

Table 1: Worldwide distribution of the species observed in the Gulf of Guinea, with depths and type of habitat. CS: cold-seeps; RS: reduced sediment; HV: hydrothermal vents**.**

Species	Locations	Sites	Ecosystem	Depth (m)	Total depth range	References
Abyssogena southwardae	East Atlantic– Gulf of Guinea	Worm Hole pockmark	CS	3090	2950-5100	Teixeira et al. 2013
		Lobes of the Congo deep-sea fan	RS	5000		
	Atlantic - MAR	Logatchev 14°N	HV	2950-3050		Krylova et al. 2010
	Atlantic - MAR	Wideawake Field (5°S)	HV	2985		Krylova et al. 2010
	West Atlantic	Florida	CS	3313		Krylova et al. 2010
	West Atlantic	Barbados	CS	2985-5100		Krylova et al. 2010
Calyptogena valdiviae	East Atlantic– Gulf of Guinea	Guiness pockmarks	CS	580-670	500-2500	Cosel & Olu 2009
		Gulf of Guinea (off Cameroon)	CS	2492		Krylova & Sahling 2006
	East Atlantic	South African Republic (Off Knysna)	CS	500		Krylova & Sahling 2006
	East Atlantic	Rio de Oro (off Morocco)	CS	2480		Krylova & Sahling 2006
Elenaconcha guiness	East Atlantic– Gulf of Guinea	Guiness pockmarks	CS	580-1200	580-1200	Cosel & Olu 2009
Laubiericoncha chuni	East Atlantic– Gulf of Guinea	Astrid pockmark	CS	2820	2800-3200	Cosel & Olu 2009
		Regab pockmark	CS	3170-3200		Cosel & Olu 2009
Christineconcha regab	East Atlantic– Gulf of Guinea	Regab pockmark	CS	3170-3200	3000-5000	Cosel & Olu 2009
		Baboon - Kouilou pockmark	CS	3000		This study
		Lobes of the Congo deep-sea fan	RS ?	5000		This study
		Bay of Biscay		4125		Krylova & Cosel 2011
Wareniconcha guineensis	East Atlantic– Gulf of Guinea	ZC	RS	3960	4000	Cosel & Olu 2009
		ZD	RS	4020		Cosel & Olu 2009
Isorropodon bigoti	East Atlantic– Gulf of Guinea	BSR	RS	500-800	150-1200	Cosel & Olu 2009
		Guiness pockmarks	CS	580-670		Rodriguez et al. 2012
	East Atlantic	off the Congo Republic and off Mauritania	RS	150-1200		Cosel & Salas 2001

Isorropodon striatum	East Atlantic– Gulf of Guinea	ZC	RS	3960	4000	Cosel & Olu 2009
		ZD	RS	4020		Cosel & Olu 2009
Pliocardia atalantae	East Atlantic	Regab pockmark	CS	3170-3200	3200	Cosel & Olu 2009

MAR, Mid-Atlantic Ridge

Table 2: Location of study sites, cruises, dives and species sampled for this study. Cv: *Calyptogena valdiviae,* Eg: *Elenaconcha guiness,* Cr: *Christineconcha regab* and As: *Abyssogena southwardae.* Specimens studied for hemoglobin structure are shown in bold.

Site (Marker)	Latitude	Longitude	Depth (m)	Cruise	Dive Number	Species sampled for this study
Guiness area – Kilkenny pockmark	S 1° 34.592	E 8° 32.910	581	WACS	433	Cv, **Eg**
Guiness area – Harp pockmark	S 1° 34.6482	E 8° 31.822	780	WACS	433	**Cv**
Worm Hole pockmark	S 4° 45.5607	E 9° 56.4811	3089	WACS	431	**As**
Lobe area – site B (CoL11)	S 6° 25.395	E 5° 49.816	4720	CONGOLOBE	492	**As**, Cr
Lobe area – site A (CoL02)	S 6° 28.275	E 6° 02.145	4769	CONGOLOBE	484	Cr, As
Lobe area – site C (CoL09)	S 6° 42.084	E 5° 29.288	4846	CONGOLOBE	491	Cr, As
Lobe area – site F (CoL03)	S 6° 35.431	E 5° 41.411	4873	CONGOLOBE	486	Cr, As

Table 3: Chemical characterization of the biotopes in the studied vesicomyid beds at each site ordered by increasing depth. Mean density of vesicomyid bivalves in the beds; proportions of each species and mean shell length of the sampled specimens. N: number of samples, n: number of individuals, ND: no data. a: Khripounoff et al. 2015, b: Khripounoff et al. submitted, c: Decker et al. 2012, d: Pop Ristova et al. 2012, e: Caprais J.C. pers. comm.; *Mean density in the bed was estimated from blade-core samples (Olu et al. this issue); **Species proportions in samples were estimated from blade-core and net samples.

Site in the Gulf of Guinea (Marker)	Depth (m)	[O_2] in the water (~15 cm above vesicomyid bed) (N) (µM)	O_2 penetration depth (cm)	[H_2S] at 1 cm depth (mM)	Max [H_2S] in the sediment (max) (mM)	Depth of max [H_2S] (cm)	H_2S flux (mmol .m^{-2}. d^{-1})	CH_4 flux (mmol .m^{-2}. d^{-1})	Vesicomyid species	Mean density in bed (N)*	Species proportion in samples (%)**	Mean individual shell length (cm) (n)
Guiness-Kilkenny pockmark	580	144.8 ± 7.2 (3) [a]	0.65	1.95	24.86	13	158	ND	*E. guiness*	165 (1)	80	6.5 ± 2.0 (11)
									C. valdiviae		20	5.4 ± 1.3 (5)
Guiness - Harp pockmark	680	136.9 [a]	ND	ND	ND	ND	0	ND	*C. valdiviae*	561 (1)	100	4.8 ± 0.4 (17)
Worm Hole pockmark (W11)	3089	ND	ND	0.048	11.44	14	ND	ND	*A. southwardae*	3 (1)	100	10.6 ± 0.3 (2)
Lobe area - Site B (CoL11)	4720	226.3 ± 6.3 (3) [b]	0.74	0	2.65	17	0	8.4 [b]	*A. southwardae*	132 (1)	67	12.1 ± 1.5 (28)
									C. regab		33	7.1 ± 0.5
Lobe area - Site A (CoL02)	4769	214.8 ± 3.3 (3) [b]	0.08	0.008	8.17	10	0	108.5 [b]	*C. regab*	751 ± 355 (4)	93	7.3 ± 0.9 (140)
									A. southwardae		7	12.2 ± 1.9 (11)
Lobe area - Site F (CoL03)	4785	239.0 ± 5.0 (2) [b]	0.36	0.14	1.42	2.5	0	208.9 [b]	*C. regab*	126 ± 132 (2)	61	6.5 ± 0.6 (11)
									A. southwardae		39	10.1 ± 1.4 (7)
Lobe area - Site C (CoL09)	4946	236.5 ± 3.7 (3) [b]	0.1	0.001	2.24	7	0	139 [b]	*C. regab*	1386 ± 429 (3)	98	6.0 ± 1.2 (132)
									A. southwardae		2	5.3 ± 0.3 (4)
Regab pockmark – Site South-West (W03/M10)	3154	242.5 ± 10.7 (3) [a]	no data	0 [d]	3 [d]	8 [d]	0 [a]	0.3 [c] - 12.3 [a]	*C. regab*	1056 ± 218 (3)	91	90.2 ± 2.2 (128)
									L. chuni		9	91.3 ± 8.0 (11)

Regab pockmark – Site Centre (W05/M7)	3171	221.3 ± 17.9 (3)[a]	0.09[e]	0[e]	13.22[e]	6[e]	0[e]	14.6[c] - 45.8[a]	*C. regab*	681 ± 297 (3)	98.5	84.3 ± 10.2 (137)
									L. chuni		1.5	95.2 ± 14.8 (2)

Table 4: Comparison of the structure and masses of the hemoglobins (Hb) in the hemolymph of vesicomyid species known to date.

Vesicomyid species	Sites	Depth of sites (m)	Number of distinct monomers	Molecular state of each native Hb	Molecular mass of native Hb and of (monomer)	Comments	Author
C. valdiviae	Guines: Harp pockmark	580-680	4	Monomer	G1: 15 158 ± 1 Da G2: 15 407 ± 1 Da G3: 15 464 ± 1 Da G4: 15 925 ± 1 Da	Mass spectrometry on native Hb	This work
E. guiness	Guines: Kilkenny pockmark	580	4	Monomer	G'1: 15 152 ± 1 Da G'2: 15 400 ± 1 Da G'3: 15 458 ± 1 Da G'4: 15 921 ± 1 Da	Mass spectrometry on native Hb	This work
A. southwardae	Worm Hole pockmark	3089	4	Monomer	G''1a: 15 169 ± 1 Da G''2a: 15 183 ± 1 Da **G''3: 16 004 ± 1 Da** **G''4: 16 020 ± 1 Da**	Mass spectrometry on native Hb**, identical chains in both sites in bold**	This work
A. southwardae	Lobes A, B, C, F	4946	4	Monomer	G''1b: 15 487 ± 1 Da G''2b: 15 180 ± 1 Da **G''3: 16 004 ± 1 Da** **G''4: 16 020 ± 1 Da**	Mass spectrometry on native hemoglobin**, identical chains between both sites in bold**	This work
L. chuni	Regab	3154	3	Monomer	G'''1: 15 361 ± 1 Da G'''2: 15 668 ± 1 Da G'''3: 15 682 ± 1 Da	Mass spectrometry on native hemoglobin, Mb also present in foot.	Decker et al. 2014
C. regab	Regab, Lobes ABCF	3154 4946	0	–	–	No circulating Hb, Mb present in foot muscle	Decker et al. 2014; This work

Table 4 (continued): Comparison of the structure and masses of the hemoglobins in the hemolymph of the vesicomyid species known to date.

Vesicomyid species	Sites	Depth of sites (m)	Number of distinct monomers	Molecular state of each native Hb	Molecular mass of native Hb and of (monomer)	Comments	Author
C. soyoae	Sagami Bay (Japan)	1160	2	Dimer: HbI and HbII	32 or 64; (16)	SEC and sequencing *Tetramers when concentrated in the blood*	Suzuki et al. 1989
C. tsubasa	Nankai Trough (Japan)	3570	2	Dimer: HbI, HbII	30; (15)	SEC and sequencing	Kawano et al. 2003
C. nautilei	Nankai Trough (Japan)	3570	2	Monomer: HbIII, HbIV	(18)	SEC and sequencing	Kawano et al. 2003
C. kaikoi	Nankai Trough (Japan)	3761	2	Dimers and tetramers	32 or 64; (16)	SEC and sequencing; *Tetramers when concentrated in the blood*	Suzuki et al. 2000
C. magnifica	EPR vents	2600	2	Monomer: α and dimer: βγ combined as tetramers	68 kDa (α: 16 0134.3 ± 1.2 Da βγ: 32 513.1 ± 2.8 Da)	Mass spectrometry on native hemoglobin	Zal et al. 2000

EPR: East Pacific Rise

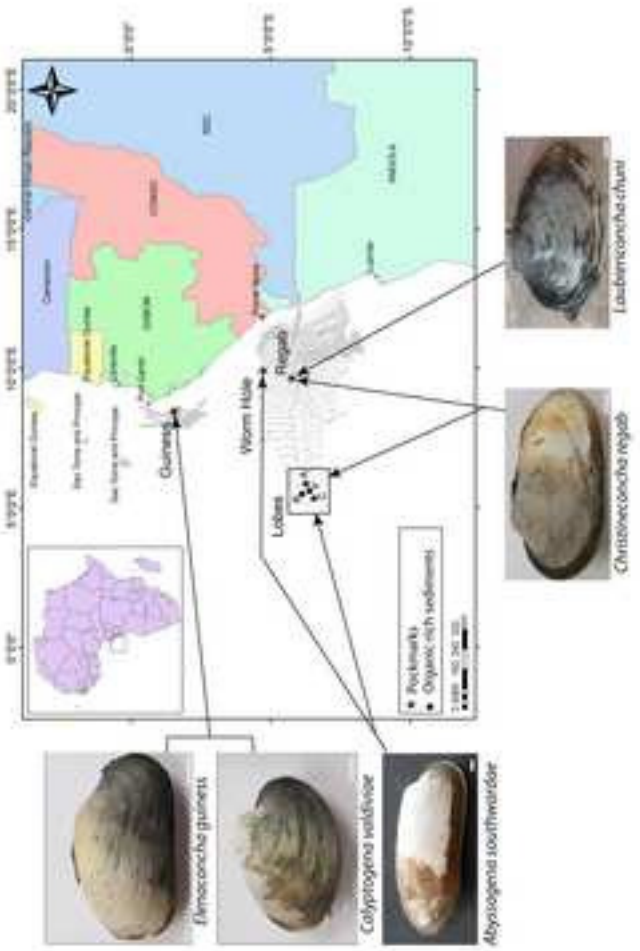
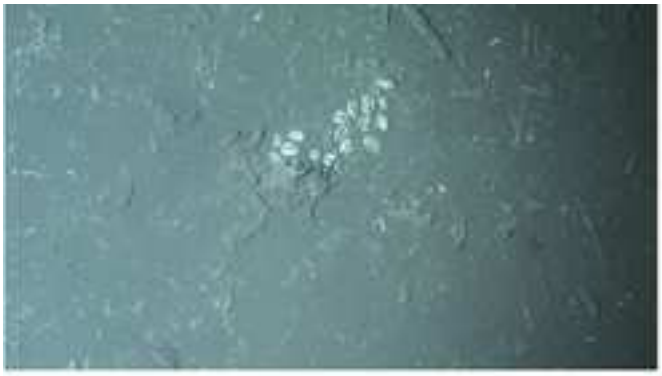


Figure1

Figure2



a

b

c

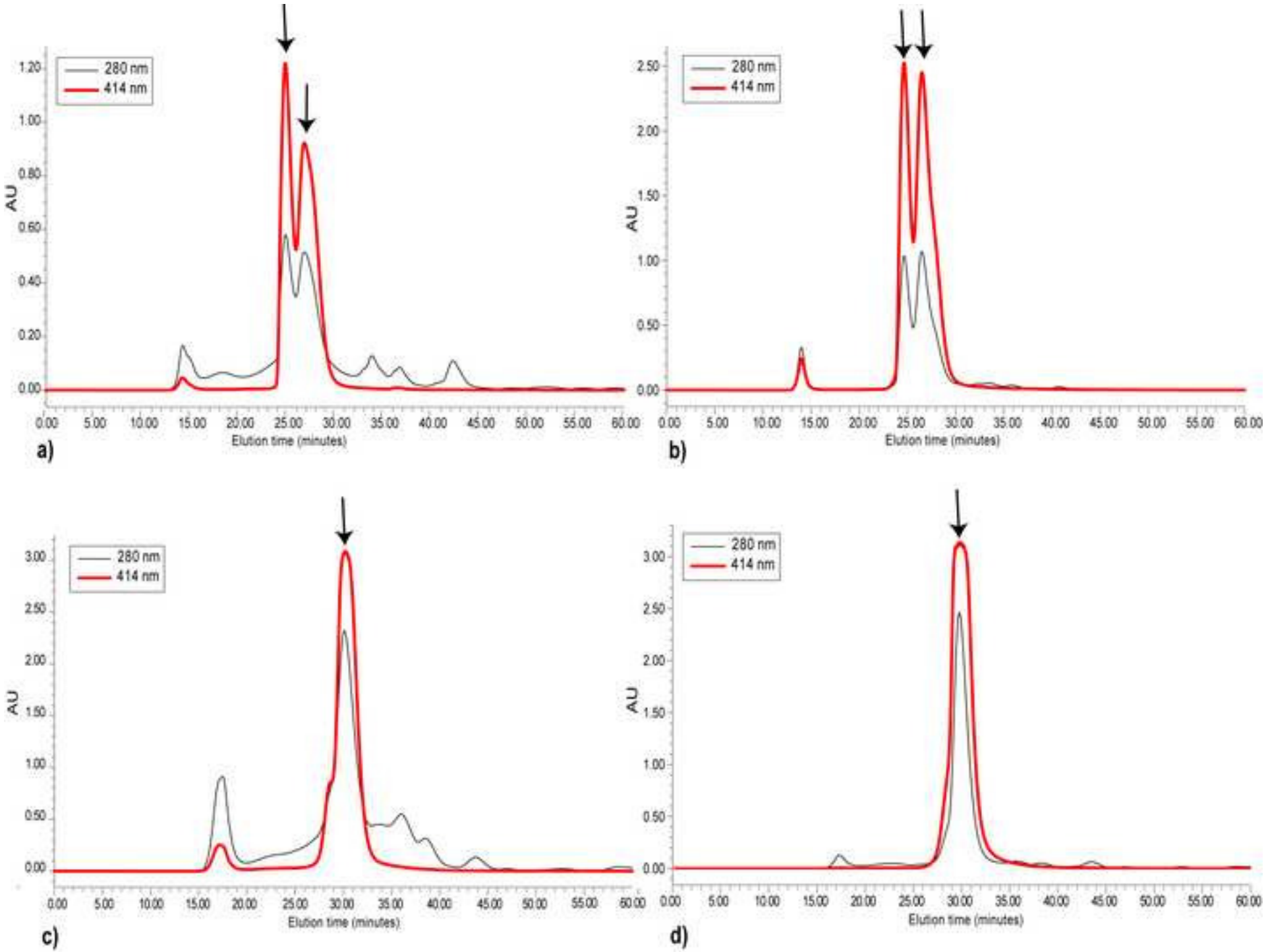
d

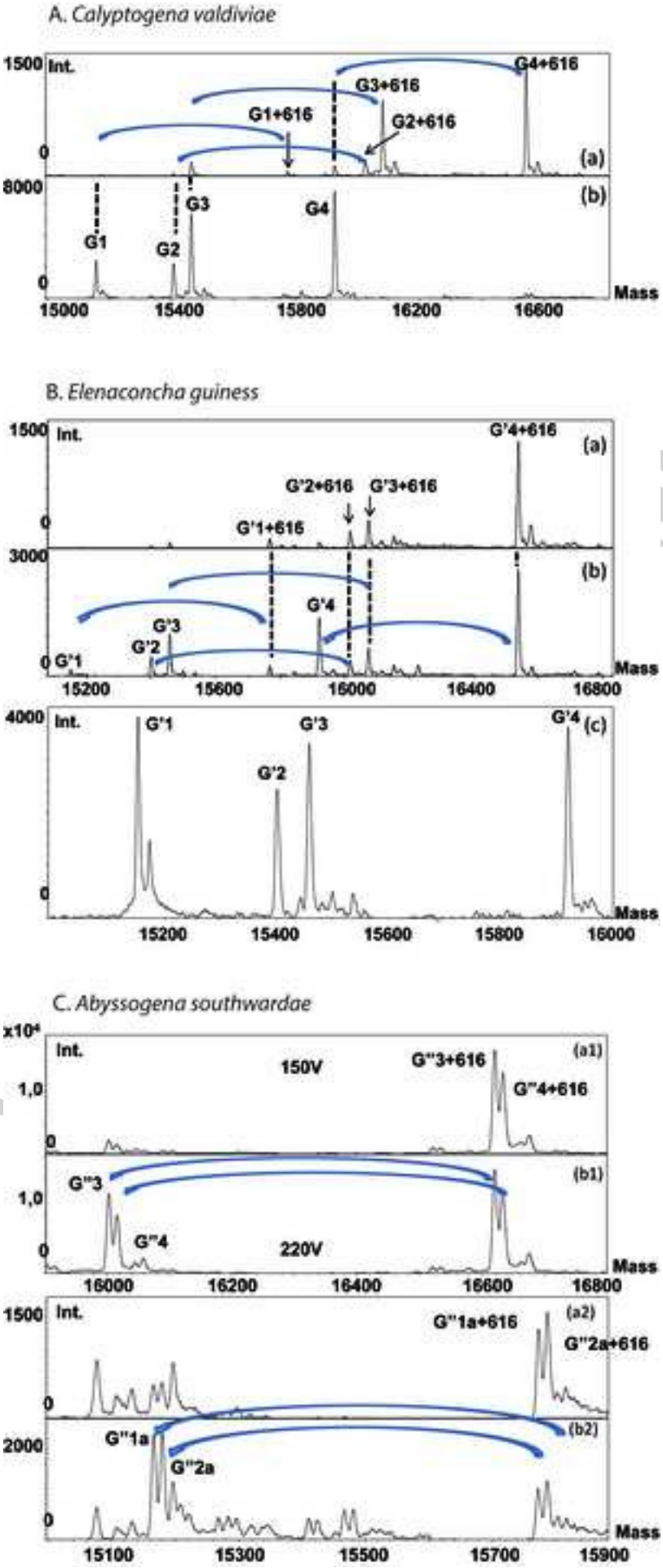
e

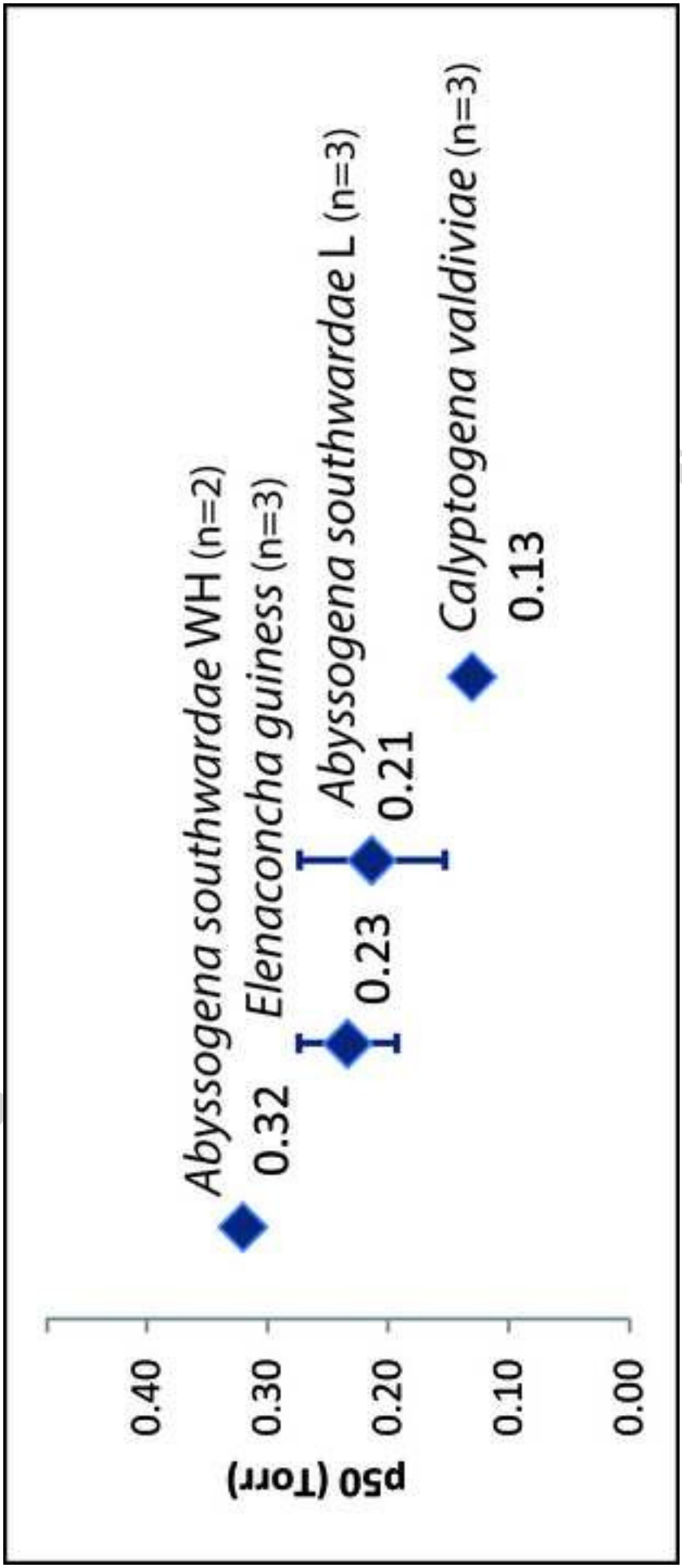
f

g

Figure3






P50 (Torr)
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0.30
0.20
0.10
0.00
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Elenaconcha guiness (n=3)
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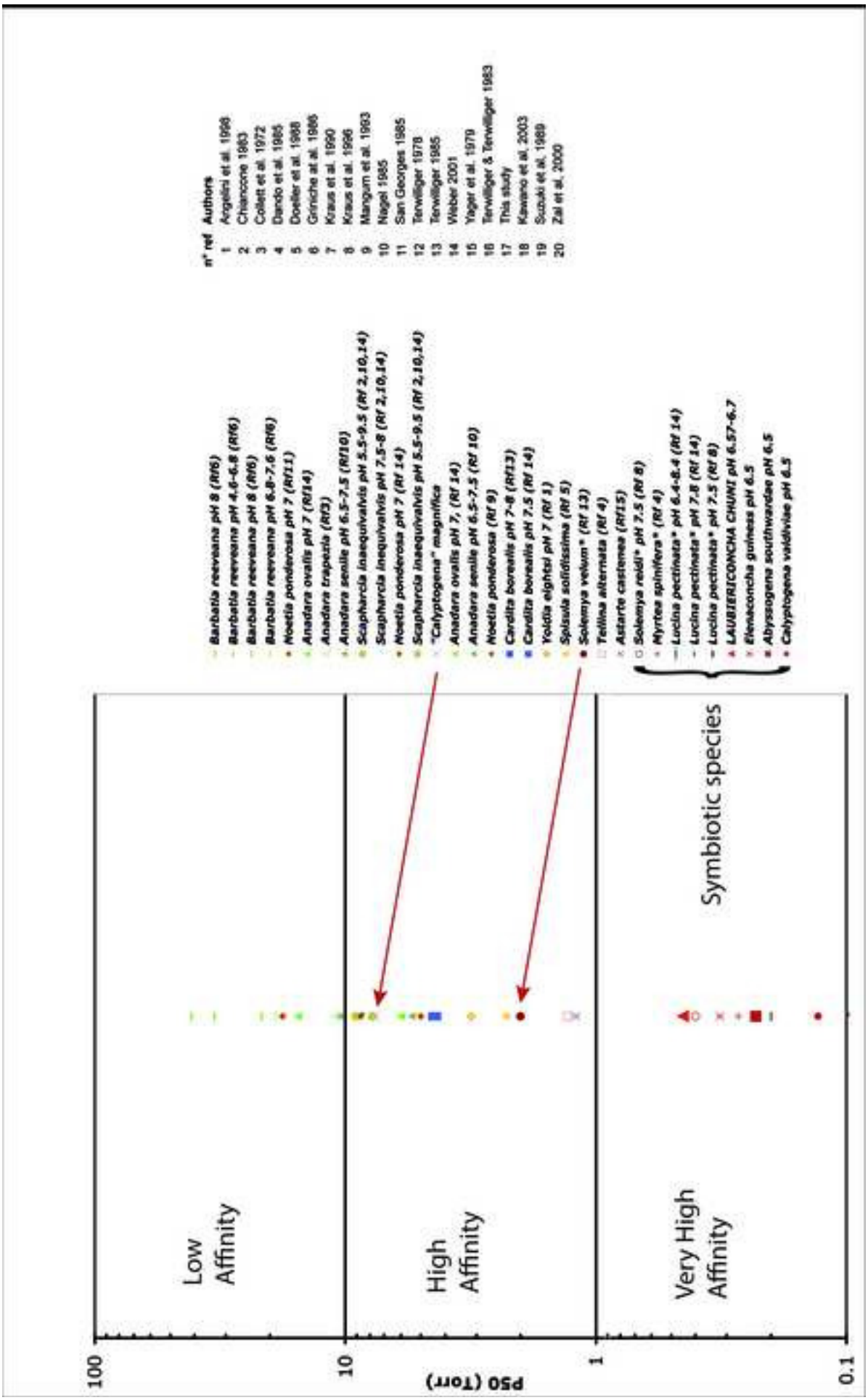
Abyssogena southwardae L (n=3)
0.21
Calyptogena valdiviae (n=3)
0.13

Figure5


n° ref Authors
1 Angelini et al. 1988
2 Chiancone 1983
3 Collett et al. 1972
4 Dando et al. 1985
5 Doeller et al. 1988
6 Griniche et al. 1986
7 Kraus et al. 1990
8 Kraus et al. 1996
9 Mangum et al. 1993
10 Nagel 1985
11 San Georges 1985
12 Terwilliger 1978
13 Terwilliger 1985
14 Weber 2001
15 Yager et al. 1979
16 Terwilliger & Terwilliger 1983
17 This study
18 Kawano et al. 2003
19 Suzuki et al. 1989
20 Zal et al. 2000
Barbatia reevesiana pH 8 (Rf6)
Barbatia reevesiana pH 4.6-6.8 (Rf6)
Barbatia reevesiana pH 8 (Rf6)
Barbatia reevesiana pH 6.8-7.6 (Rf6)
Noetia ponderosa pH 7 (Rf11)
Anadara ovalis pH 7 (Rf14)
Anadara trapezia (Rf3)
Anadara senilis pH 6.5-7.5 (Rf10)
Scapharcia inaequivalvis pH 5.5-9.5 (Rf 2,10,14)
Scapharcia inequivalvis pH 7.5-8 (Rf 2,10,14)
Noetia ponderosa pH 7 (Rf 14)
Scapharcia inaequivalvis pH 5.5-9.5 (Rf 2,10,14)
"Calyptogena" magnifica
Anadara ovalis pH 7, (Rf 14)
Anadara senilis pH 6.5-7.5 (Rf 10)
Noetia ponderosa (Rf 9)
Carelita borealis pH 7-8 (Rf13)
Carelita borealis pH 7.5 (Rf 14)
Yoldia eightsi pH 7 (Rf 1)
Spisula solidissima (Rf 5)
Solemya velum* (Rf 13)
Tellina alternata (Rf 4)
Astarte castanea (Rf15)
Solemya reidi* pH 7.5 (Rf 8)
Myrtea spinifera* (Rf 4)
Lucina pectinata* pH 6.4-6.4 (Rf 14)
Lucina pectinata* pH 7.8 (Rf 14)
Lucina pectinata* pH 7.5 (Rf 8)
LAUBIERICONCHA CHUNI pH 6.57-6.7
Elenaconcha guineea pH 6.5
Abyssogena southwardae pH 6.5
Calyptogena valdiviae pH 6.5
Symbiotic species
Low Affinity
High Affinity
Very High Affinity
P50 (Torr)
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10
1
0.1

Figure6

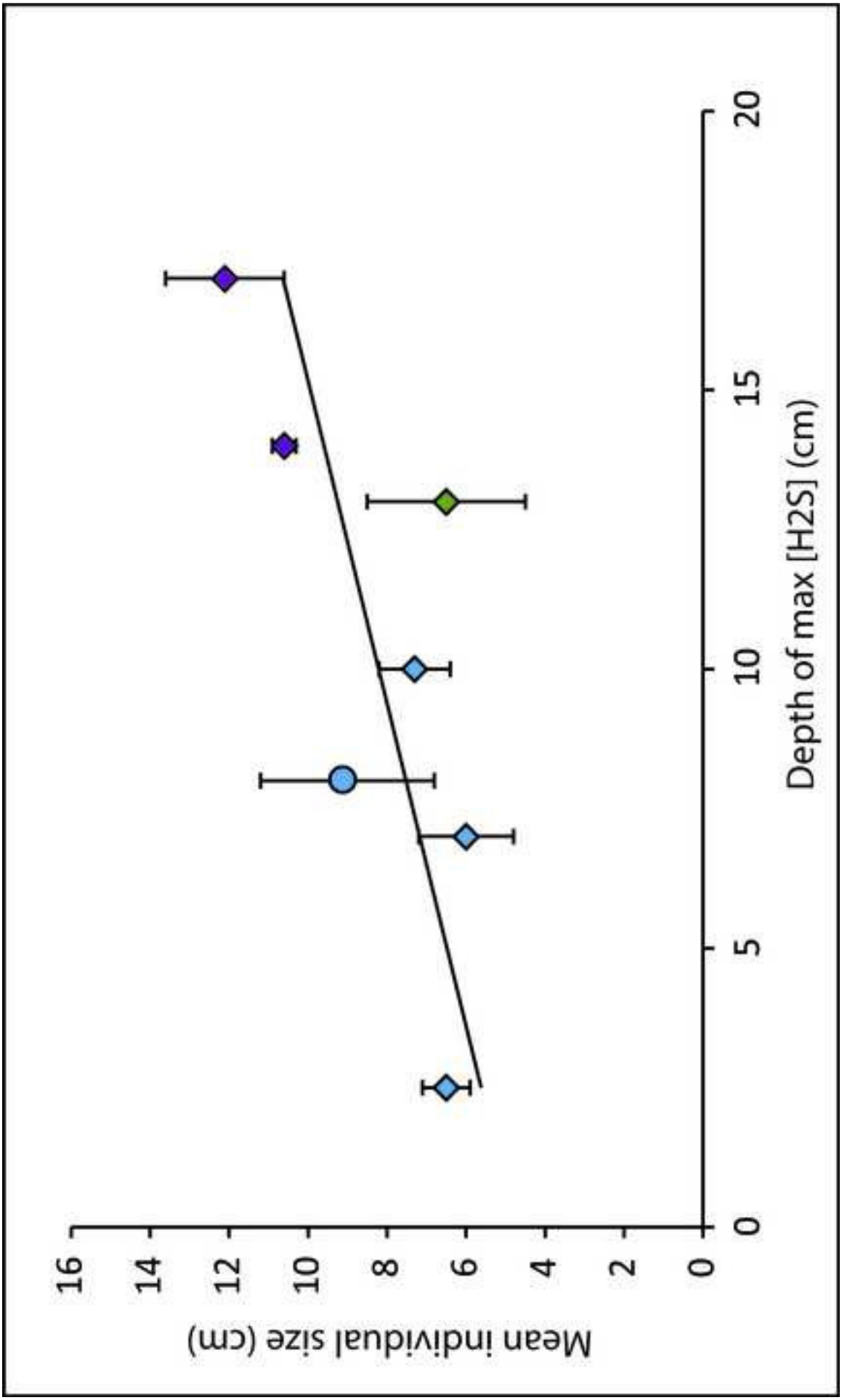
Mean individual size (cm)
16
14
12
10
8
6
4
2
0
0
5
10
15
20
Depth of max [H2S] (cm)

Figure7

Figure8