Globin's Structure and Function in Vesicomyid Bivalves from the Gulf of Guinea Cold Seeps as an Adaptation to Life in Reduced Sediments

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Abstract

Vesicomyid bivalves form dense clam-beds both at deep-sea cold-seeps and hydrothermal vents. The species diversity within this family questions about niche separation and specific adaptations. To compare their abilities to withstand hypoxia we have studied the structure and function of erythrocyte hemoglobin (Hb) and foot myoglobin (Mb) from two Vesicomyid species : *Christineconcha regab* and *Laubiericoncha chuni*, collected from the Regab pockmark in the Gulf of Guinea at a depth of 3000 m. *L. chuni* possesses three monomeric globins, G1 (15361 Da), G2 (15668) and G3 (15682) in circulating erythrocytes (Hb), and also three globins, G1, G3 and G4 (14786) in foot muscle (Mb). Therefore globins G2 and G4 appears to be specific for erythrocytes and muscle, respectively, but globins G1, and G3 are common. In contrast, *C. regab* lacks erythrocyte Hb completely, and only possesses globins monomers G1' (14941 Da), G2' (15169 Da) and G3' (15683 Da) in foot muscle. Thus two Vesicomyid species, *C. regab* and *L. chuni*, show a remarkable diversity in globin expression when examined by ESI-MS. Oxygen binding affinities reveal extremely high oxygen affinities (P_{50} <1 Torr, from 5 to 15°C at pH7.5), in particular *L. chuni* globins, which might be an advantage for *L. chuni* to dig deeply for sulfides and remain buried for long time in reduced sediments.

Keywords: hemoglobin, myoglobin, mass-spectrometry, oxygen-affinity, symbiosis, clams

Footnote :

Abbreviations: Hb = hemoglobin, Mb = myoglobin

Introduction

Vesicomyid clams are heterodont bivalve mollusks that live worldwide on the deep-sea floor from 100 to 9500 m depth (Krylova and Sahling 2010). Most of the 120 vesicomyid species described to date are found in reducing environments on the continental margins or along the oceanic ridges (Krylova and Sahling 2010). They also form dense patches around seepages both at hydrothermal vents, cold seeps and whale falls (Sibuet and Olu 1998). Some species have been found in a single site only, raising questions on niche separation and specific adaptations, but very few studies have been dedicated so far to clam physiology (Barry and Kochevar 1998; Goffredi and Barry 2002). Vesicomyids have only rudimentary guts and rely instead on thioautotrophic gill endosymbionts for nutrition (Cavanaugh 1983; Fiala-Médioni and Le Pennec 1987). The bivalve host fuels its symbionts with sulfides taken up by its foot that can extend in the sediment (or in basaltic crevices at vents), while oxygen and carbon dioxide from the seawater above enters through the vertical siphons (Arp et al. 1984). Most Vesicomyids live in sulfide rich sediment but concentrations may be quite variable (Hashimoto et al. 1989; Levin et al. 2003; Sahling et al. 2002). For example, at seeps in Monterey Bay, *P. kilmeri* dominates in areas with sulfide levels between 4 and 18 mM, whereas *C. pacifica* prefers lower sulfide concentration (0 to 4 mM) (Barry et al. 1997).

Whereas most coastal bivalves have clear hemolymph with no respiratory pigments, all vesicomyids from chemosynthetic environments known to date are called “blood-clams” due to their red hemolymph. Hemolymphs of all five blood-clam species analyzed to date contain intracellular hemoglobin (Hb) in erythrocytes (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). The blood-clam “*Calyptogena” magnifica* Boss & Turner 1980, from the hydrothermal vents on the East-Pacific Rise, has an intracellular hemoglobin (Hb) of 68kDa which transports oxygen to both host and symbionts (Terwilliger et al. 1983; Zal et al. 2000), while sulfide is carried by a lipoproteic sulfide-binding component dissolved in the hemolymph (Childress et al. 1993; Zal et al. 2000). Hb of this clam that is exposed to variable environmental conditions has a relatively high affinity for oxygen (Terwilliger et al. 1983), but may nevertheless greatly facilitate oxygen transport needed for the chemoautotrophic

hydrogen sulfide metabolism. Indeed, *"C". magnifica* lives wedged into fissures of the basaltic rock in a variable environment ranging between that of mixed vent fluid (2.3-14°C, 2-40 µM H_2S, no O_2) and the surrounding deep-sea seawater (2°C, no H_2S and 110 µM O_2) (Arp et al. 1984; Fisher et al. 1988; Scott and Fisher 1995). Under such conditions an oxygen-carrier that transports and stores oxygen may favor a consistent oxygen delivery to the host and its symbionts, especially if oxygen affinity is independent of the temperature (Terwilliger et al. 1983). To date this is the only vesicomyid Hb whose functional properties have been analyzed to some extent.

The structure of the intracellular Hbs has been studied in four blood-clam species collected from cold seeps around Japan. *Phreagena soyoae, "Phreagena" tsubasa* and *Abyssogena kaikoi* (formely *Calyptogena soyoae, Calyptogena tsubasa* and *Calyptogena kaikoi* (Audzijonyte et al. 2012; Krylova and Sahling 2010) all have two homo-dimeric Hbs of 32 kDa, termed HbI and HbII with more than 90% sequence identity, revealing their close relationship (Kawano et al. 2003). In *A. kaikoi* both Hbs might also appear as homo-tetramers, presumably when their concentration is large enough (Suzuki et al. 2000). Moreover, *A. kaikoi* adductor muscles also contain two cytosolic homo-dimeric myoglobins (Mb), MbI and MbII, which appear to be identical to HbI and HbII dimers, respectively (Suzuki et al. 2000). This identity suggests that the role of Hbs in *A. kaikoi* is merely oxygen storage under low oxygen conditions in the deep-sea, rather than oxygen transport in the hemolymph (Suzuki et al. 2000). The fourth species, "*Calyptogena" nautilei*, has two monomeric Hbs of 18 kDa, HbIII and HbIV, which have only 33 and 42% identity with the sequences of HbI and HbII respectively (Kawano et al. 2003). As all vesicomyid clam species seem to originate from a common ancestor, these sequence variations and their monomeric/dimeric quaternary structures suggest that hemoglobin expression might be influenced by habitat conditions of each clam species and specifically by oxygen concentration (Kawano et al. 2003).

Christineconcha regab (Krylova and Cosel, 2011) and *Laubiericoncha chuni* Thiele and Jaeckel, 1931 are two clam species discovered at Regab pockmark in the Gulf of Guinea (Cosel and Olu -Le Roy 2008; Cosel and Olu 2009; Olu-Le Roy et al. 2007). *C. regab* was formerly described as

104 *Calyptogena regab* (Cosel and Olu, 2009), but Krylova and Cosel showed that this species differs
105 morphologically from the other *Calyptogena* and in particular lacks their red hemolymph. Moreover
106 *C. regab* is also phylogenetically apart from the great majority of the vesicomyid species (Decker et
107 al. 2012b). On the contrary *L. chuni* appears to be a true blood-clam and is included within a clade of
108 several species from Japan's Trench (Decker et al. 2012b). In the Gulf of Guinea, at Regab pockmark,
109 *C. regab* and *L. chuni* live sympatrically within the same bivalve patches. However, *C. regab* is very
110 abundant and widely distributed all over the pockmark, while *L. chuni* has been sampled sporadically
111 in its most peripheral, South-Western part only. Physico-chemical conditions in Regab pockmark are
112 relatively stable showing a water-temperature around 2.6°C all year round, with an oxygen
113 concentration about 215-220 µmol $.L^{-1}$ (Decker et al. 2012a). However within the sediment oxygen is
114 only present in the uppermost millimeters, and sulfide concentration can reach 5 mM (Pop Ristova et
115 al. 2012). Sulfides are produced close to the surface between 3 and 6 cm depth. Although difficult to
116 measure precisely, the depth of the sulfide peak (or sulfate-methane transition zone) seems to vary
117 at the pockmark scale (Pop Ristova et al. 2012). This implies that vesicomyids have to cope with very
118 hypoxic, almost anoxic conditions to reach sulfides more or less deeply within the sediment.
119 The aim of this study was to search for oxygen-binding proteins in these two vesicomyid
120 species and to compare their structural and functional properties. We used HPLC to purify the
121 globins and ESI-MS mass spectrometry to analyze their quaternary structure and composition. We
122 measured oxygen-binding properties under various pH and temperature conditions. These results are
123 discussed in terms of adaptation to hypoxic conditions and in relation to the specific distribution of
124 the two species at Regab pockmark.
125
126 **Methods**
127 **Animal collection**
128 During three scientific cruises in the Gulf of Guinea, vesicomyid bivalves were collected at Regab
129 pockmark, 8 km North of the Congo canyon, at a depth of 3100 m (Ondreas et al 2005). The cruises

were: (1) GUINECO M76/3 -cruise in July-August 2008 (2) WACS-cruise (West African Cold Seeps) in January-March 2011, and (3) CONGOLOBE-cruise in December 2011-January 2012. Vesicomyid specimens were sampled at three different sites within the Regab pockmark: site 1 / North (Guineco Marker 3), site 2 / Center (WACS Marker W05 / Guineco Marker 7) and site 3 / South-West (WACS Marker W03 / Guineco Marker 10) (Figure 2), using a fishing net pulled over the mud surface by the ROV. Two different vesicomyid species were sampled: *Christineconcha regab* living at all three sites and *Laubiericoncha chuni* found at site 3 and very occasionally at site 2; After retrieval onboard the nets with bivalves were quickly transferred to a cold room at 4°C before sorting and dissection.

Hemolymph and tissue collection

Hemolymph was sampled in *L. chuni* using a 1 mL syringe, inserting the needle between the valves at the anterior end of the clam, or after opening it (Table S1), and its foot was also dissected. As *C. regab* had no red blood, only the deep-red foot was cut after opening of the shells. All samples were frozen in liquid nitrogen and stored at -80°C in the laboratory until use.

Globin purification by HPLC

Hemolymph samples:

Hemolymph samples of *L. chuni* were thawed to lyse the erythrocytes and centrifuged at 10 000 rpm for 10 minutes at 4°C to recover the Hb. Proteins in the hemolymph were separated by analytical Size-Exclusion Chromatography (SEC) performed on a Superose 12 - 10/300 GL Tricorn column (GE Healthcare), with a fractionation range from 1 to 300 kDa. The column was equilibrated with a saline HEPES-buffer as those prepared for *"Calyptogena" magnifica* by Childress et al (1991). The elution rate was 0.5 $mL.min^{-1}$, and the absorbance of the eluate was monitored at 280 nm and 414 nm indicative of protein and heme concentrations respectively. Every heme-containing protein peak was collected separately and concentrated in micro-centrifugal filter devices Centricon-3kDa (Millipore).

Foot tissue samples:

Feet of *C. regab* and *L. chuni* were weighed, and crushed in 1 mL/g ice-cold HEPES-buffer as those prepared by Childress et al. (1991). The homogenate was centrifuged 3 times at 10000 rpm for 10 minutes at 4°C, and the supernatant was kept after each centrifugation. When the volume of homogenate was insufficient for analysis, we pooled and crushed the feet of 3 specimens from a same collection site (Table S1). The feet homogenates supernatant of *L. chuni* were directly purified on serially connected Superose 12 and Superdex 75 columns, the latter with a fractionation range of 3 to 70 kDa. Each heme-containing peak was collected separately and concentrated in a micro-centrifugal filter device Centricon-3kDa (Millipore).

Mass Spectrometry of the purified globins

Samples of hemolymph and foot tissue for mass spectrometry were purified as described previously, but in an ammonium acetate buffer at 50 mM pH 6.2. ESI-MS analyses were performed in denaturing conditions to search for eventual disulfide bonds and in non-denaturing conditions to preserve the native structure. Ten individuals of each species (*L. chuni* or *C. regab*) were analyzed.

For the denaturing analyses, each sample was dissolved at a final concentration of about 0.1 mg/ml in a mix of water/acetonitrile/formic acid at (50:50:1 in volume). Mass data were obtained using a MicroTOFLC (Bruker-Daltonik GmbH, Bremen, Germany) mass spectrometer fitted with Z-spray electrospray ion source, and operated in positive ion mode. MS data were acquired from 500 to 2000 m/z. Mass calibration was performed using a mix of Tune Low (Agilent Technologies # G1969-8500) and a solution of horse heart-myoglobin at 20 fmol/µL (Sigma, # M-1882). Data were processed with the Data Analysis software: Bruker Daltonik (GmbH, Bremen, Germany) and multiple ionized spectra were deconvoluted with MaxEnt tool to generate molecular mass spectra. All molecular masses were calculated from the raw data multiple ionized peaks.

For non-denaturing analyses, samples were purified using mini-gel filtration columns (Microbiospin 30, Biorad) in 200 mM or 50 mM ammonium acetate (pH7.5). ESI-MS measurements were performed on an electrospray time-of-flight mass spectrometer (MicrOTOF$_{LC}$, Bruker Daltonic, Germany). The capillary exit voltage was set to optimize the desolvation process while preventing dissociation of the non-covalent species (values from 100 to 200 V were tested). Samples were diluted 10 times in 100 mM ammonium acetate and continuously infused into the ESI ion-source at a flow rate of 3 µl/min through a Harvard syringe pump (Harvard Apparatus model 11). Acquisition mass range was 1000-10,000 mass/charge-units. Calibration was performed using a concentrated solution of Cesium Iodide (1mg/ml in water: isopropanol 1:1). Masses were calculated and averaged for all ions distributed in the ionized state.

Direct infusion ESI-MS data were complex to interpret, as globin masses were similar and thus generated very close and multiple charged ions. To simplify our interpretations we had to separate the different globin chains, using reverse phase-Liquid Chromatography Mass Spectrometry (rp-LCMS). Samples were injected into a HP1100 HPLC (Agilent Technologies). The globins were separated on a 2,1 x 125 mm, 5 µm, 300 Å, Nucleosil® C_{18} column (Macherey-Nagel) prior to introduction into the mass spectrometer. Mobile phases were: A: 0.1% TFA, and B: 0.08% TFA in acetonitrile. A linear gradient from 5% B to 60% B in 65 min, then to 80% B in 5 min, was used for separation, at a flow rate of 300 µL/min. Column temperature was kept at 25°C. UV trace was monitored at a wavelength of 214 nm, typical for peptide-bond absorption. 1/20th of the eluted solution coming out of the UV detector was sent to the mass spectrometer by a T-split. The other 19/20th were manually collected each time a globin was eluted from the column (shown by the UV detector); These collected fractions, were systematically re-injected on a MicrOTOF-Q II (Bruker-Daltonik GmbH, Bremen, Germany) to confirm our LCMS measurements.

Heme-assay analyses: heme and protein concentrations

206 10 µL of purified hemoglobin were diluted in 2mL of the purification HEPES-buffer (Childress et al.
207 1991). 10 µL of potassium ferricyanide solution (0.01 mol. L^{-1}) were added to 2ml of this diluted
208 solution, and incubated for 5min at room temperature to oxidize the hemoglobin completely into
209 met-hemoglobin. 10µL of potassium cyanide (0.05 mol. L^{-1}) was then added to obtain the cyan-met-
210 hemoglobin derivative. The solution was analyzed in a spectrophotometer at 540 nm. Absorbance
211 was measured at 540 nm and the extinction coefficient of 11.0 $L.mmol^{1}cm^{1}$ was used to calculate
212 heme concentration. The Bradford method was used for protein quantification (Bradford 1976).
213
214 **Oxygen binding measurements**
215 Oxygen-binding affinities of hemoglobins and myoglobins were obtained in a gas-diffusion chamber
216 (Sick and Gersonde 1969). Small samples (5µL) of purified globin solution were equilibrated with pure
217 N_2 for the deoxygenated state, and pure O_2 for the fully oxygen saturated state, and mixtures of
218 these gases were added stepwise by the Muti-gaz-controller mks 647C (Instruments Deutschland) to
219 determine the partial oxygen pressure (P_{50}) corresponding to 50% O_2-saturation. The pH was
220 measured with a microelectrode Thermo Orion 3 Star (Electron Corporation) on an aliquot of the
221 sample incubated in the water bath, at the same temperature as the measured P_{50}. Oxygen-binding
222 properties and pH measurements were carried out at 5, 10 and 15°C for both Mbs and Hb.
223 Technically 5°C is the lowest possible temperature to measure P_{50} and avoid water condensation in
224 the sample, so this is the closest possible temperature to the deep-seawater temperature around the
225 clams (2.6°C) (Decker et al. 2012a).
226 Values of P_{50} and n_{50} (Hills cooperativity coefficient at P_{50}) were derived from the linear regression of
227 the Hill plots ($\log(S/1-S)=f(\log(PO_2))$) for saturation values in the linear portion of the curve i.e. at
228 saturation values between 30-70%. Bohr effect was tested on *L. chuni* hemoglobin and *C. regab*
229 myoglobin at 5°C with pH values ranging from 5.2 to 7.2. The pH was adjusted by diluting the
230 hemoglobin solutions with the Vesicomyid buffer at the chosen pH at a ratio of 1:1.
231

232 **Statistical methods**

233 Temperature and Bohr effects were tested using non-parametric tests [Wilcoxon–Mann–Whitney
234 test for (n = 2), and Kruskal–Wallis test when (n > 2)] and a post-hoc test: Behrens–Fisher (BF) for
235 non-parametric multiple comparisons. All analyses were performed using the free open source R
236 Environment (R Development Core team 2010). The NPMC library was used for non-parametric
237 multiple comparisons (Helms and Munzel 2008).

238

239

240 **Results**

241 **Structural analysis of the globins**

242 *L. chuni* circulating hemoglobin

243 From 1 ml to 10 ml hemolymph were collected per individual of *L. chuni*. Heme concentration was
244 0.4 ± 0.1 mM. (n=3). Gel filtration of the hemolymph resulted in a major heme-containing peak
245 (Figure 3a). Mass spectrometry was performed on this fraction in order to determine the number of
246 constituents of this heme-protein sample. The expected error on a mass result is about 0.005 %
247 according to ESI-MS instrument specifications. On each sample several mass measurements were
248 performed and the results were averaged in order to estimate mass precision. Taken together,
249 percentages of error and precision allowed to identify similar masses with about 1 Da uncertainty as
250 being identical molecules, termed Globins G1, G2 etc... The spectra of *L. chuni*'s Hb revealed the
251 presence of three different monomeric globin chains called G1 to G3 with experimental masses of
252 15361 ±1 Da, 15668 ±1 Da and 15682 Da ±1 Da respectively (Table 1, Figure 4a). In addition, two
253 individuals, one from each site, had a minor covalent homo-dimeric chain of about 30724 Da named
254 G1-G1 (Figure not shown). The number of constituents in the hemolymph varied between
255 individuals: in particular G2 was only present in the hemolymph of five individuals out of seven.

256

257 *L. chuni* foot myoglobin

The gel filtration of the foot homogenate gave one major fraction corresponding to a heme-protein and several non heme-proteins lacking absorption peaks at 414 nm (Figure 3b). The second purification on a Superdex 75 enabled to collect a single pure heme-protein fraction. Mass spectrometry performed on this heme fraction revealed three monomeric chains (Table 1, Figure 4b). Two chains had masses identical to G1 and G3 from the purified hemolymph. The last globin chain (G4) was lighter, with a mass of 14786 ± 1 Da. In order to determine the stoichiometry, non-denaturing mass spectrometry was performed. The ESI-MS conditions were optimized using a solution of human hemoglobin. While human hemoglobin reference was observed as a α2β2 tetramer, as expected, masses of *L.chuni's* globins corresponded to those of monomers bound to a single heme unit: at 15401 ± 1 Da for G4, 16297± 1 Da for G3 and 15977 ± 1 Da for G1 (Figure 5). Comparing the masses of the globins found in the circulating blood (Fig 4a) with those found in the foot extract (Fig 4b) of *L. chuni*, globins G2 appears specific of hemolymph, whereas G4 appears to be specific for muscle, but globins G1, and G3 are found both in hemolymph and muscle.

C. regab foot myoglobin

As for *L. chuni* foot homogenate, the gel filtration of *C. regab*'s Mbs provided one major fraction and several non-heminic proteins (Figure 6a). The second purification of the heme-proteins allowed an apparent complete elimination of the non-heminic proteins (Figure 6b).

Mass spectrometry analyses on the final purification of the second fraction revealed three different monomeric chains of 15139 ±1 Da (G1'), 15169 ±1 Da (G2') and 15683 ±1 Da (G3') . The globin chain G1' was found in all specimens from all sites, whereas G2' was only present in specimens from the central and South-Western site, and chain G3' in specimens from the Northern site, (Table 1). Some of these monomeric chains (G1' and G2') were also found in their oxygenated state (results not shown). Two homo-dimeric chains of G1'-G1' and G2'-G2' were also observed in two specimens from the central site (Table 1).

Oxygen-binding properties

Oxygen binding properties were measured on the purified Hbs and Mbs of *L. chuni*, (Table 2). At 5°C, the value of the P_{50} was significantly higher for circulating Hb (0.45 ± 0.18 Torr at pH 6.65 ± 0.07, *n*=3), than for foot Mb (0.19 ± 0.02 Torr at pH 6.27 ± 0.4, n=3) (Wilcoxon Mann-Whitney, W=9, p<0.08). Consequently, the oxygen affinity is higher for cytosolic Mb than for circulating Hb in *L. chuni*. As expected for monomeric globins, there was no cooperativity in any of the molecules as n_{50} was close to 1 (Table 2).

A slight temperature effect was observed for the hemoglobin with a tendency of the affinity to decrease with a temperature rise from 5 to 15°C, (Figure 7a), however not statistically significant in our sampling (Kruskal-Wallis: X^2=4.7, p=0.1). Foot myoglobin affinity remained stable with an increase of temperature (Kruskal-Wallis: X^2=3.7, p=0.2) (Figure 7a). Variation of pH had no effect on hemoglobin affinity (Figure 7b), (Kruskal-Wallis, X^2=13.37, p=0.42)

C. regab myoglobin affinity was about 0.85 Torr ± 0.09 at pH= 6.63 and T°=5°C, n=3 and with this high value its affinity was lower than the affinity of *L. chuni* Mb and Hb (Wilcoxon Mann-Whitney: W=9, p<0.08). As for *L. chuni* no cooperativity was measured (Table 2) and a drop of affinity was observed with an increase of temperature (Figure 8a), but no Bohr effect was observed (Figure 8b).

Discussion

At Regab pockmark, the dominant species *Christineconcha regab* is the first recorded vesicomyid clam from a chemosynthetic environment with clear hemolymph, i.e that is not a “blood-clam”. However the deep-red foot of this species contains myoglobin. The other vesicomyid clam found at regab, *Laubiericoncha chuni*, is a regular blood-clam having both intracellular circulating hemoglobin and also myoglobin in its foot muscle. Both species also have deep-red adductor muscles, probably containing Mb too.

***L. chuni* and *C. regab* have monomeric globins**

In *L.* chuni, both circulating Hb and cytosolic Mb are monomeric globins of about 15 kDa. A monomeric structure is also found in the vesicomyid *P. nautilei* from Japan's trench, but its molecular mass is higher: about 18kDa, as determined from cDNA sequence (Kawano et al. 2003). In fact, compared to the stable molecular mass of vertebrate globins, those of invertebrates vary much more: from 14 to 19 kDa (Bruneaux et al. 2008; Weber and Vinogradov 2001). Thus our results fit within the normal mass range for invertebrate globins. The three other cold seep vesicomyid species have likely globins of about 15-16kDa but they are associated as homodimers (Kawano et al. 2003; Suzuki and Ohta 2000; Suzuki et al. 1989a).

In our sampling of *L. chuni* hemolymph has two or three types of globin monomers of slightly increasing masses that apparently do not dimerize naturally. As Hb is circulating through the blood vessels and capillaries irrigating the foot muscle, it might be difficult to sort out the circulating globins from the cytosolic muscle globins, and this might explain the similarity of the globins found in both hemolymph and foot muscle. However, G4, with a lighter mass of about 14 kDa is only observed in the foot muscle, and thus seem to be a specific cytosolic Mb, whereas globin G2, when present, is only found as circulating Hb. In purified circulating Hb the lightest chain G1 can form a homodimer. This covalent homodimer is probably due to the presence of cysteine residues in the primary sequence, forming disulfide bridges between two globin chains, which has to be confirmed by sequencing. On the contrary no dimerization is observed in *L. chuni's* foot homogenate. In *L. chuni* and *C regab*, the number or globin chains of different masses vary according to the specimens. Having various globins within an organism might enable a fine-tuning of the O_2 binding, as the various globins might differ in their O_2 affinities or in other functional properties. Moreover, most of the bivalves possess two hemoglobins, one with a higher affinity than the second one (Nagel 1985; Weber and Vinogradov 2001). The globin diversity within an organism could thus enable a modulation of the oxygen affinity even as monomers, to fit to the oxygen levels of the surrounding environment. This diversity could either result from a structural genetic diversity at the population level, or come from modulations in the transcription of the globin genes, due to individual metabolic

variations, and/or due to environmental changes. The latter hypothesis has previously been assessed for the differential expression of globin messenger RNA in the vent tubeworm *Ridgeia piscesae,* according to its habitats: on the hot anoxic vent chimney the short-fat *Ridgeia* morphotype shows levels of gene expression up to 12-times greater, and also much higher Hb concentrations, compared to the long-skinny *Ridgeia* morphotype living in the cooler and more oxygenated surrounding basaltic fields (Carney et al. 2007). In a comparable way, as we observed differences between sampling sites, we may hypothesize that, triggered by the oxygen conditions in their surrounding microhabitat, the vesicomyids could express different proportions of their various globin monomers to regulate the bivalve's oxygen supply and storage at a fine level.

L. chuni and _C. regab_ globin functional properties

Coastal and shallow water bivalves from the Arcidae family having intracellular Hb show oxygen affinities, measured within the same pH range of 6.8-7 and at a temperature of 20°C that vary according to the species from 7.8 to about 33 Torr (Chiancone et al. 1993; Nagel 1985; Weber and Vinogradov 2001). The globins of the two vesicomyid species we analyzed show a very high affinity (P_{50} <1 Torr from 5 to 15°C at pH about 7.5) for circulating hemoglobin and foot myoglobin with a significantly higher affinity for *L. chuni* globins than for *C. regab* Mb, despite slight individual variations. These values are thus among the highest levels found in bivalves Hb, but within the range of (0.1-3 Torr) for the globin affinities of the symbiotic bivalves living in reduced (shallow water) sediments, such as solemyids and lucinids (Kraus et al. 1996; Terwilliger and Terwilliger 1985; Weber and Vinogradov 2001). In particular *Solemya reidi* has an intracellular Hb with a P_{50} of about 0.3-0.5 Torr at pH 7.5 and 20°C (Kraus et al. 1996), which is similar to the values we measured in the two seep vesicomyids. Within the same pH and temperature conditions, the affinity of the branchial cytosolic globins of *Lucina pectinata* is even higher since the P_{50} is about 0.1 Torr (Kraus and Wittenberg 1990). Compared at the same temperature of 10°C and within the same pH range, the values of *L. chuni*'s monomeric Hb is 0.22 Torr, and corresponds surprisingly to a much higher affinity

than that of the tetrameric Hb of the vent clam *"Calyptogena" magnifica* (P_{50}= 7.6 ± 0.23 Torr at pH 6.78 and 10°C) (Terwilliger et al. 1983). However a tetrameric Hb transports 4 times more O_2 per molecule than a monomer, but the oxygen supply finally relies on their respective heme concentration in the hemolymph that is from 0.1-0.4mM in *L. chuni*, which seems rather comparable to those measured in "*C". magnifica* (<0.4mM) (Powell and Arp, 1989). Although tetrameric, the vent clam Hb has a low cooperativity, (n=1.24), and both the affinity and the cooperativity vary little with a temperature increase from 2° to 11°C (Terwilliger et al. 1983). These figures contrast with the properties found in the monomeric Hb of *L. chuni*. The globins of *L. chuni* and *C. regab* do not show any cooperativity (n_{50} =1) at all the temperatures tested and no Bohr effect, i.e. no drop of affinity when the pH drops, aligns with the monomeric state of the globins that do not interact together in the hemolymph. This is also consistent with what is observed in most bivalves' red blood cell Hb (Weber and Vinogradov 2001). Functionally it means that a pH drop in the tissues when they become acidic will not result in an increase of oxygen delivery from the globins to the tissues.

On the contrary, when the temperature increases, the affinity of Hb in *L. chuni* drops. However, based on the exothermic nature of heme-oxygenation, P_{50} must increase at higher temperatures. The heat or enthalpy change (ΔH) due to the oxygen binding on the globins can be directly derived from the integrated Van't Hoff equation: $\Delta H = -2.303R * \Delta \log P50/\Delta(1/T)$, where R is the universal gas constant and T is the absolute temperature. Using this equation, we have calculated the enthalpy of the Hb-O_2 complex derived from our P_{50} data, at various temperatures. Between 5 and 15°C, at pH 7.5, the enthalpy changes for the Hb of *L. chuni* is -69 $kJ.mol^{-1}$. Moreover this high negative value is consistent with an absence of Bohr effect, as oxygenation-linked proton dissociation is endothermic, and would lower the overall enthalpy value. Comparatively, the heat liberated upon oxygen binding to alpha or beta human Hb monomers alone, is about -66 $kJ.mol^{-1}$ (Ackers 1980). Our enthalpy value is very close to that value and also comparable to those from the large Hb molecules in the pulmonate mollusk *Biomphalaria glabra* (ΔH=-49 to -77 $kJ.mol^{-1}$) (Bugge and Weber, 1999), and in an hydrothermal copepod (ΔH=-68.7 $kJ.mol^{-1}$) (Hourdez et al, 2000).

388 Oxygen affinity is higher in *L. chuni* (circulating hemoglobin and foot myoglobin: 0.45 and 0.19 Torr
389 respectively) than in *C. regab (*0.85 Torr). It is noteworthy that the affinity of the myoglobin in *L.*
390 *chuni* is particularly high, even higher than that of the circulating Hb, and surprisingly less variable to
391 temperature differences (as there is no Bohr effect to create an endothermic dissociation of protons
392 to compensate for the natural exothermic oxygen binding to the heme). The higher oxygen affinity in
393 the Mb than in the Hb indicates a biologically advantageous oxygen transfer from Hb to Mb to enable
394 oxygen storage in the muscle, as known in vertebrate animals and described in literature for some
395 invertebrates that have both these pigments (Wittenberg, 1970). The high affinity of Mb to oxygen
396 might also indicate a great importance of binding oxygen within the foot, in particular during sulfide
397 uptake from the sediments, to avoid oxidation of sulfide before they are transported through the
398 hemolymph and reach the symbionts, in the gills.
399 In conclusion the two sympatric species at the cold seep pockmark Regab vary greatly in their
400 modes to bring oxygen to their symbionts: *C. regab* transports the oxygen dissolved in its hemolymph
401 as a regular coastal bivalve, whereas *L. chuni* has a greatly enhanced transport capacitance with its
402 Hb as oxygen-carrier within red blood cells, as all the blood-clams. In fact Hb can both ensure the
403 oxygen transport and its storage in the hemolymph during hypoxic periods when the clam remains
404 closed, or when a greater oxygen demand occurs during burying. This oxygen storage might enable *L.*
405 *chuni* to bury deeper into the sediment to reach a larger source of sulfides. This hypothesis would be
406 in favor of the deep position of *L. chuni* which siphons are only rarely seen at the sediment surface,
407 while it could nonetheless ensure an aerobic metabolism. This also could explain the distribution of
408 both species along the pockmark according to the depth of the sulfide production. Indeed *L. chuni* is
409 more abundant in the southwestern part of the pockmark (site 3), where the lower methane fluxes
410 (Decker et al. 2012a; Pop Ristova et al. 2012) and deeper AOM maximum rates (Pop Ristova et al.
411 2012) may drive sulfide production deeper in the sediment, than at the most active central venting
412 site 2, as observed at other seep sites (Fischer et al. 2012). Whether its Hb could bind sulfide as those
413 of the lucinids and solemyids (Kraus 1990; Kraus et al. 1996) remains to be investigated. However it

414 is possible that the great molecular mass molecule, eluted in the void volume of the column during
415 the purification of the hemolymph, could be a Sulfide Binding Component comparable to the SBC
416 lipoprotein isolated in *"Calyptogena" magnifica* (Zal et al. 2000).
417
418 Compared to the majority of the blood clams found at vent and seeps, it is questionable how *C.*
419 *regab* can cope without Hb in its hemolymph. It might be explained by its different symbionts that
420 could have a different energy metabolism (Decker et al. 2013), or simply because this species always
421 position its shell and siphons well above the sediments surface to avoid hypoxy and get enough
422 oxygen. It would be interesting to see, whether hypoxic conditions would lead to anaerobic
423 metabolism in this species. Anyway *C. regab*'s foot myoglobin allows an oxygen supply during its
424 burying or moving laterally to find richer sulfide areas.
425
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440

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567

Figure Captions

Figure 1: Mixed clam aggregate of *Christineconcha regab* and *Laubiericoncha chuni.* The arrow points to *L. chuni*, from which only the siphons are visible. Photo © IFREMER, WACS cruise 2011, ROV Victor 6000

Figure 2: Sites studied within the Regab pockmark: Northern site (M3), Centre site (W05/M7) and South-Western site (W03/M10) . Photos © MARUM, GUINECO cruise 2008, ROV Quest 4000.

Figure 3: HPLC spectra of *L. chuni* hemolymph a) hemoglobin peak after SEC filtration on a Superose 12 column and b) myoglobin peak after SEC filtration on Superose 12 and Superdex 75 columns in series.

Figure 4 : Comparison between (a) *L.chuni* circulating hemoglobin and (b) *L.chuni* foot myoglobin (MaxEnt deconvolution spectra) : masses calculated on multiply charged spectrum were : (a): G1 : 15361.3 ± 0,3 Da; G2 : 15668.3 ± 0.2 Da; G3 : 15682.4 ± 0.3 Da and (b) G1 : 15362,8 ± 0,2 Da; G3 : 15682.1 ± 0.5 Da; G4 : 14786.8 ± 0.2 Da

Figure 5: Comparison of non-denaturant ESMS between (a,c) human hemoglobin and (b,d) *L.chuni* foot myoglobin (a and b : multiply charged spectra, c and d : MaxEnt deconvolution spectra). The human sample showed tetrameric α2β2 hemoglobin as expected (essentially with 16,17,18 and 19 H^+), whereas no multimeric form was found in the samples of *L.chuni*. The masses obtained for *L. chuni*'s globins show they are associated to their heme molecule : (▲)15401±1 Da : G4 + heme; (●)16297±1 Da : G3 + heme; (★)15977±1 Da : G1 + heme ; (charges from 6 to 9 H^+).

Figure 6: a) Elution profile of *C. regab* myoglobin after Size Exclusion Chromatography on a Superose 12. Arrows show the collected peak then purified on a Superdex 75 (b). Both columns were equilibrated with the Vesicomyid buffer (pH 6.2) described in Childress et al. (1991).

Figure 7: Temperature effect on *L. chuni* hemoglobin and foot myoglobin (a) and Bohr effect on *L. chuni* hemoglobin (b).

Figure 8: Temperature effect on *C. regab* foot myoglobin (a) and Bohr effect on *C. regab* myoglobin (b)

620 Table 1: Summary of globin chains identified in *L. chuni* circulating Hb and foot muscle samples, and
621 in *C. regab* foot homogenate.
622
623 Table 2: Oxygen binding properties of *L. chuni* hemoglobin and myoglobin and *C. regab* myoglobin at
624 15°C.
625

Tables :

Table 1: Summary of globin chains identified in *L. chuni* circulating Hb and foot muscle samples, and in *C. regab* foot homogenate.

Species	Chains found in all analyzed samples	Chains specific to…
L. chuni	G1 : 15361 ± 1 Da G3 : 15682± 1 Da	Foot muscle: G4 : 14786 ± 1 Da Circulationg Hb: G2 : 15668 ± 1 Da
C. regab	G1' : 15139 ± 1 Da	Site 1: G3' : 15683 ± 1 Da Site 2 : G'1-G'1: 30278 ± 1 Da G'2-G'2: 30340 ± 1 Da Sites 2 and 3: G2' : 15169 ± 1 Da

Table 2: Oxygen binding properties of *L. chuni* hemoglobin and myoglobin and *C. regab* myoglobin at 5°C.

Species	Specimens or samples	Type	pH	p50	n50
L. chuni	425-V1	Hemoglobin	6.7	0.26	0.83
	425-V2		6.57	0.48	0.91
	425-V3		6.67	0.61	0.71
	Mean (SD)		**6.65 (0.07)**	**0.45 (0.18)**	**0.82 (0.1)**
	Lc1	Myoglobin	6.02	0.17	1
	Lc2		6.05	0.2	1.09
	Lc3		6.74	0.2	1.55
	Mean (SD)		**6.27 (0.40)**	**0.19 (0.02)**	**1.20 (0.29)**
C. regab	Chr1	Myoglobin	6.78	0.95	0.98
	Chr2		6.55	0.8	0.92
	Chr3		6.55	0.79	0.82
	Mean (SD)		**6.63 (0.13)**	**0.85 (0.09)**	**0.91 (0.08)**

Figure 1

Figure 2

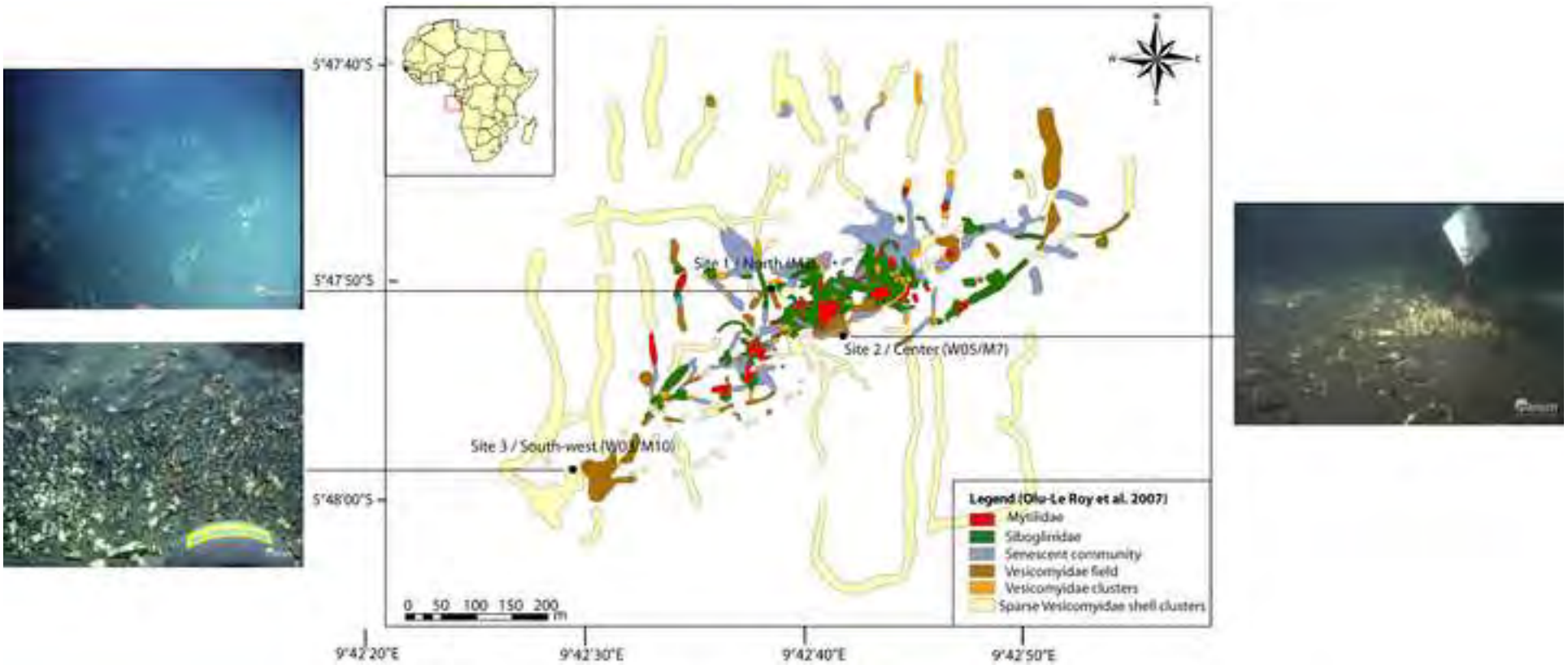
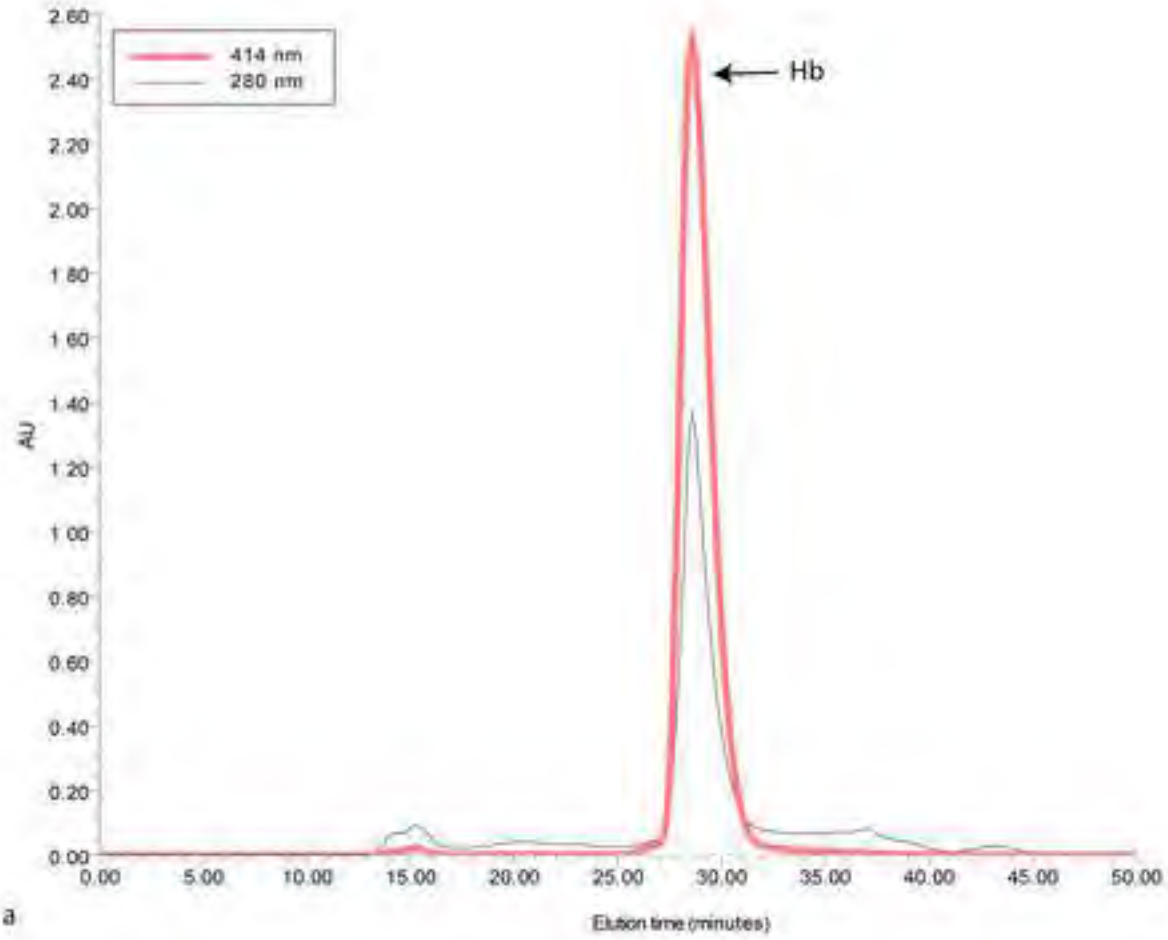

5°47'40"S
5°47'50"S
5°48'00"S
9°42'20"E
9°42'30"E
9°42'40"E
9°42'50"E
Site 2 / Center (W05/M7)
Site 3 / South-west (W03/M10)
0 50 100 150 200 m
Legend (Olu-Le Roy et al. 2007)
Mytilidae
Siboglinidae
Senescent community
Vesicomyidae field
Vesicomyidae clusters
Sparse Vesicomyidae shell clusters

Figure 3


414 nm
280 nm
Hb
AU
Elution time (minutes)
a

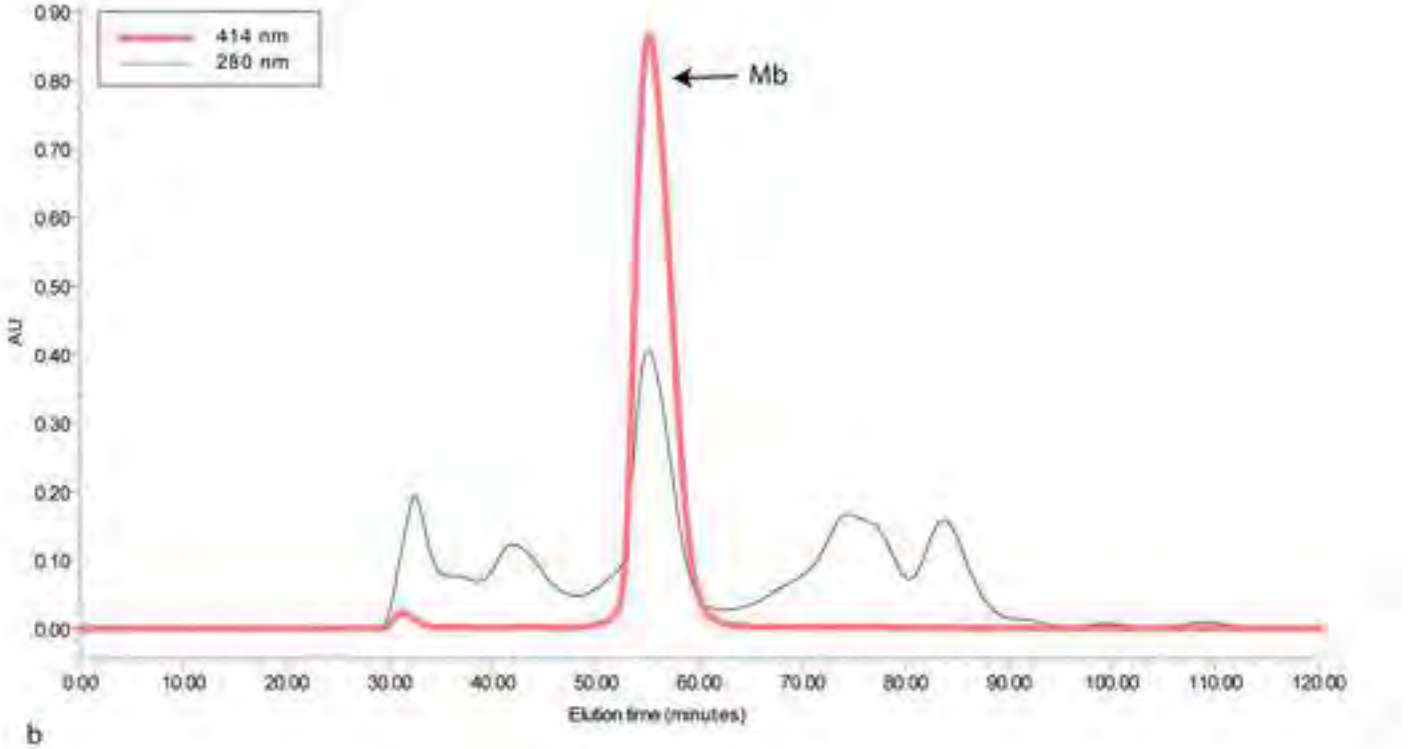

414 nm
280 nm
Mb
AU
Elution time (minutes)
b

Figure 4

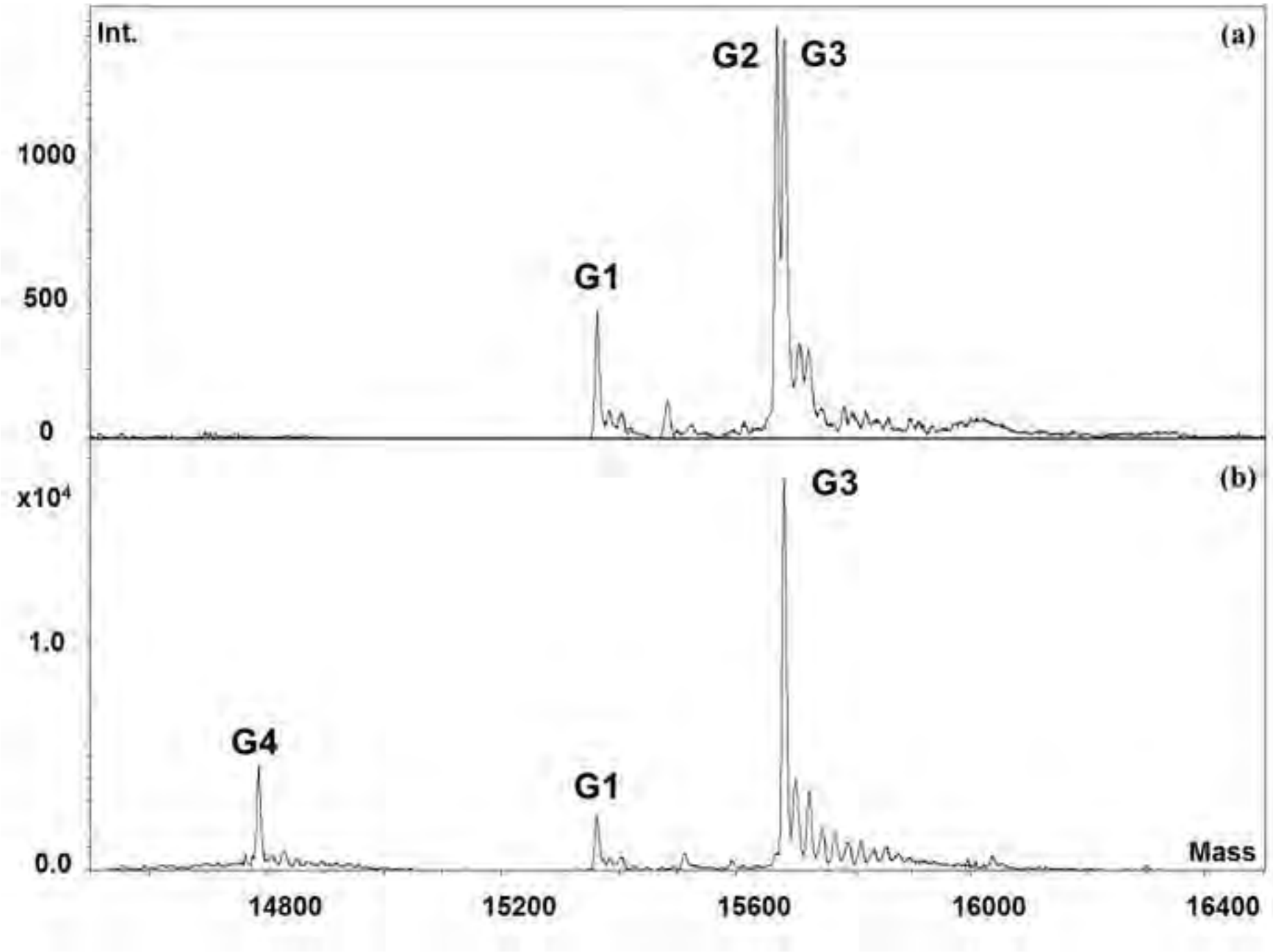

Int.
(a)
G2
G3
1000
500
0
G1
x10⁴
(b)
G3
1.0
G4
G1
0.0
Mass
14800
15200
15600
16000
16400

Figure 5

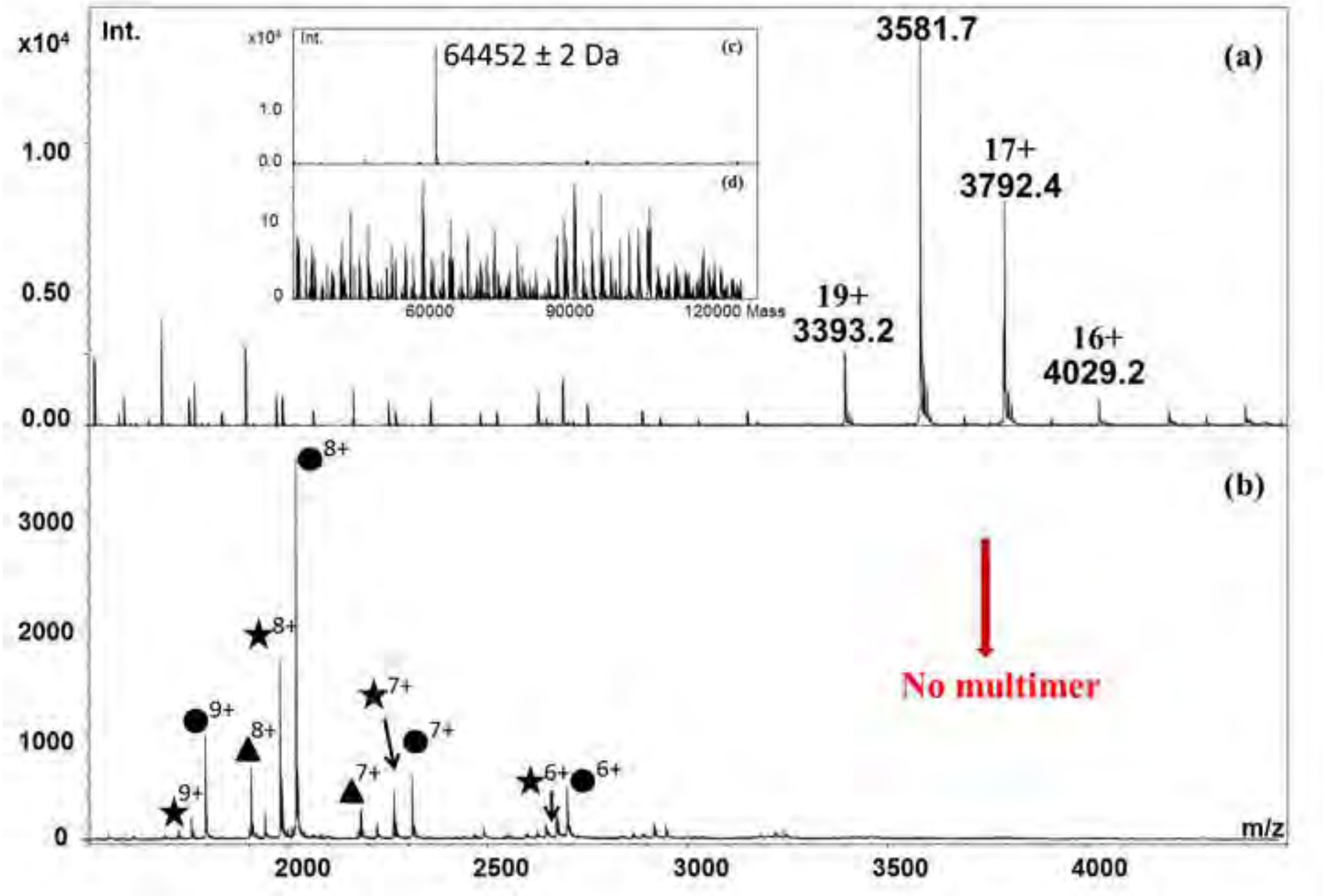
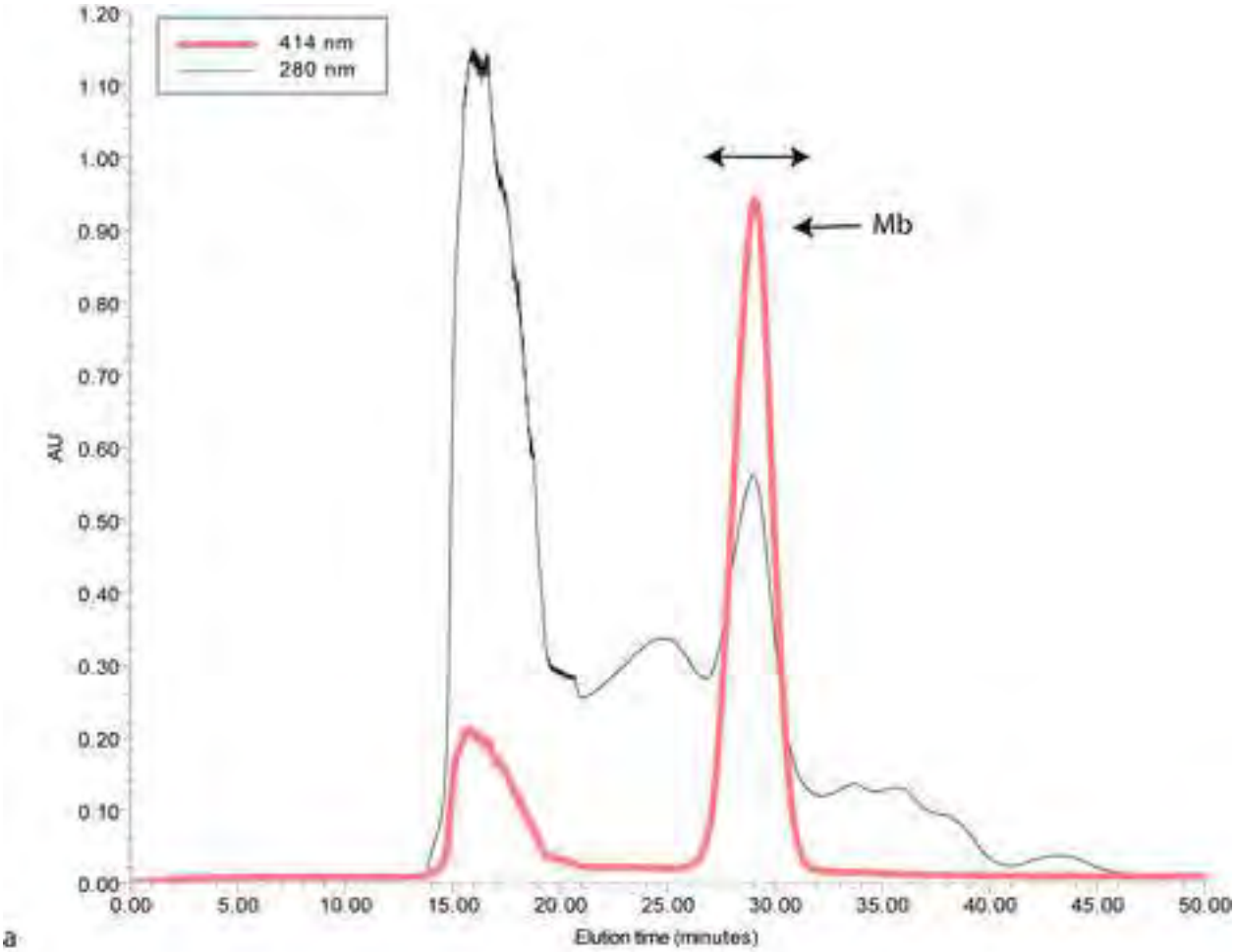

x10⁴
Int.
1.00
0.50
0.00
3581.7
(a)
17+
3792.4
19+
3393.2
16+
4029.2
x10⁴
Int.
1.0
0.0
(c)
64452 ± 2 Da
(d)
10
0
60000
90000
120000 Mass
3000
2000
1000
0
(b)
8+
8+
9+
8+
7+
7+
9+
7+
6+
6+
No multimer
m/z
2000
2500
3000
3500
4000

Figure 6


414 nm
280 nm
Mb
AU
Elution time (minutes)
a

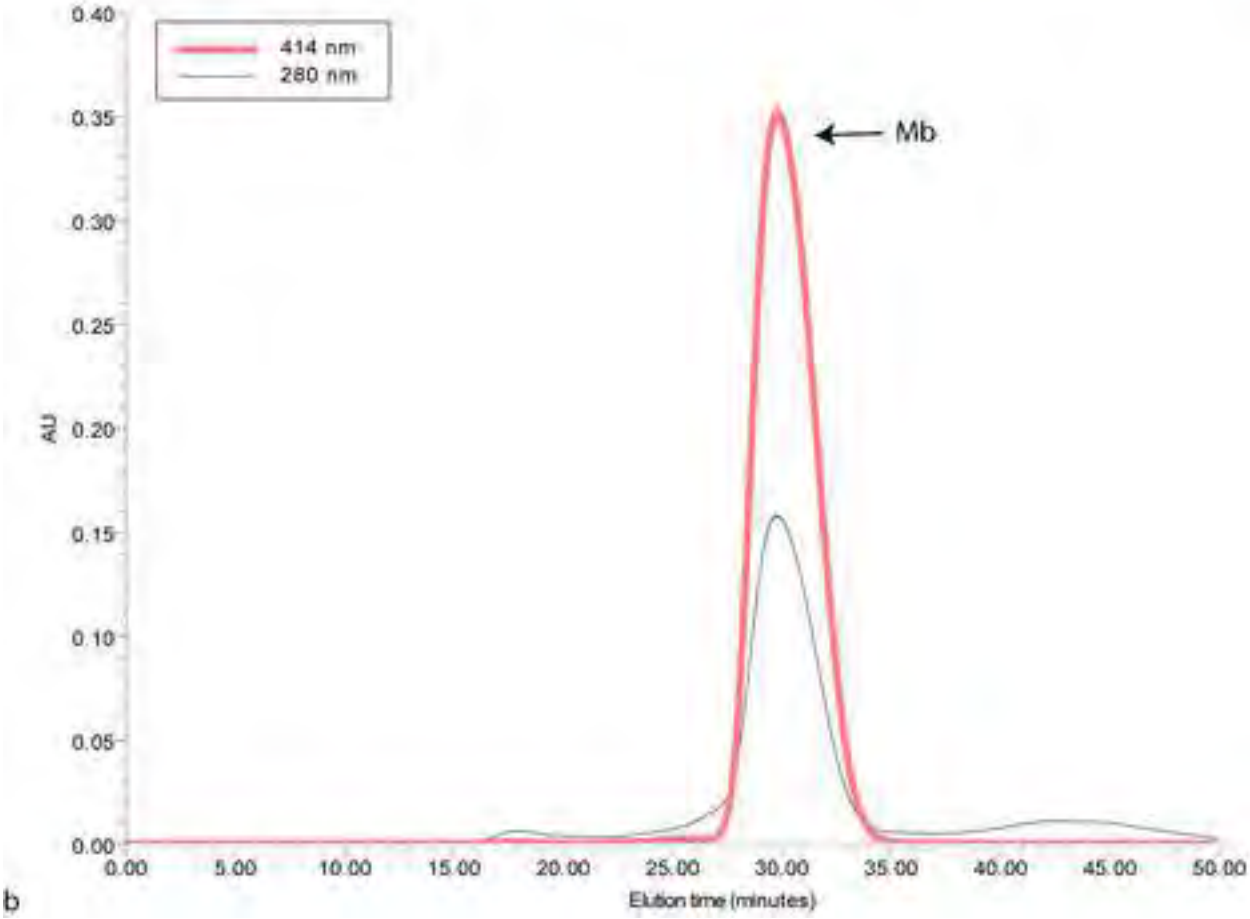
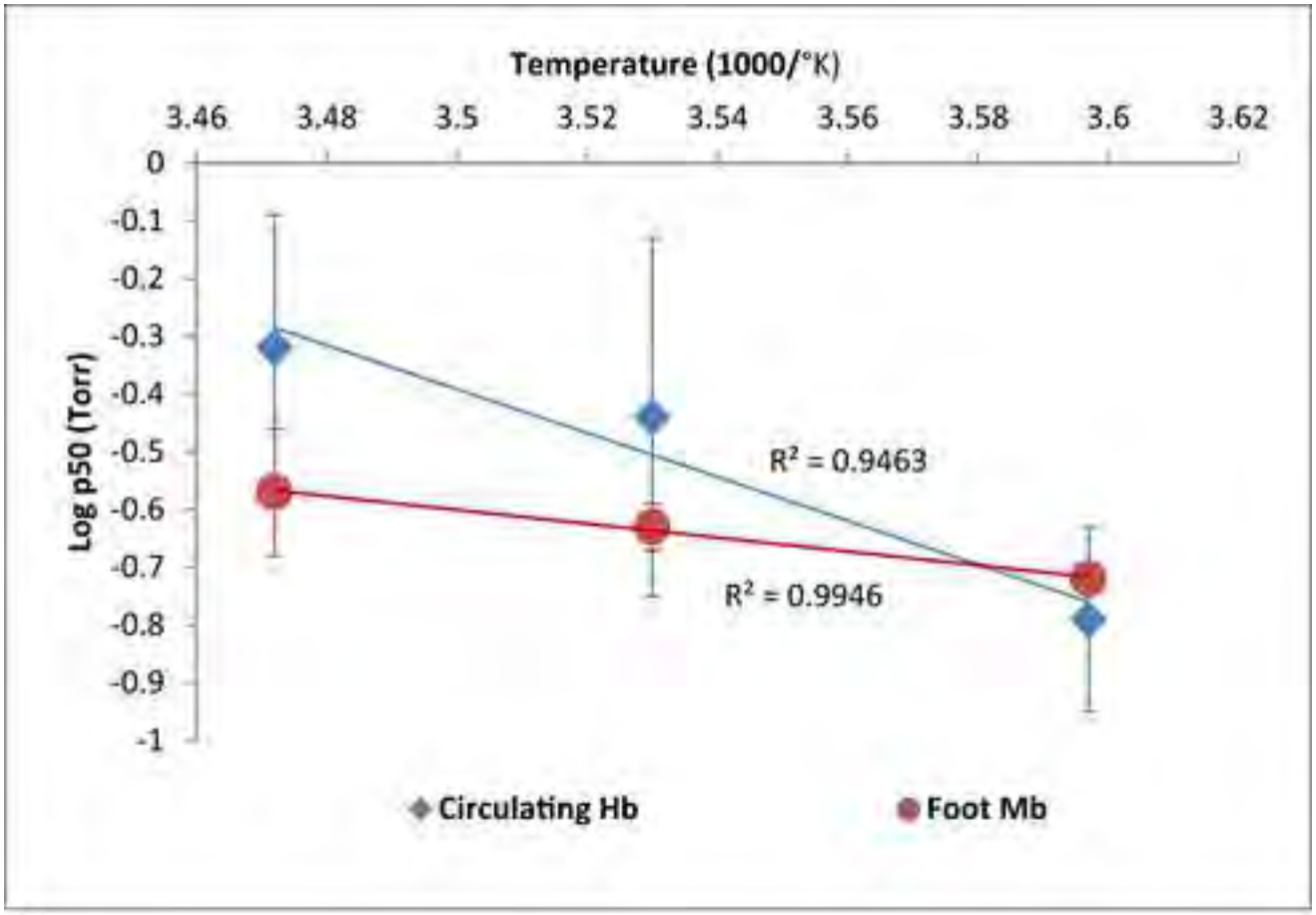
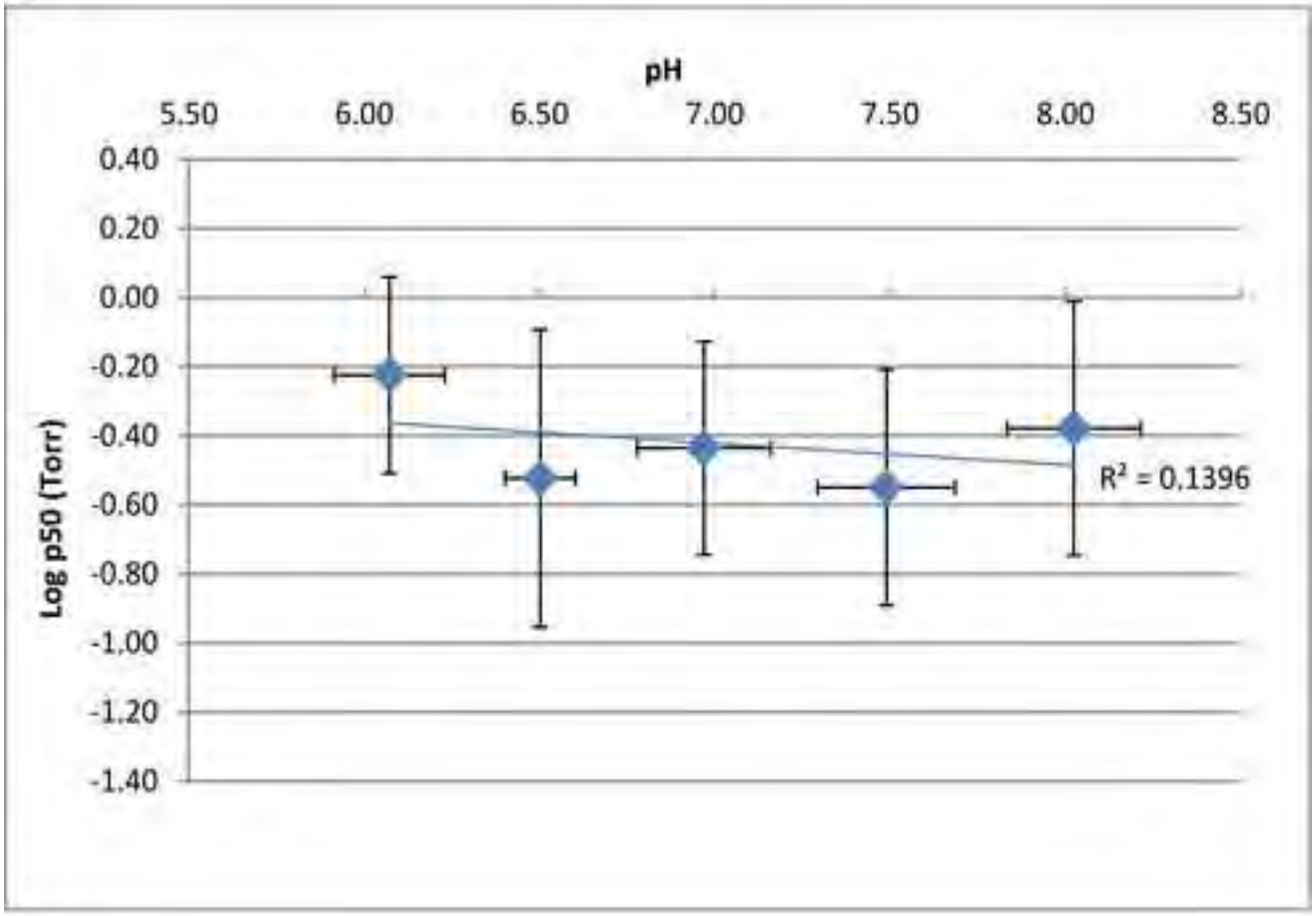

414 nm
280 nm
Mb
AU
Elution time (minutes)
b

Figure 7

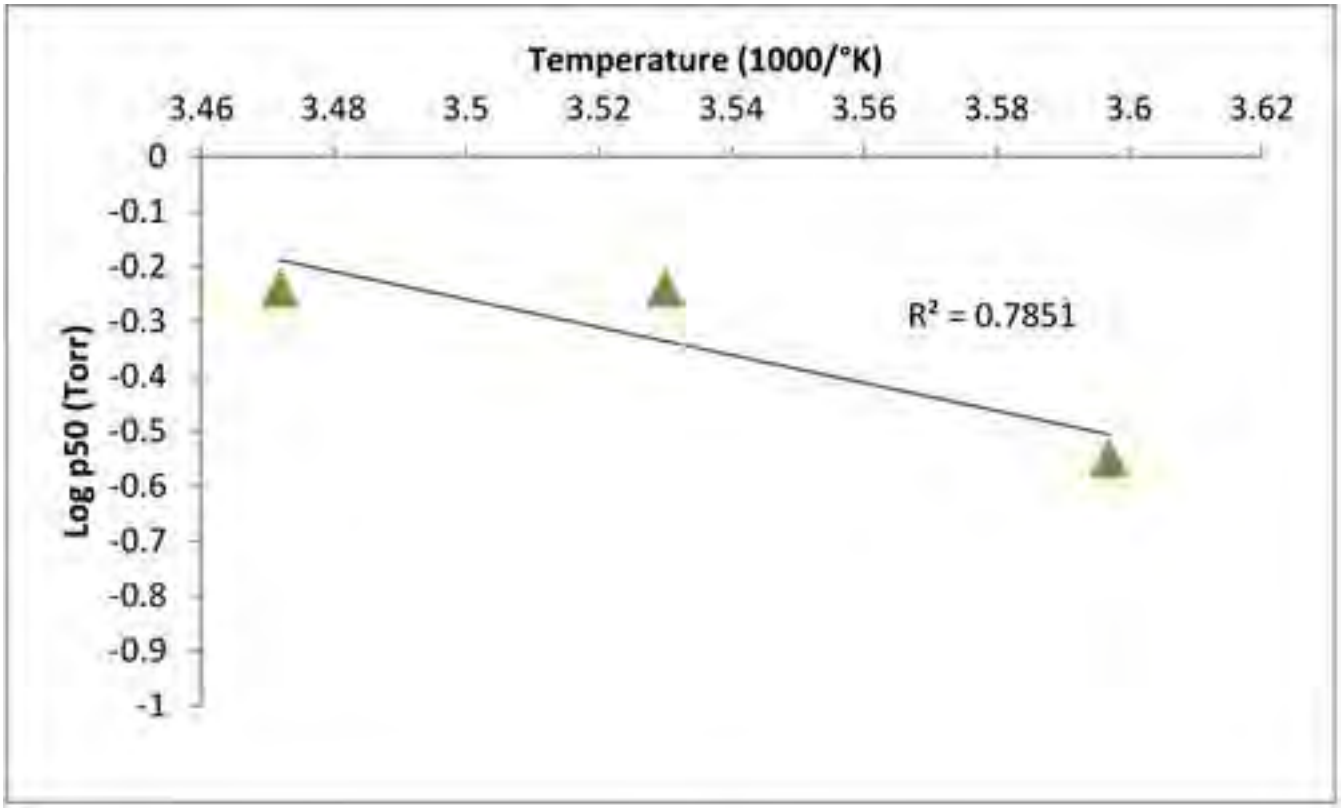

Temperature (1000/°K)
3.46
3.48
3.5
3.52
3.54
3.56
3.58
3.6
3.62
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1
Log p50 (Torr)
R² = 0.9463
R² = 0.9946
Circulating Hb
Foot Mb

a

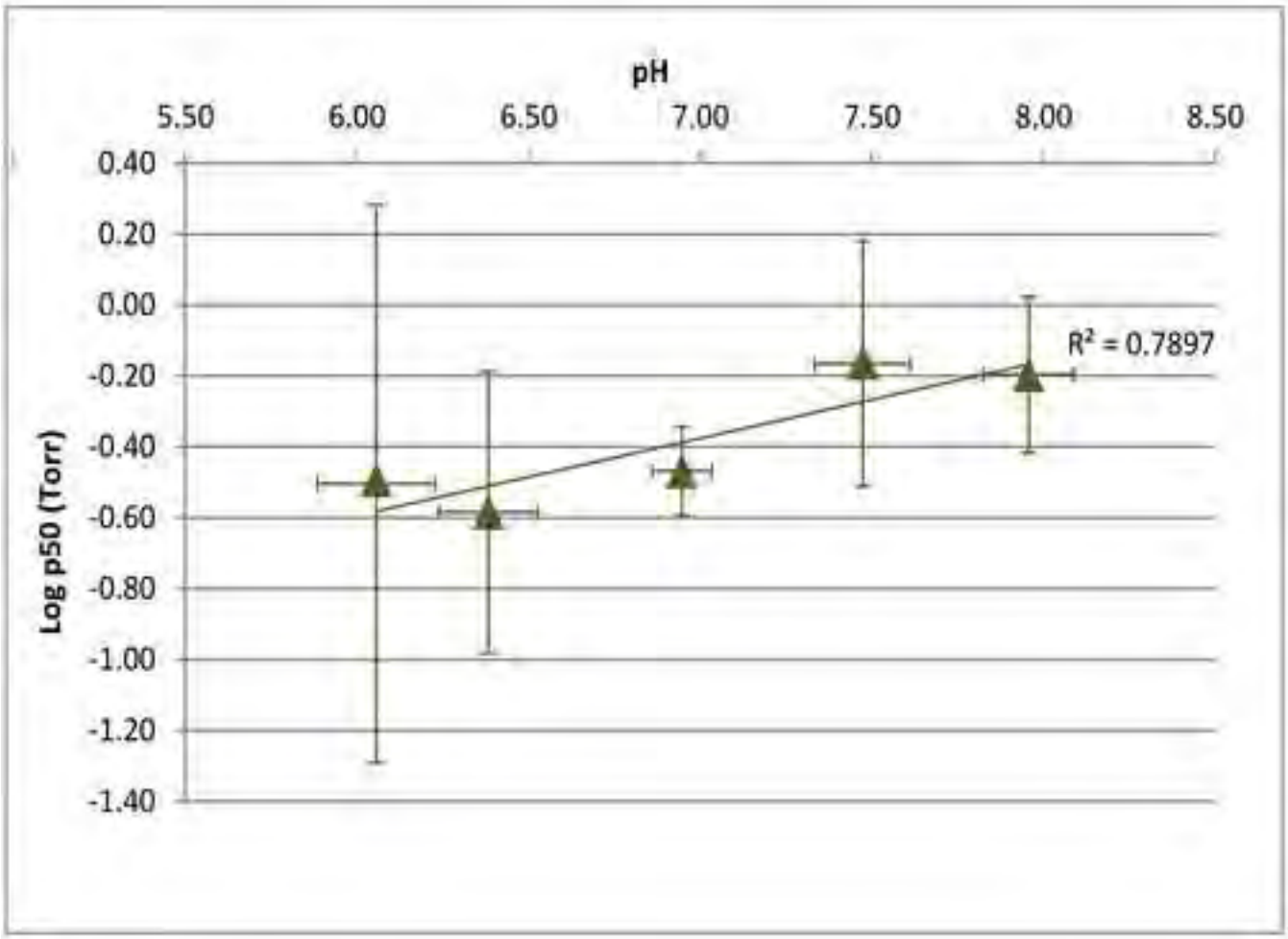

pH
5.50
6.00
6.50
7.00
7.50
8.00
8.50
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
-1.20
-1.40
Log p50 (Torr)
R² = 0.1396

b

Figure 8


Temperature (1000/°K)
3.46
3.48
3.5
3.52
3.54
3.56
3.58
3.6
3.62
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1
Log p50 (Torr)
R² = 0.7851

a


pH
5.50
6.00
6.50
7.00
7.50
8.00
8.50
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
-1.20
-1.40
Log p50 (Torr)
R² = 0.7897

b

Supporting information:

Table S1: Localization of studied sites, cruises, dives, specimen identification, sampled tissues and sample processing. * Foot tissue pooled before analyses.

PANGAEA Labels: [a] M76_3b_312_NET1, [b] M76_3b_323_PUC12, [c] M76_3b_379_NET2

Sites - Marker	Cruise	Dive n°- Specimen n°	Species	Tissue collected	Globin purification	Mass spectrometry	Heme assay analysis	Oxygen binding measurements
Site 1, North-M3	GUINECO 2008	209 [a]-V3	*C. regab*	Foot	x			
		209 [a] -V5	*C. regab*	Foot	x	x		
		211 [b] -PC12-V2	*C. regab*	Foot	x	x		
		211 [b] -PC12-V3	*C. regab*	Foot	x	x		
Site 2, Centre - W05/M7	WACS 2011	427-V118	*C. regab*	Foot	x	x		
		427-V119	*C. regab*	Foot	x	x		
		427-V120	*C. regab*	Foot	x	x		
		427-V122	*L. chuni*	Hemolymph	x	x		
Site 3, South-West - W03/M10	WACS 2011	425-V8	*C. regab*	Foot	x	x		
		425-V9	*C. regab*	Foot	x	x		
		425-V10	*C. regab*	Foot	x	x		
		425-V11*	*C. regab*	Feet pooled	x			x
		425-V12*						
		425-V13*						
		425-V14*	*C. regab*	Feet pooled	x			x
		425-V15*						

Chr1

Chr2

		425-V17*	Chr3					
		425-V18*						
	GUINECO 2008	225 [c] -V1		*L. chuni*	Hemolymph	x	x		x
		225 [c] -V6		*L. chuni*	Hemolymph				x
		225 [c] -V7		*L. chuni*	Hemolymph				x
	WACS 2011	425-V1		*L. chuni*	Hemolymph	x	x	x	x
		425-V2		*L. chuni*	Hemolymph	x	x	x	x
		425-V3		*L. chuni*	Hemolymph	x	x	x	x
		425-V1*	Lc1	*L. chuni*	Feet pooled	x	x		x
		425-V2*		*L. chuni*					
		425-V3*		*L. chuni*					
		425-V4*	Lc2	*L. chuni*	Feet pooled	x	x		x
		425-V5*							
		425-V6*							
		426-V57*	Lc3	*L. chuni*	Feet pooled	X	x		x
		426-V58*							
		426-V59*							
	CONGOLOBE 2012	481-V4		*L. chuni*	Hemolymph		x		