Temperature effects on hemocyanin oxygen binding in an Antarctic cephalopod

S. Zielinski, F.J. Sartoris and H.O. Pörtner

Alfred-Wegener-Institute for marine and polar research, Ecophysiology and Ecotoxicology,
Columbusstrasse, 27568 Bremerhaven, Germany

running head: Antarctic cephalopod hemocyanin function

Correspondence address:

Prof. Dr. H.O. Pörtner
Alfred-Wegener-Institut
für Polar- und Meeresforschung
Ökophysiologie und Ökotoxikologie
Columbusstr.
27568 Bremerhaven
Germany

Phone: ++49 471 4831 1307

FAX: ++49 471 4831 1149

email: hpoertner@awi-bremerhaven.de

Abstract

The functional relevance of oxygen transport by hemocyanin at the low temperatures of the Antarctic was investigated in the cold-stenothermal octopod *Megaleledone senoi*. The temperature dependence of oxygen binding to hemocyanin was analyzed by continuous simultaneous recordings of changes in pH and hemocyanin oxygen saturation. These data were compared to the thermal sensitivity of oxygen binding to the hemocyanin of other temperate and cold water cephalopods (octopods and giant squid according to the literature; eurythermal cuttlefish *Sepia officinalis* according to this study). Similar to observations in temperate octopods oxygen affinity of *Sepia officinalis* hemocyanin changed at $\Delta P_{50}/^{\circ}\text{C} = 0.12 \text{ kPa}$ (pH 7.4) with increasing temperatures, whereas in *Megaleledone senoi* this ratio was much smaller ($< 0.01 \text{ kPa}$, pH 7.2). Furthermore, *Megaleledone senoi* hemocyanin displayed one of the highest levels of oxygen affinity ($P_{50} < 1 \text{ kPa}$, pH 7.6, 0°C) found so far in cephalopods and a very low cooperativity ($n_{50} = 1.4$ at 0°C). pH sensitivity of oxygen binding indicated by the Bohr effect ($\Delta \log P_{50}/\Delta \text{pH}$) increased with increasing temperature in both, the cuttlefish and the Antarctic octopod. However, a pH independent venous reserve was visible below 43% saturation at 1.0 kPa O_2 in *Megaleledone senoi* at 0°C and below 19% at 1.7 kPa O_2 in *Sepia officinalis* at 20°C . In the context of literature data these findings confirm that the lower metabolic rate of octopods and cuttlefish compared to squid is reflected in less pH dependent oxygen transport. Findings in the Antarctic octopod were similar to a recent report on *Vampyroteuthis* hemocyanin with an extremely high oxygen affinity supporting a very low metabolic rate. Whereas in cold adapted giant squid, the extreme thermal sensitivity of oxygen transport may limit thermal tolerance, the minimized thermal sensitivity of hemocyanin oxygen binding in Antarctic octopods will reduce metabolic scope and thereby contribute to their stenothermality.

Introduction

Cephalopods are found throughout the seas of the world, from warm tropical waters to polar oceans (Roper *et al.*, 1984). Representatives of this group, especially squids, usually display the highest metabolic rates among marine invertebrates, even higher than those of fishes of similar size and mode of life (Webber and O'Dor, 1985; O'Dor and Webber, 1986). Oxygen delivery via the blood is maximized to cover metabolic requirements (Pörtner, 1994). However, oxygen carrying capacity by their blood pigment, hemocyanin, is constrained by the low maximum concentration of an extracellular pigment. This limitation is due to the unfavorable increase in colloidal osmotic pressure and blood viscosity at high pigment concentrations (Mangum, 1983; 1990). In consequence, the hearts of cephalopods must pump large amounts of blood (Wells *et al.*, 1988; Shadwick *et al.*, 1990) and the tissue extracts most of the oxygen on each return (Pörtner, 1994). Therefore, although squid display the highest hemocyanin concentrations in the animal kingdom, the level of bound oxygen around 1 - 2 mmol l⁻¹ (Brix *et al.*, 1989) is below the typical 4 - 5 mmol l⁻¹ found in active fishes (Urich, 1990). An even lower oxygen binding capacity than in squids results in some octopods at somewhat lower hemocyanin levels (Miller and Mangum, 1988; Senozan *et al.*, 1988, Brix *et al.*, 1989).

Cooperativity as well as adjustments of affinity according to changes in temperature and blood pH are the only mechanisms used to modulate hemocyanin function in most cephalopods for an adjustment of oxygen transport (e.g. Mangum, 1990; Pörtner, 1990; Brix *et al.*, 1989). Low molecular weight organic substances contributing to blood pigment function in vertebrates or crustaceans are not found in cephalopods. In consequence, extremely high Bohr coefficients ($\Delta \log P_{50} / \Delta \text{pH} < -1$) are common (Bridges, 1994) and pH dependent, very high levels of cooperativity are found in several cephalopod species (Miller, 1985; Pörtner, 1990). Binding of CO₂ together with O₂ in arterial blood has been suggested to support pigment function on the venous side in sepioid species (Brix *et al.*, 1981) where both O₂ and CO₂ are released and this CO₂ helps to exploit the large Bohr effect. In squid, supplementary oxygen uptake via the skin

supports the excessive oxygen demand and provides the excess CO₂ required for the Bohr effect to function (Pörtner, 1994).

A change in ambient temperature has a large effect on hemocyanin oxygen transport of some cephalopods, caused by an increase in cooperativity and a fall in oxygen affinity with rising temperature (Brix *et al.*, 1989; 1994; Mangum, 1990). If a rise in metabolic rate with temperature is supported by an adequate rise in P₅₀ the respective species should be able to live in a greater temperature range than a species where P₅₀ remains constant or where the change in P₅₀ is too large. For example, the high thermal sensitivity of hemocyanin oxygen affinity in the giant squid *Architeuthis monachus* suggests that arterial saturation becomes impossible at high temperatures (Brix, 1983). This question has gained general importance since comparative studies in Antarctic and temperate fish and invertebrates (including cephalopods, Pörtner and Zielinski, 1998) revealed that the limits of thermal tolerance are characterized by oxygen limitation, owing to insufficient capacity of circulation and/or ventilation to provide sufficient oxygen at extreme temperatures (for review see Pörtner *et al.*, 2000). Comparison of hemocyanin oxygen binding in cephalopods of different metabolic rates and from various latitudes should show how hemocyanin oxygen transport has adapted to different temperature regimes at various levels of metabolic activity and how blood pigment function contributes to the oxygen limitation of thermal tolerance.

These questions are especially interesting for an understanding of physiological adaptations to life in Antarctica. Here the marine environment is characterized by low and very stable water temperatures close to freezing (Clarke, 1988). Under these conditions more oxygen is physically dissolved thereby facilitating oxygen supply. At the same time metabolic rate is reduced with the consequence that in some species blood pigments may be less important at lower temperatures (for hemocyanin see Mauro and Mangum, 1982b; Bumett *et al.*, 1988). Some Antarctic fishes have even lost their respiratory pigments (Ruud, 1954). These relationships have not yet been investigated in Antarctic cephalopods.

Live specimens of the octopod *Megaleledone senoi* became available during a recent expedition to Antarctica with RV 'Polarstern'. This species is found in the indo-atlantic sector of the Antarctic ocean (Taki, 1961; Kubodera and Okutani, 1986; 1994). In our study we investigated oxygen binding to the hemocyanin of this stenothermal Antarctic octopod by using a technique which allows continuous simultaneous recordings of blood pH and oxygenation and construction of pH saturation diagrams (Pörtner, 1990). Such an approach is most suitable for cephalopod blood owing to the extremely large pH dependence of oxygen binding (see above). It avoids the use of artificial buffers which may lead to a change in oxygen binding properties (Pörtner, 1990; Brix et al. 1994). At the same time, the required amount of blood is reduced such that more sophisticated data can be collected from the very few animals accessible in remote and logistically difficult environments like the Antarctic. Oxygen binding properties of *Megaleledone senoi* hemocyanin were compared with those from other eury- and stenothermal cephalopods. For eurythermal octopods some literature data were available. Temperature effects on oxygen binding were studied experimentally in the cuttlefish *Sepia officinalis* to complement the data set available in the literature (Lykkeboe *et al.*, 1980; Johansen *et al.*, 1982a). To some extent, cuttlefish display a similar mode of life as octopods close to the bottom of the sea (von Boletzky, 1983), however, at a larger metabolic scope. Therefore, the role of metabolic scope in thermal adaptation of hemocyanin function should become visible.

Materials and Methods

Animals

Antarctic octopods (*Megaleledone senoi*, up to 9 kg body weight) were caught in November 1996 north to Elephant Island Antarctica during expedition ANT XIV/2 of RV 'Polarstern'. The animals were collected from bottom trawls. Samples were taken immediately after capture.

Cuttlefish *Sepia officinalis* (470 to 960 g body weight) were obtained from the Marine Biomedical Institute of the University of Texas, Galveston, Texas, USA, where this species has been bred and grown for several consecutive generations. They were kept at a salinity of 35‰ at temperatures of 20 to 22°C.

Sampling procedure

Animals were anaesthetized by transferring them into seawater which contained 2% ethanol (v/v). After full anaesthesia was reached, the animals were removed from the seawater and the mantle was opened by a ventral incision. Blood was collected from the vena cava, the systemic heart and the gill hearts. Blood samples from all animals were pooled, frozen and stored for up to one year at close to -20°C until utilized for in vitro studies of oxygen binding.

Analysis of oxygen binding

Oxygen binding characteristics of cephalopod hemocyanin were studied using a specially constructed cuvette, built by Hellma GmbH & Co (Mülheim, Germany; Fig. 1). The cuvette contained an upper and a lower compartment connected via two shafts (1.5 and 2 mm in diameter) in the left and right periphery of the cuvette, as well as a central compartment between the shafts, where blood formed a thin layer of only 0.45 mm. Stirring bars operating in the upper and lower compartments ensured continuous exchange of blood between all compartments and thus uniform mixture of the blood. Oxygen saturation was monitored continuously by measuring absorbance at 345 nm through the thin layer using a diode array spectro-photometer with fibre optics (X-dap, IKS Optoelektronik Meßgeräte GmbH, Waldbronn, Germany). Blood samples were equilibrated by introducing humidified gas mixtures through a hole in the lid of the cuvette. Gas mixtures of variable P_{O_2} (between 1.0 and 20.0 kPa) were prepared from pure O_2 , CO_2 and N_2 by gas-mixing pumps (type 2M303/a-F, Wösthoff, Bochum, Germany), complete deoxygenation occurred under pure N_2 . Blood pH was varied by changing P_{CO_2} (between 0.09 and 1.01 kPa) or by replacing small volumes (<10 µl per 2 ml of blood) of supernatant plasma after ultracentrifugation (1 h at 120000g; Beckman Airfuge, Beckman Instruments Inc, Fullerton Ca, USA) with fixed acid (1 mol l⁻¹ HCl) or base (2 mol l⁻¹ NaOH; Morris *et al.*, 1985; Pörtner, 1990). Changes in blood pH during oxygenation/ deoxygenation of hemocyanin were measured continuously by using a needle pH-electrode (long micro needle

electrode #811, Diamond General Corp, Ann Arbor MI, USA) which was introduced into one of the shafts via a second hole in the lid. Total CO₂ was analyzed at 0°C in 50 µl blood samples of *Megaleledone senoi* using the gas chromatography method of Lenfant and Aucutt (1966) modified after Boutilier *et al.* (1985). Measurements of oxygen binding properties were carried out at 0, 5 and 10°C for samples of *Megaleledone senoi* and at 0, 10 and 20°C for samples of *Sepia officinalis*. 10° C was chosen since this temperature can be reached in the northern part of sepia's distribution range depending on the season (Isemer and Hasse, 1985).

Graphical analysis and calculations

Hemocyanin concentrations were calculated according to Nickerson and van Holde (1971). Oxygen capacity was estimated using the molecular weights for octopods and sepia as compiled by Miller (1994) and the assumption that there are 70 O₂ binding sites per hemocyanin molecule in octopods and 80 in sepia. For the evaluation of hemocyanin oxygen saturation, constant absorbance levels in the range of the highest Po₂- and pH-values were set to 100% saturation. Changes in hemocyanin oxygenation and pH were plotted in a pH/saturation diagram according to Pörtner (1990). The resulting oxygen-binding curves represent isobars delineating the change in oxygenation with pH at constant Po₂. The points of intersection of the oxygen isobars with the line of half saturation quantify P₅₀ as it depends on pH. These P₅₀ and pH values were used to evaluate the Bohr coefficient, $\Delta \log P_{50} / \Delta \text{pH}$ by linear regression analysis. For comparison and owing to the presence of a large pH insensitive reserve at low temperatures, the coefficient $\Delta \log P_{80} / \Delta \text{pH}$ was evaluated following the same procedure. The Haldane coefficient ($\Delta \text{HCO}_3^- / \Delta \text{HcyO}_2$) was evaluated from the vertical distance between buffer lines in a pH/bicarbonate diagram (as used by Brix *et al.* 1981). For an evaluation of whether oxygen linked CO₂ binding to the hemocyanin occurs (Lykkeboe *et al.*, 1980), "measured" and calculated apparent bicarbonate levels were compared. Apparent "bicarbonate" (the sum of HCO₃⁻ and CO₃²⁻ levels)

was calculated from measured CO₂ concentrations (Cco₂) using the Pco₂ applied and the measured pH according to

$$[\text{HCO}_3^-] = \text{Cco}_2 - \alpha \text{Pco}_2 \quad (1)$$

where α is the solubility of CO₂. For comparison, bicarbonate levels were also calculated according to equation (1) with Cco₂ values derived from the Henderson-Hasselbalch equation:

$$\text{Cco}_2 = \text{Pco}_2 \cdot (\alpha \cdot 10^{\text{pH} - \text{pK}'''} + \alpha) \quad (2)$$

α and pK''' were calculated according to Heisler (1986).

Along each isobar in the pH/saturation diagram, values of saturation S depend on pH values and the Po₂ of the isobar. The pH/saturation diagram allows comparison of S and logPo₂ with P₅₀ at the same pH (= pH₅₀). This leads to an analysis of cooperativity at a specific pH. If this is done in the range of saturation S between 0.4 and 0.6, the analysis leads to an estimate of Hill coefficients (n₅₀) according to

$$\log(S/1-S) = n_{50}(\log \text{Po}_2 - \log \text{P}_{50}) \quad (3)$$

where Po₂ is the Po₂ of the isobar, S results from Po₂ at a specific pH (pH₅₀) and P₅₀ is the Po₂ for S = 0.5 at the same pH (Pörtner, 1990).

Results

The concentration of hemocyanin in native blood (hemolymph) was 93 g l⁻¹ for *Megaleledone senoi* and 142 g l⁻¹ for *Sepia officinalis*. This is equivalent to a maximum level of hemocyanin bound oxygen of 1.86 mmol O₂ l⁻¹ in the octopod and of 2.84 mmol l⁻¹ in sepia. For *Megaleledone senoi* hemocyanin the highest pH sensitivity of oxygen binding was found at 10°C as indicated by maximum slopes $\Delta S/\Delta \text{pH}$ (Fig. 2). Lower temperatures resulted in a somewhat decreased pH sensitivity of oxygen affinity with a maximum of $\Delta S/\Delta \text{pH} = 13\%$ per pH-unit at 10°C compared to a maximum of 10% per pH-unit at 0°C. Saturation at 0°C did not fall below 43% even at low pH (6.4 and 6.6) and low Po₂ (1 kPa). At 10°C, saturation dropped

to a minimum of 32% at the same P_{O_2} . Intermediate values of pH sensitivity and maximum unloading were found at 5°C.

A high Bohr coefficient of -2.33 was found at 10°C, similarly high as the coefficient $\Delta \log P_{80} / \Delta pH$ (Table 1). The experimental evaluation of the Bohr coefficient was not possible at lower temperatures due to the fact that pH dependent saturation did not drop below 50% at most oxygen partial pressures. An extrapolation of binding data to very low oxygen partial pressures revealed a Bohr coefficient of approximately -0.9 at 0°C, below the level of $\Delta \log P_{80} / \Delta pH$. Furthermore, oxygen affinity (P_{50}) at pH 7.2 changed only at $\Delta P_{50} / ^\circ C < 0.01$ kPa from 0.98 kPa at 0°C to 1.1 kPa at 10°C (Table 1).

For *Sepia officinalis* hemocyanin, pH sensitivity was high at 20°C reaching a maximum $\Delta S / \Delta pH$ of 41% per pH-unit (Fig. 3). Especially in the pH-range between 7.4 and 7.8 very small pH-changes were sufficient to cause maximal unloading of oxygen down to 19% saturation. pH sensitivity at 20°C was higher than found for *Megaleledone senoi* at all temperatures. Similar to *Megaleledone senoi* lower temperatures resulted in a decreased pH sensitivity of oxygen binding with a decreased Bohr factor and level of $\Delta \log P_{80} / \Delta pH$ (Table 1) and an increase in the pH-insensitive reserve at the same P_{O_2} . At 0°C, $\Delta S / \Delta pH$ reached only a maximal value of 7% per pH-unit. Oxygen saturation remained above 50% at all investigated oxygen partial pressures and pH values. At pH 7.4 oxygen affinity fell from $P_{50} = 5.3$ kPa at 10°C to $P_{50} = 6.5$ kPa at 20°C ($\Delta P_{50} / ^\circ C = 0.12$ kPa, Table 1).

The change in cooperativity with pH and temperature for *Sepia officinalis* is shown in Fig. 4. At 20°C, the largest Hill coefficient (n_{50}) of 5.9 was found at a pH (7.48) where pH sensitivity ($\Delta S / \Delta pH$) was also high. A decrease in temperature to 10°C resulted in a decrease of the maximal Hill coefficient to $n_{50} = 4.6$ (pH 7.29). The maximum was shifted to lower pH. In contrast to cuttlefish, much lower Hill coefficients were found for *Megaleledone senoi* (Table 1). At 0°C n_{50} was 1.4 (pH 7.43), while it varied between 1.0 (pH 6.83) and 1.4 (pH 7.31) at 10°C. In contrast to the cuttlefish no clear maximum could be found.

Analysis of total CO_2 in *Megaleledone senoi* blood during variations of Po_2 and Pco_2 yields the buffer lines depicted in the pH/bicarbonate diagrams (Fig. 5). The position of the buffer line shifts between oxygenated and deoxygenated blood according to the quantity of H^+ bound by the pigment. The vertical distance between the buffer lines yields the Haldane coefficient ($\Delta\text{HCO}_3^-/\Delta\text{HcyO}_2$). For *Megaleledone senoi* hemocyanin it rose with falling pH at 0°C (Table 1). The diagram (Fig. 5) also includes the calculated apparent bicarbonate levels for oxygenated and deoxygenated blood. These theoretical values diverge only slightly from the measured values and suggest that O_2 linked CO_2 binding does not exist. Non-bicarbonate buffer values (β_{NB}) of $4.25 \text{ mmol l}^{-1} \text{ pH-units}^{-1}$ result for *Megaleledone senoi* blood at 0°C a value in the same range as in the squids *Illex illecebrosus* and *Loligo pealei* (5.0 and $5.8 \text{ mmol l}^{-1} \text{ pH-units}^{-1}$, respectively; Pörtner, 1990).

Discussion

At the low temperatures of Antarctica icefishes rely exclusively on the transport of physically dissolved oxygen in the blood. The presence of hemocyanin bound oxygen in *Megaleledone senoi* blood (hemolymph) at levels similar to those seen in squids and temperate octopods (cf. Brix et al., 1989) suggests that oxygen transport via hemocyanin in this Antarctic species is not less important than in temperate and warm water cephalopods. This may be related to the low level of capillarisation of cephalopod musculature compared to fish muscles (Bone et al. 1981).

Sensitivities to pH and temperature are most important in the modification of hemocyanin oxygen binding in cephalopods (Bridges, 1994; Pörtner, 1994; Brix et al., 1989). The temperature dependence of P_{50} varies between species. For example, the oxygen affinity of giant squid (*Architeuthis monachus*) hemocyanin decreases at $\Delta P_{50}/\Delta T = 1.89 \text{ kPa per } ^\circ\text{C}$ temperature increment (pH 7.4), while only $\Delta P_{50}/\Delta T = 0.20 \text{ kPa}/^\circ\text{C}$ (pH 7.4) was found for the octopod *Octopus vulgaris* (calculated after Brix et al., 1989). A lower value of $\Delta P_{50}/\Delta T = 0.10 \text{ kPa}/^\circ\text{C}$ (pH 7.4) was found for the octopod *Eledone cirrhosa* (calculated after Bridges, 1994).

similar to the value of $\Delta P_{50}/\Delta T = 0.12 \text{ kPa}/^\circ\text{C}$ (pH 7.4) calculated for *Sepia officinalis* hemocyanin in the present study. In eurythermal cephalopods like *Sepia officinalis*, *Octopus vulgaris* or *Eledone cirrhosa* and some squids a moderate rise in P_{50} with temperature occurs (cf. Brix et al., 1994) such that P_{O_2} is buffered at a high enough capillary P_{O_2} . This is required for elevated diffusive oxygen flux to mitochondria when increasing temperature causes a rise in metabolic rate. This feature allows *Sepia officinalis* to cover a wide range of distribution from the Mediterranean to the North Sea (von Boletzky, 1983). The extreme thermal sensitivity of P_{50} seen in the giant squid may eliminate hemocyanin oxygen transport and contribute to low heat tolerance (Brix, 1983). In contrast, the very small adjustments of P_{50} found in *Megaleledone senoi* hemocyanin ($\Delta P_{50}/\Delta T = 0.01 \text{ kPa}/^\circ\text{C}$, pH 7.2) may be detrimental since blood P_{O_2} will be buffered at too low levels when temperature rises, not being able to adequately support an increase in metabolic rate. This may contribute to the stenothermality of Antarctic octopods like *Megaleledone senoi*.

For *Sepia officinalis* at 20°C maximum pH sensitivity (Fig. 3) was found in the range of *in vivo* blood pH (7.4 - 7.8 at 17 - 19°C; Johansen *et al.*, 1982a). The same observation is valid for squid (Pörtner, 1990). The fact that the oxygen transport system is designed to respond to even small changes in extracellular acid-base status emphasizes the pH dependent P_{O_2} buffer function of the hemocyanin (Pörtner, 1994). Accordingly, cephalopods have been suggested to regulate primarily extracellular, not intracellular, acid-base balance (Pörtner, 1994).

The *in vivo* blood pH of the Antarctic octopod is not known. Assuming that this parameter follows α -stat predictions (Reeves, 1972) and is high in the cold as seen in *Loligo pealei* blood *in vitro* (Howell and Gilbert, 1976) the *in vivo* pH range for *Megaleledone senoi* will be located between pH 7.7 and 7.9 at 0°C. With the highest pH sensitivity in this pH range, oxygen unloading would occur at very low oxygen tensions (< 1 kPa) supporting only very low metabolic rates (Fig. 2). A P_{50} below 1 kPa (pH 7.6; 0°C) reflects one of the highest oxygen

affinities reported so far for cephalopods. This value is close to the P_{50} of 0.47 to 0.55 kPa evaluated for the cold water vampire squid *Vampyroteuthis infernalis* (5°C; Seibel *et al.*, 1999). These findings suggest that *Megaleledone senoi* displays a metabolic rate similarly low as found for the Antarctic octopod *Pareledone charcoti* (0.3 $\mu\text{mol g}^{-1} \text{h}^{-1}$ at 0°C and about 50 g body weight; H.O. Pörtner, T. Hirse, V. Wegewitz, unpublished data, 15 times lower than similar sized *Sepia officinalis* at 17°C, 4.4 $\mu\text{mol g}^{-1} \text{h}^{-1}$, Johansen *et al.*, 1982b) or even lower and close to the 0.1 $\mu\text{mol g}^{-1} \text{h}^{-1}$ measured at 5°C in the deep sea squid *Vampyroteuthis infernalis*, Seibel *et al.*, 1997).

Similar to findings in the crustaceans *Cancer magister*, *Cancer anthonyi* (Burnett *et al.*, 1988) and the octopod *Eledone cirrhosa* (Bridges, 1994), the Bohr coefficient evaluated for both investigated cephalopod species dropped when temperature decreased. In *Sepia officinalis* $\Delta\log P_{50}/\Delta\text{pH}$ decreased moderately from -1.33 at 20°C to -0.99 at 10°C (Table 1). The Bohr coefficient fell drastically from an extremely high value of -2.33 at 10°C to a much smaller value evaluated by extrapolation as -0.9 at 0°C in the hemocyanin of *Megaleledone senoi* (Table 1). The Bohr factor in the vampire squid was found even lower at -0.22 (Seibel *et al.*, 1999). These findings indicate that the Bohr effect becomes less important at low temperature and low metabolic rate.

A mechanism of oxygen-linked CO_2 -binding has been proposed for *Sepia* hemocyanin which transports both O_2 and CO_2 to the tissues. CO_2 produced in metabolism and the CO_2 released during deoxygenation would elicit a drop in pH as required for the large Bohr effect (<-1.0) to function normally (Lykkeboe *et al.*, 1980; Brix *et al.*, 1981). No oxygen-linked CO_2 -transport was found in *Megaleledone senoi* (fig. 5). At 0°C, the estimated Bohr coefficient of $\Delta\log P_{50}/\Delta\text{pH} \approx -0.9$ would reflect normal function of the Bohr effect, while the extremely high Bohr coefficient at 10°C would be counterproductive for oxygen transport, a finding in line with the stenothermality of Antarctic animals.

A reduced pH sensitivity of hemocyanin oxygen binding in *Megaleledone senoi* and other octopods compared to *Sepia officinalis* and squids is reflected in the magnitude of the pH-

independent venous reserve which rises as temperature falls (Figs. 2, 3). This reserve represents the amount of oxygen which remains bound to the respiratory pigment at constant P_{O_2} even when pH falls to very low values. Comparison of this venous reserve for several cephalopod species at normal environmental temperatures and low P_{O_2} shows that it is below 5% (at 1.7 kPa) for the squid *Illex illecebrosus* (15°C; Pörtner, 1990), 19% (at 1.7 kPa) for *Sepia officinalis* (20°C; this study), and 43% (at 1 kPa) for the Antarctic octopod *Megaleledone senoi* (0°C; this study). A value of below 10% results for the haemocyanin of the octopod *Octopus dofleini* (at 1.7 kPa and 20°C; Pörtner, 1990; calculated after Miller and Mangum, 1988), however, the *in vivo* value may be higher since this octopod lives at lower temperatures. The high pH sensitivity of squid hemocyanins maximizes the release of oxygen in the tissues and supports their high metabolic rate (Pörtner 1990, 1994). Sepioids and even more so, octopods display a less active life style with lower metabolic rates (for example: Houlihan *et al.*, 1982; Webber and O'Dor, 1985; 1986; Finke *et al.*, 1996; Seibel *et al.*, 1997). This may eliminate the necessity to maximize pH dependent oxygen transport to the extent seen in squids.

The cooperativity of respiratory pigments is characterised by the Hill coefficient (n_{50}). In *Sepia officinalis* at 20°C (fig. 4) and in the squids *Illex illecebrosus* and *Loligo vulgaris* the maximum cooperativity correlates with the highest pH sensitivity of oxygen binding ($\Delta S/\Delta pH$) in the range of *in vivo* pH (Pörtner, 1990). This reflects maximal deoxygenation at minimal pH change (Pörtner, 1990; 1994). A decrease in temperature caused the maximal Hill coefficient of *Sepia officinalis* hemocyanin to drop from $n_{50} = 5.9$ at 20°C to $n_{50} = 4.6$ at 10°C. At the same time, this maximal value was shifted to lower pH values, when *in vivo* pH should rise according to α -stat predictions (Reeves, 1972). A similar temperature dependence of the Hill coefficient was found for several crustaceans (Mauro and Mangum, 1982a,b). Like with the Bohr effect, this suggests that cooperativity is less important in oxygen transport at lower temperatures. Accordingly, a low cooperativity of $n_{50} = 1.4$ was found for *Megaleledone senoi*, at 0°C (Table 1) and of $n_{50} = 2.2$ for the vampire squid (Seibel *et al.*, 1999). Surprisingly, cooperativity did not increase with temperature in *M. senoi* hemocyanin.

The question arises why thermal sensitivity is so low in *Megaleledone senoi* hemocyanin whereas it is so high in the blood pigment of cold adapted giant squid (cf. Brix, 1983). A high value of $\Delta P_{50}^{\circ C^{-1}}$ reflects a high heat of oxygenation (cf. Brix et al., 1994) or Arrhenius activation energy. Giant squid likely display higher metabolic rates and thus require to maintain higher P_{50} levels than *Megaleledone senoi*. A high heat of oxygenation may be required for high P_{50} values at low temperature as in giant squid. In that respect the low thermal sensitivity of hemocyanin in the Antarctic octopod is again in accordance with the low metabolic rate of this group.

In summary, the pH sensitivity of oxygen binding in cephalopod hemocyanins is adjusted to metabolic rate. The pH insensitive reserve was largest in *Megaleledone senoi* and intermediate in *Sepia officinalis* hemocyanin, if compared to squids (this study and Pörtner, 1990). Furthermore, the Bohr effect is reduced and the pH insensitive reserve rises during cooling suggesting that pH-sensitivity falls in the cold. The temperature dependence of the Bohr factor was less pronounced in the eurythermal *Sepia officinalis*, which would, together with an appropriate change in P_{50} , ensure oxygen supply at changing ambient temperatures in their natural environment. In *Megaleledone senoi*, a high oxygen affinity of the hemocyanin, a moderately high Bohr coefficient and a low cooperativity cause P_{O_2} to be buffered at low values matching a low rate of oxygen consumption. The low thermal sensitivity of oxygen affinity prevents an upward shift of the buffered P_{O_2} at higher temperatures, suggesting that oxygen transfer to tissues may become limiting when oxygen demand rises. This contrasts the findings in giant squid where an excessive drop in oxygen affinity hampers arterial oxygen uptake and thereby contributes to limiting heat tolerance (Brix, 1983). Accordingly, hemocyanin likely contributes to an oxygen limitation of heat tolerance which sets in early and characterizes thermal tolerance in Antarctic octopods (Pörtner and Zielinski, 1998) and probably also in giant squid.

Acknowledgments:

The authors like to thank Iris Hardewig and Boris Klein for sampling of blood from *Megaleledone senoi* during the expedition with RV 'Polarstern'. The technical and logistical help by the staff of the Marine Biomedical Institute is gratefully acknowledged. Supported by grants of the Deutsche Forschungsgemeinschaft to HO Pörtner (Po 278).

Literature Cited

- Bone, Q., A. Pulsford, and A. D. Chubb. 1981. Squid mantle muscle. J. mar. biol. Ass. U.K. **61**: 327-342
- Boutilier, R. G., G. K. Iwama, T. A. Heming, and D. J. Randall. 1985. The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15°C. Respir. Physiol. **61**: 237-254.
- Bridges, C. R. 1994. Bohr and Root effects in cephalopod haemocyanins - paradox or pressure in *Sepia officinalis*? Pp. 121-130 in Physiology of cephalopod molluscs - lifestyle and performance adaptations, H. O. Pörtner, R. K. O'Dor and D. MacMillan, eds. Gordon and Breach Publishers, Basel.
- Brix, O. 1983. Giant squids may die when exposed to warm water currents. Nature **303**: 422-423.
- Brix O., G. Lykkeboe, and K. Johansen. 1981. The significance of the linkage between the Bohr and Haldane effects in cephalopod bloods. Respir. Physiol. **44**: 177-186.
- Brix O., A. Bardgard, A. Cau, A. Colosimo, S. G. Condò, and B. Giardina. 1989. Oxygen-binding properties of cephalopod blood with special reference to environmental temperatures and ecological distribution. J. Exp. Zool. **252**: 34-42.
- Brix O., A. Colosimo, and B. Giardina. 1994. Temperature dependence of oxygen binding to cephalopod haemocyanins: ecological implications. Pp. 149-162 in Physiology of cephalopod molluscs - lifestyle and performance adaptations, H. O. Pörtner, R. K. O'Dor and D. MacMillan, eds. Gordon and Breach Publishers, Basel.

- Burnett L. E., D. A. Scholnick, and C. P. Mangum. 1988. Temperature sensitivity of molluscan and arthropod hemocyanins. Biol. Bull. **174**: 153-162.
- Clarke A. 1988. Seasonality in the Antarctic marine environment. Comp. Biochem. Physiol. **90B**: 461-473.
- Finke E., H. O. Pörtner, P. G. Lee, and D. M. Webber. 1996. Squid (*Lolliguncula brevis*) life in shallow waters: oxygen limitation of metabolism and swimming performance. J. Exp. Biol. **199**: 911-921.
- Heisler N. 1986. Buffering and transmembrane ion transfer processes. Pp. 3-47 in Acid-base regulation in animals, N. Heisler, ed. Elsevier Science Publishers, Amsterdam.
- Houlihan D. G., A. J. Innes, M. J. Wells, and J. Wells. 1982. Oxygen consumption and blood gases of *Octopus vulgaris* in hypoxic conditions. J. Comp. Physiol. **148**: 35-40.
- Howell B. J., and D. L. Gilbert. 1976. pH-temperature dependence of the hemolymph of the squid, *Loligo pealei*. Comp. Biochem. Physiol. **55A**: 287-289
- Isemer H. J., and L. Hasse. 1985. The Bunker climate atlas of the North Atlantic ocean. Vol I: observations. Springer Verlag, Berlin, Heidelberg, New York, Tokyo. 218 pp.
- Johansen K., O. Brix, and G. Lykkeboe. 1982a. Blood gas transport in the cephalopod, *Sepia officinalis*. J. Exp. Biol. **99**: 331-338.
- Johansen K., O. Brix, S. Kornerup and G. Lykkeboe. 1982b. Factors affecting O₂ uptake in the cuttlerfish, *Sepia officinalis*. J. mar. Biol. Ass. U.K. **62**: 187-191.
- Kubodera T., and T. Okutani. 1986. New and rare cephalopods from the Antarctic waters. Mem. Natl. Inst. Polar Res., Spec. Issue **44**: 129-143.
- Kubodera T., and T. Okutani. 1994. Eledonine octopods from the Southern Ocean: systematics and distribution. In Southern Ocean cephalopods: life cycles and populations, P. G. Rodhouse, U. Piatkowski and C.C. Lu, eds. Ant. Sci. **6**: 205-214.
- Lenfant C., and C. Aucutt. 1966. Measurement of blood gases by gas chromatography. Respir. Physiol. **1**: 398-407.
- Lykkeboe G., O. Brix, and K. Johansen. 1980. Oxygen-linked CO₂ binding independent of pH in cephalopod blood. Nature **287**: 330-331.

- Mangum C. P. 1983.** Oxygen transport in the blood. Pp. 373-429 in The biology of Crustacea Vol. 3, L. H. Mantel, ed. Academic Press, New York, London.
- Mangum C. P. 1990.** Gas transport in the blood. Pp. 443-468 in Squid as experimental animals, D. L. Gilbert, E. J. Adelman jr and J. M. Arnold, ed. Plenum Publishing Corporation, New York.
- Mauro N. A., and C. P. Mangum. 1982a.** The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. I. Intraspecific responses to seasonal differences in temperature. J. Exp. Zool. **219**: 179-188.
- Mauro N. A., and C. P. Mangum. 1982b.** The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. II. Interspecific adaptations to latitudinal changes. J. Exp. Zool. **219**: 189-195.
- Miller K. I. 1985.** Oxygen equilibria of *Octopus dofleini* hemocyanin. Biochemistry **24**: 4582-4586.
- Miller K. I. 1994.** Cephalopod hemocyanins. A review of structure and function. Pp. 101-120 in Physiology of cephalopod molluscs - lifestyle and performance adaptations, H. O. Pörtner, R. K. O'Dor and D. MacMillan, eds. Gordon and Breach Publishers, Basel.
- Miller K., and C. P. Mangum. 1988.** An investigation of the nature of Bohr, Root, and Haldane effects in *Octopus dofleini* hemocyanin. J. Comp. Physiol. **158B**: 547-552.
- Morris S., A. C. Taylor, C. R. Bridges, and M. K. Grieshaber. 1985.** Respiratory properties of the haemolymph of the intertidal prawn *Palaemon elegans* (Rathke). J. Exp. Zool. **233**: 175-186.
- Nickerson K.W., and K.E. van Holde. 1971.** A comparison of molluscan and arthropod hemocyanin. I. Circular dichroism and absorption spectra. Comp. Biochem. Physiol. **39B**: 855-872.
- O'Dor R. K., and D. M. Webber. 1986.** The constraints on cephalopods: why squid aren't fish. Can. J. Zool. **64**: 1591-1605.
- Pörtner H. O. 1990.** An analysis of the effects of pH on oxygen binding by squid *Illex illecebrosus*, *Loligo pealei*) haemocyanin. J. Exp. Biol. **150**: 407-424.

- Pörtner H. O. 1994.** Coordination of metabolism, acid-base regulation and haemocyanin function in cephalopods. Pp. 131-148 in Physiology of cephalopod molluscs - lifestyle and performance adaptations, H. O. Pörtner, R. K. O'Dor and D. MacMillan, eds. Gordon and Breach Publishers, Basel.
- Pörtner H. O. 1997.** Oxygen limitation of metabolism and performance in pelagic squid. Proceedings of the 30th European Marine Biological Symposium, 45-56.
- Pörtner H.O. and S. Zielinski. 1998.** Environmental constraints and the physiology of performance in squids. in Cephalopod Biodiversity, Ecology and Evolution, A.I.L Payne, M.R. Lipinski, M.R. Clarke and M.A.C. Roeleveld, eds. South Afr. J. Mar. Sci. **20**: 207-221.
- Pörtner H.O., P.L.M. van Dijk, I. Hardewig and A. Sommer. 2000.** Levels of metabolic cold adaptation: tradeoffs in eurythermal and stenothermal ectotherms. in Antarctic Ecosystems: models for wider ecological understanding, W. Davison and C. Howard Williams eds; Caxton Press, Christchurch New Zealand, in press
- Reeves R. B. 1972.** An imidazole alaphastat hypothesis for vertebrate acid-base-regulation: tissue carbon dioxide content and body temperature in bullfrogs. Respir. Physiol. **81**: 255-274.
- Roper C. F. E., M. J. Sweeney, and C. E. Nauen. 1984.** FAO species catalogue. Vol. 3: Cephalopods of the world. An annotated and illustrated catalogue of species of interest to fisheries. FAO Fisheries synopses 125, 277 pp.
- Ruud J. T. 1954.** Vertebrates without erythrocytes and blood pigment. Nature **173**: 848-850.
- Seibel B. A., E. V. Thuesen, J. J. Childress, and L. A. Gorodezky. 1997.** Decline in pelagic cephalopod metabolism with habitat depth reflects differences in locomotory efficiency. Biol. Bull. **192**: 262-278.
- Seibel B. A., F. Chausson, F. H. Lallier, F. Zal, and J. J. Childress. 1999.** Vampire blood: respiratory physiology of the vampire squid (Cephalopoda: Vampyromorpha) in relation to the oxygen minimum layer. Exp Biol Online **4**: 1.

- Senozan N. M., A. Avinc, and Z. Unver. 1988.** Hemocyanin levels in *Octopus vulgaris* and the cuttlefish *Sepia officinalis* from the Aegean sea. Comp. Biochem. Physiol. **91A**: 581-585.
- Shadwick, R. E., R. K. O'Dor, and J. M. Gosline. 1990.** Respiratory and cardiac function during exercise in squid. Can. J. Zool. **68**: 792-198.
- Urich, K. (1990)** Vergleichende Biochemie der Tiere. Gustav Fischer Verlag, Stuttgart, New York. 708 pp.
- Taki I. 1961.** On two new eledonid octopods from the Antarctic sea. J. Fac. Fish. Anim. Husb. Hiroshima Univ. **3**: 297-316.
- von Boletzky S. 1983.** *Sepia officinalis*. Pp. 31-52 in Cephalopod life cycles Vol. I, Boyle P.R., ed. Academic Press, London.
- Webber D. M., and R. K. O'Dor. 1985.** Respiration and swimming performance of short-finned squid (*Illex illecebrosus*). NAFO Sci. Coun. Studies **9**: 133-138.
- Webber D. M., and R. K. O'Dor. 1986.** Monitoring the metabolic rate and activity of free-swimming squid with telemetered jet pressure. J. Exp. Biol. **126**: 203-224
- Wells M. J., R. T. Hanlon, P. G. Lee, and F. P. Dimarco. 1988.** Respiratory and cardiac performance in *Lolliguncula brevis* (Cephalopoda, Myopsida): the effects of activity, temperature and hypoxia. J. exp. Biol. **138**: 17-36.

Figure Legends

Fig. 1 Cuvette used for the measurement of oxygen binding properties. Dashed areas show the compartments of the cuvette filled with blood. Absorbance was measured through a thin layer, 0.45 mm thick, in the centre part of the cuvette.

Fig. 2 Continuous analysis of the relationships between oxygen binding, pH and P_{O_2} in whole blood of the Antarctic octopod *Megaleledone senoi* at temperatures between 0 and 10°C (S - saturation; points were chosen at regular intervals from the continuous recordings).

Fig. 3 Depiction of oxygen binding properties of *Sepia officinalis* hemocyanin at temperatures between 0 and 20°C in a pH/saturation diagram. Each line indicates an oxygen isobar and shows the changes in oxygen binding depending on pH (S - saturation; points were chosen at regular intervals from the continuous recordings).

Fig. 4 Hill coefficients (n_{50}) of *Sepia officinalis* hemocyanin depending on pH and temperature.

Fig. 5 pH/bicarbonate diagram for whole blood of *Megaleledone senoi*. Experimental buffer lines of oxygenated and deoxygenated blood match those derived from calculated apparent bicarbonate levels (broken lines; see text).

Table 1 Oxygen affinity (P₅₀), Bohr, Haldane and Hill coefficients evaluated for the hemocyanins of the Antarctic octopod *Megaleledone senoi* and of the cuttlefish (*Sepia officinalis*) at different temperatures. P₅₀, Haldane and Hill coefficients are valid for the pH values given in brackets (nd - not determined; HcyO₂ - concentration of oxygenated hemocyanin).

Temperature (°C)	P ₅₀ (kPa)	Bohr co- efficient (ΔlogP ₅₀ / ΔpH)	(ΔlogP ₈₀ / ΔpH)	Haldane coefficient (ΔHCO ₃ ⁻ / ΔHcyO ₂)	Hill coefficient (n ₅₀)
<i>Megaleledone senoi</i>					
0	0.98 (pH 7.2)	≈ -0.9	-1.51	0.66 (pH 6.2) 0.50 (pH 6.8) 0.39 (pH 7.2)	1.4 (pH 7.43)
5		nd	-1.46	nd	1.5 (pH 7.24) 1.0 (pH 7.31)
10	1.10 (pH 7.2)	-2.33	-2.13	nd	1.2 ± 0.1 (pH 6.83-7.31; n = 6)
<i>Sepia officinalis</i>					
10	5.3 (pH 7.4)	-0.99	-1.44		4.6 (pH 7.29)
20	6.5 (pH 7.4)	-1.33	-1.94		5.9 (pH 7.48)

Fig. 1

fig.3

Fig. 4

Fig. 5