Role of blood-oxygen transport in thermal tolerance of the cuttlefish, *Sepia officinalis*

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Synopsis Mechanisms that affect thermal tolerance of ectothermic organisms have recently received much interest, mainly due to global warming and climate-change debates in both the public and in the scientific community. In physiological terms, thermal tolerance of several marine ectothermic taxa can be linked to oxygen availability, with capacity limitations in ventilatory and circulatory systems contributing to oxygen limitation at extreme temperatures. The present review briefly summarizes the processes that define thermal tolerance in a model cephalopod organism, the cuttlefish *Sepia officinalis*, with a focus on the contribution of the cephalopod oxygen-carrying blood pigment, hemocyanin. When acutely exposed to either extremely high or low temperatures, cuttlefish display a gradual transition to an anaerobic mode of energy production in key muscle tissues once critical temperatures (T_{crit}) are reached. At high temperatures, stagnating metabolic rates and a developing hypoxemia can be correlated with a progressive failure of the circulatory system, well before T_{crit} is reached. However, at low temperatures, declining metabolic rates cannot be related to ventilatory or circulatory failure. Rather, we propose a role for hemocyanin functional characteristics as a major limiting factor preventing proper tissue oxygenation. Using information on the oxygen binding characteristics of cephalopod hemocyanins, we argue that high oxygen affinities (= low P_{50} values), as found at low temperatures, allow efficient oxygen shuttling only at very low venous oxygen partial pressures. Low venous PO_2s limit rates of oxygen diffusion into cells, thus eventually causing the observed transition to anaerobic metabolism. On the basis of existing blood physiological, molecular, and crystallographical data, the potential to resolve the role of hemocyanin isoforms in thermal adaptation by an integrated molecular physiological approach is discussed.

Introduction

Temperature as a key environmental factor shapes the physiology of ectothermic marine animals, and thereby their biogeography and mode of life in various climates and ecosystems. As a principle background of these ecological patterns, animals specialize on limited thermal windows. The mechanisms defining this level of specialization have regained interest in recent years in the light of ongoing climatic change and its effects in marine ecosystems (Walther et al. 2002). Comparative study of physiological mechanisms setting thermal limits in marine ectotherms from various phyla led to the recent concept of an oxygen-limited thermal tolerance as a unifying principle in water-breathing metazoans (Pörtner 2001, 2002). This concept implies that upper and lower limits of thermal tolerance are set by limitations in aerobic scope, due to onset of a mismatch between oxygen supply and demand and reduced capacity to supply oxygen. The limitations in aerobic scope arise before thermal stress causes transition to anaerobic metabolism and further effects at the cellular, biochemical level (Pörtner 2002). The reduction in aerobic scope lowers functional capacity and fitness and may lead to reduced survival in the field during thermal extremes, as shown in fish (Pörtner and Knust 2007).

The oxygen-limitation hypothesis has also recently been investigated in detail for a common cephalopod of European coastal waters, the cuttlefish *Sepia officinalis* (Melzner et al. 2006a, 2006b, 2007b; Melzner et al., unpublished data). This short review will try to integrate our current understanding of the mechanisms defining thermal tolerance in cuttlefish and especially focus on the role of the blood pigment, hemocyanin, in thermal limitation.

This paper summarizes one of the 22 symposia that constituted the "First International Congress of Respiratory Biology" held August 14–16, 2006, in Bonn, Germany.
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Physiology of the oxygen transport system of the cuttlefish

Coleoid cephalopods represent a highly evolved and energetic invertebrate group that acquired its high level of performance and sophistication as a result of the co-evolution and continued competition with fish. The two groups developed similar performance characteristics and had to overcome the specific structural constraints characteristic of each phylum, with the result that for the same level of performance, the active cephalopods display higher metabolic rates than do fish with comparable modes of life (for a review, see O'Dor and Webber 1991). While both groups use a high-pressure closed circulatory system, oxygen delivery occurs by use of erythrocytes and cellular hemoglobin in the case of fishes and by use of extracellular hemocyanin in the case of cephalopods. In the latter case, oxygen delivery via the blood is maximized to cover metabolic requirements, especially during exercise in squid (Pörtner 1994). However, the capacity of hemocyanin for carrying oxygen is limited. This is due to the unfavorable increase in colloidal osmotic pressure and blood viscosity at high pigment concentrations (Mangum 1983, 1990). At an oxygen-binding capacity of only 3 mM (as opposed to 10 mM in fish) (Urich 1990), cephalopods rely on fully oxygenating their pigment at the gills and on releasing the majority of bound oxygen during each passage through the tissue capillary beds. Johansen et al. (1982) determined that under resting conditions, about 80% of bound oxygen are being released in the tissues at 17°C ambient temperature in the cuttlefish *S. officinalis*. Such peformance can only be achieved in combination with finely tuned ventilatory and circulatory systems.

While active squid respire oxygen from a stream of water that also fuels swimming movements, the more sedentary cuttlefish have successfully decoupled their ventilatory water pumps from locomotory pumping systems (Wells 1990). By extracting high proportions of dissolved oxygen, relatively small volumes of water have to be pumped through the mantle cavity (Wells and Wells 1985, 1991); during jet locomotion requirements go in the opposite direction. Jet propulsion is most efficient when a large volume of water is ejected at low velocity (O'Dor and Webber 1991). Thus, squid only extract 5–10% of dissolved oxygen from their ventilatory stream, while they eject seawater equivalent to 20–30% of their body mass per jet (Wells and Wells 1991). At a long-term acclimation temperature of 15°C, cuttlefish of 105 g wet mass can extract 80% of dissolved oxygen from their ventilatory stream (Melzner et al. 2006b). Combined action of the ventilatory muscles, the collar flaps of the funnel apparatus and the radial mantle muscle fibers (Bone et al. 1994) generate a water current through the cuttlefish mantle cavity at relatively low mean pressures of <0.02 kPa. Low flow requirements and low pressures lead to a low power output of the ventilatory system of 0.1–0.2 mW kg^{-1} animal, which results in very low cost for ventilation mechanics in cuttlefish of 1–1.5% of routine energy expenditure (Melzner et al. 2006b).

The circulatory system supports these impressive figures: venous return through the anterior cephalic vein (AVC), the most important cuttlefish vein, is obligatorily coupled to ventilatory pressure oscillations in the mantle cavity: short blood-flow pulses in the vein are elicited exactly at the maximum increase in mantle-cavity pressure (Melzner et al. 2007a). As mantle-cavity pressure in cephalopods is directly correlated to respiratory water movements through the mantle cavity (Shadwick 1994), this apparent connection between circulatory and ventilatory systems might enable efficient gas exchange at the gills, by exactly timing blood flow within gill vessels and water flow around the latter. Even more important are the low venous PO_2 values of 2–4 kPa (Johansen et al. 1982; Melzner et al. unpublished observations) that enable high oxygen-transfer rates from the ventilatory water stream into the blood during countercurrent gas exchange at the gills.

Oxygen limitation of thermal tolerance in cuttlefish

While oxygen transfer functions nicely in a thermal window between 11°C and 23°C for 15°C-acclimated cuttlefish, further acute warming or cooling of the organism leads to progressive internal hypoxia (hypoxemia), time-limited survival, and, eventually, death. Figure 1 illustrates the thermal dependency of several components of the cuttlefish oxygen-transfer apparatus as correlated with temperature-dependent changes in cellular energy parameters. Using *in vivo* ^{31}P NMR techniques, we were able to continuously monitor the energy status of mantle muscle, whose radial fibers are important for refilling the mantle cavity with water during ventilation. While concentrations of cellular high—energy phosphate compounds (ATP, PLA = Phospho-L-arginine) remained at high and constant levels at temperatures between 11°C and 23°C, an accumulation of inorganic phosphate was observed at average temperatures below 8°C and above 26°C (Fig. 1C).

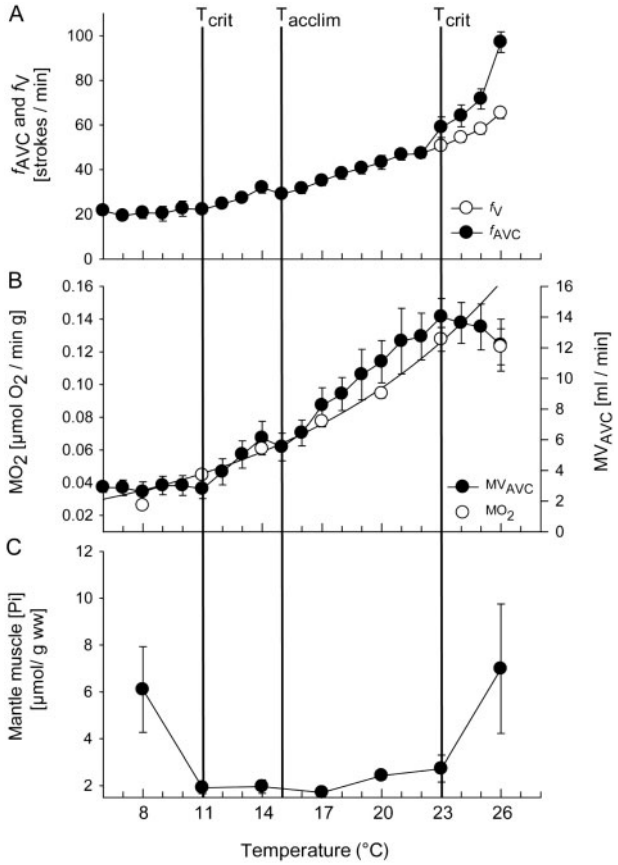

Fig. 1 Oxygen limitation of thermal tolerance in the cuttlefish *Sepia officinalis* (100–250g body mass). Note: All experiments were conducted on animals long-term acclimated to 15°C (T_{acclim}) and acutely exposed to changing temperatures (at a rate of 1°C h^{-1}). Determined threshold temperatures (T_{crit}) thus are only valid for this specific acclimation temperature; acclimation to lower (higher) temperatures would likely alter the thermal tolerance window (e.g., see Sommer et al. 1997). (**A**) Ventilation frequency (f_V) and *anterior cephalic vein* (AVC) pulse rate (f_{AVC}). Note the splitting of frequencies above 23°C; (**B**) AVC minute volume (MV_{AVC}) and routine metabolic rate (MO_2); (**C**) Inorganic phosphate (Pi) concentration in mantle muscle fibers. Accumulation of Pi results from phosphagen use to buffer cellular ATP levels. (Data from: Melzner et al. 2006a, 2006b, Melzner et al., unpublished data; all data points are mean values obtained from $N = 5$–10 animals, error bars represent standard errors)

These increases were caused by PLA being used in a transphosphorylation reaction to buffer cellular ATP levels, in order to compensate for a failure of aerobic energy provision (Melzner et al. 2006a). However, progressive depletion of the cellular PLA pool (of about 34 µmol g ww^{-1}) (Storey and Storey 1979) led to a rapid decline of the Gibb's free energy of ATP hydrolysis ($|dG/d\xi|$) in radial mantle muscle fibers, especially in the warm. Values were modelled to decrease from control values of about 55 to below 44 kJ per mol ATP hydrolized at temperatures >26°C. These drastic changes in the cellular thermodynamic environment went along with stagnating ventilation pressures at temperatures >26°C. In addition to capacity limitations of the musculature involved, thresholds for the functioning of vital ATPases may have been reached (see Melzner et al. 2006a for a detailed discussion). Kammermeier et al. (1982) and Jansen et al. (2003) determined critical thresholds for $|dG/d\xi|$ for a variety of vital cellular ATPases of between 45 and 53 kJ mol^{-1}.

Whole animal metabolic rates (MO_2, Fig. 1B) appeared to deviate from a regular exponential pattern within a thermal window of 11°C and 23°C, in that below 11°C and above 23°C, less oxygen was being consumed than expected (Melzner et al. 2006b). This indicated that the observed transition towards an anaerobic mode of energy production by means of phosphagen utilization was most likely caused by an insufficient capacity of ventilatory and/or circulatory systems to provide the required amounts of oxygen to tissues.

The cuttlefish ventilatory system is very cost effective, as these animals are able to extract a large percentage of oxygen from the ventilatory current, while transporting only low water volumes at low pressure through their mantle cavities. During acute increases in temperature, cuttlefish are able to drop oxygen extraction rates from the ventilatory current to about 35% at 26°C. Thus, they increase oxygen diffusion gradients across the gills in order to match increasing oxygen demand. The opposite happens at decreasing temperatures, at which we found oxygen extraction rates to increase to >90% (Melzner et al. 2006b). Most importantly, model calculations revealed that the ventilatory capacity displayed should suffice at all experimental temperatures to provide arterial PO_2 values of >14 kPa in the gills (Melzner et al. 2006b), as necessary for full oxygenation of the blood pigment (Johansen et al. 1982). Although ventilatory power output changes more than 80-fold across the temperature range of 8–26°C, costs for ventilation mechanics most likely remain below 10% of the animals' metabolic rate even at the highest temperatures, illustrating the efficient ventilatory design of the cuttlefish ecotype.

In contrast, the circulatory system suffered from capacity limitation at high temperatures: blood minute volume (MV_{AVC}) of the AVC increased with temperature up to 23°C and levelled off beyond, correlating with the mentioned stagnation of metabolic rate at the same temperature (Fig. 1B). Increased AVC peak blood velocity (v_{AVC})

and blood pulse frequency (f_{AVC}) contributed to a 2.5 fold increase in MV_{AVC} between 15°C and 23°C (Melzner et al., unpublished data), while MO_2 rose 2.2 fold in the same temperature interval (Fig. 1B). As oxygen extraction from the blood is already very high (80%) in control cuttlefish (Johansen et al. 1982), temperature-dependent increments in oxygen demand are likely provided by tantamount increases in blood perfusion rather than by increasing hemocyanin-bound oxygen transport. Hemodynamic patterns in the AVC at maximum blood flow at 20–23°C matched those observed under recovery from exercise surprisingly well (Melzner et al. 2007a), leading us to conclude that the *S. officinalis* circulatory system is designed in mechanical terms to sustain 2–2.5-fold increases in metabolic rate, regardless of the nature of the specific aerobic challenge (exercise or acute thermal change). Oxygen demand beyond maximum sustainable rates led to a progressive disintegration of correlated ventilatory and circulatory convection systems (Melzner et al. 2007a): the ventilatory system depends on steadily rising ventilation frequency to increase perfusion of the gills, which, on the other hand, negatively affects the correlated AVC blood pulse mechanics. Starting at temperatures of 20–21°C, peak blood velocity (v_{AVC}) cannot be increased any more, while from 23°C upwards, a disintegration of the (usually) coupled AVC-ventilation pulse system (Fig. 1A) results in stagnating MV_{AVC}. Other cuttlefish circulatory organs (branchial/systemic hearts) have also been observed to functionally disintegrate at about the same temperature range as the AVC–ventilatory system (Mislin 1966; Fiedler 1992).

Thus, at high temperatures we witnessed a clear limitation of the capacity of the circulatory system, thereby preventing a further increase in oxygen consumption rates and causing progressive tissue hypoxia and, finally, anaerobic metabolism beyond critical temperatures (T_{crit}, see Pörtner 2002) of about 23°C. At the cold end of the thermal window, below a T_{crit} of 11°C, we did not witness a significant change in the temperature-dependent patterns of ventilatory or circulatory activity that would explain the observed decrease in MO_2 and subsequent transition to anaerobic metabolism (Melzner et al., unpublished data, Fig. 1). However, hemocyanin functional characteristics may significantly contribute to the observed limitations in ventilatory muscle capacity and the observed transition to hypoxemia at very low temperatures.

The role of hemocyanin in cuttlefish thermal tolerance

Cephalopod hemocyanins are decameric proteins; the 4 MDa decamer consists of subunits that are 350 kDa (*Octopus*) (Miller et al. 1998) to 400 kDa (*S. officinalis*) in size. Each hemocyanin subunit is an enormous polypeptide chain containing seven or eight globular folded regions (functional units or domains), each of which carries one molecule of oxygen. As a consequence of these differences in protein composition, the oxygen transport capacity spans 70–160 O_2 molecules per decamer and didecamer, respectively. The full exploitation of oxygen transport requires rapid adjustments of oxygen affinity at all levels of oxygen saturation depending on environmental and functional conditions. In most cephalopods, cooperativity, as well as temperature-dependent and pH-dependent changes in affinity, are the only means of modulating hemocyanin function (Brix et al. 1989, 1994; Mangum 1990; Pörtner 1990). Typically, those cephalopods with the highest metabolic rates are equipped with low affinity (=high P_{50} values) hemocyanins, enabling them to buffer high PO_2 values in their venous blood, and thereby facilitate oxygen diffusion into tissues. The oceanic squid *Illex illecebrosus* is such a species (Fig. 2): high venous PO_2 values of 6 kPa (Pörtner et al. 1991) go along with a P_{50} value of about 8 kPa (at pH 7.4). However, some coastal squid species (e.g., *Lolliguncula brevis*) (Fig. 2) display opposite trends; to cope with frequent hypoxic events in their habitat (Finke et al. 1996), they possess a high-affinity hemocyanin, characterized by P_{50} values <2 kPa. This enables the species to saturate its pigment with oxygen even in very hypoxic waters. Similarly, high affinities can also be found in a low–metabolic-rate Antarctic octopod (Fig. 2) (*Megaleldone senoi*, Zielinski et al. 2001) and, taken to an extreme, in the vampire squid *Vampyroteuthis infernalis*. This organism is specialized to survive in the oxygen minimum zones of the midwater (Seibel et al. 1999), and is characterized by the highest affinities ($P_{50} < 1$ kPa) so far known for any cephalopod.

Extremely large Bohr shifts ($\Delta \log P_{50}/\Delta pH < -1$; Bridges 1994) and very high levels of pH-dependent cooperativity are common in cephalopods (Miller 1985; Pörtner 1990, 1994). In *S. officinalis*, the large Bohr-effect (<–1.0) is exploited through both CO_2 produced in metabolism, as well as CO_2 bound at the gills and released during venous deoxygenation (Lykkeboe et al. 1980; Brix et al. 1981). In some

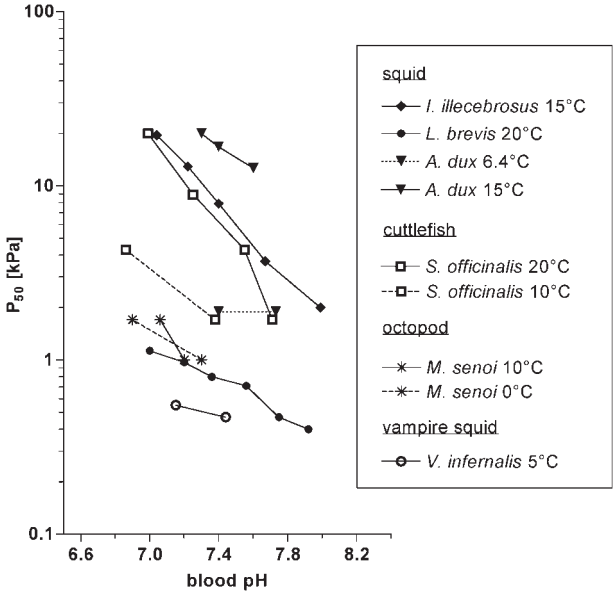

Fig. 2 pH (and temperature) dependence of hemocyanin affinity in some cephalopods. Low P_{50} values indicate a high oxygen affinity of the pigment Sources of data: *Architeuthis dux*: Brix (1983), Brix et al. (1989); *Illex illecebrosus*: Pörtner (1990); *Lolliguncula brevis*: Mangum (1991); *Sepia officinalis*: Zielinski et al.(2001); *Megaleledone senoi*: Zielinski et al. (2001); *Vampyroteuthis infernalis*: Seibel et al. (1999). Note: some of the data have not been analyzed by pH saturation analysis. Results may thus have been influenced by pH shifts during experimental analysis (Pörtner 1990).

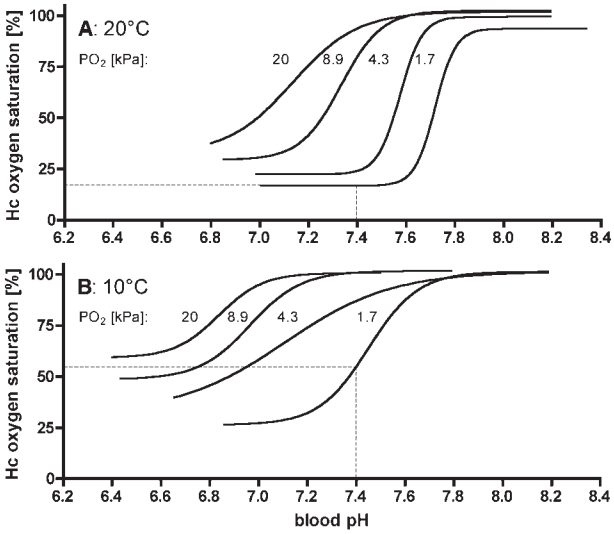

Fig. 3 pH/saturation diagram derived from *S. officinalis* whole blood samples (redrawn from Zielinski et al. 2001): hemocyanin (Hc) saturation and pH were simultaneously measured at four constant PO_2 values (1.7, 4.3, 8.9, and 20 kPa) at 20°C (**A**) and 10°C (**B**) ambient temperature. Dotted lines indicate oxygen saturation of the pigment at pH 7.4 and $PO_2 = 1.7$ kPa. See text for further explanations.

cephalopods, an increase in ambient temperature has a large effect on oxygen transport, as reflected by a change in cooperativity or a fall in oxygen affinity of the pigment during warming (Fig. 2) (Brix et al. 1989, 1994; Mangum 1990). While some eurythermic cephalopods are able to decrease affinity with temperature at a reasonable rate and thereby support higher metabolic rates (e.g., *S. officinalis*) (Fig. 2), other, more stenothermal species, lack this flexibility in hemocyanin properties. The Antarctic octopod *Megaleledone senoi* is unable to decrease affinity with increasing temperature, therefore preventing liberation of sufficient amounts of oxygen to fuel increased demand (Zielinski et al. 2001). An opposite trend can be found in the cold-adapted giant squid *Architeuthis dux*, which decreases affinity at a very high rate with rising temperature. This led Brix (1983) to conclude, that in the warm, the species may die from arterial desaturation. Clearly, lifestyle adaptations of the various cephalopod ecotypes are very well reflected in their hemocyanin functional properties, with the fine tuning of blood oxygen affinity correlating with the degree of stenothermia/eurythermia.

The strong multidimensional interaction between temperature, blood acid–base status and hemocyanin functioning reflects the pH-dependent PO_2 buffer function of the pigment (Pörtner 1994), which is adequately illustrated through pH saturation analysis (Pörtner 1990; Zielinsiki et al. 2001) (Fig. 3). In the study by Zielinsiki et al. (2001) on the functional properties of *S. officinalis* hemocyanin, the level of hemocyanin-bound oxygen was found to be 2.84 mmol l^{-1}. Especially in the pH range between 7.4 and 7.8, and with a maximum of pH-dependent cooperativity (Hill-coefficient n_{50}) of 5.9 at pH 7.48, very small pH changes were sufficient to cause maximal unloading of oxygen from the pigment. The $\triangle S/\triangle pH$ reached a maximal value of 41% per 0.1 pH unit at 20°C. Similar to the condition in squid (Pörtner 1990), pH sensitivity was found to be maximal in the range of *in vivo* pH in *S. officinalis* (Johansen et al. 1982; Zielinski et al. 2001).

Our previous study suggests that in cuttlefish acclimated to 15°C ventilatory processes do not limit hemocyanin oxygenation at the gills at least within the range of investigated temperatures (between 11°C and 26°C) (Melzner et al. 2006b). A thermal limitation of arterial oxygen uptake may only arise with inadequate functional properties of the blood pigment itself. While studies of hemocyanin functional properties at temperatures beyond high critical limits (i.e., >23°C) are currently unavailable, the data

reported by Zielinski et al. (2001) can help us understand how hemocyanin may contribute to tissue oxygen limitation at temperatures <11°C in the cuttlefish. Insufficient arterial saturation probably does not become apparent in the cold (Melzner et al. 2006b). On the venous side, the pH/saturation diagram at 20°C (Fig. 3A) and a typical venous pH value of 7.4 at 17–19°C (Johansen et al. 1982), illustrate that about 70% of the hemocyanin-bound oxygen can be released from the pigment at a venous PO_2 of 4.3 kPa and more than 80% at a venous PO_2 of 1.7 kPa. Thus, effective oxygen shuttling is achieved at relatively high venous oxygen diffusion heads of >2 kPa. However, cooling to 10°C, just below the lower T_{crit}, changes this situation (Fig. 3B): affinity increases such that at a blood pH of 7.4, <20% of bound oxygen can be liberated in the tissues at a PO_2 4.3 kPa, and only 40% at a PO_2 value of 1.7 kPa. This situation would become even worse if there were an alpha-stat pattern of pH_e regulation (Reeves 1972) as proposed for cephalopod blood (Howell and Gilbert 1976). Accordingly, pH_e would change at a rate of –0.018 pH units°C^{-1}, thus resulting in venous pH values of about 7.6 at 10°C. Figure 3B reveals, that at such a pH, <10% of bound oxygen can be released from the pigment at PO_2 values of 1.7 kPa. Obviously, venous oxygen partial pressures would have to be lowered even further to achieve proper unloading of the pigment at low temperature. Alternatively, blood perfusion requirements would rise dramatically; were oxygen utilization in the blood to drop from 80% to 20%, blood volume flow would have to increase 4-fold, in order to maintain oxygen supply to tissues despite lower oxygen demand in the cold. As we did not see any increases in blood perfusion at temperatures <11°C (Melzner et al., unpublished data), we conclude that extraction efficiencies are being maintained, however, at the cost of more than 4-fold reductions in blood PO_2, possibly to values of <1 kPa (Melzner et al., unpublished data). This trend towards progressively lower PO_2 being needed to fully deoxygenate the respiratory pigment continues at temperatures <10°C (Zielienski et al. 2001).

Low PO_2 values will most likely cause diffusion limitations and thereby contribute to the observed anaerobiosis at temperatures <8°C. Information on oxygen diffusion gradients and cellular PO_2 values is scarce for marine ectothermic animals. Inside mammalian red muscle cells oxygen partial pressures at rest range between 0.7 and 5 kPa and intracellular oxygen gradients are shallow owing to the presence of myoglobin (Mb). Minimum intracellular PO_2 required for maximum cytochrome turnover in red muscle ranges between 0.04 and 0.07 kPa. Owing to large mitochondrial surface areas in relation to capillary diffusion areas, oxygen diffusion gradients from cytosol to mitochondria are lower than 0.01 kPa (Gayeski and Honig 1986, 1988; Clark et al. 1987; Gayeski et al. 1987). As Honig et al. (1992) concluded from their studies, it is the PO_2 gradient between capillary and cytosol that is rate-limiting for oxygen transfer. In marine teleosts, in which venous PO_2 represents the pressure head for oxygen diffusion in the systemic heart, threshold PO_2 values of ~1–3.3 kPa have been demonstrated to limit cardiac performance during exercise and at the upper T_{crit} (Steffensen and Farrell 1998; Lannig et al. 2004). Venous oxygen partial pressures in fish swum to fatigue or subjected to hypoxia ranged between 0.8 and 2 kPa (Kiceniuk and Jones 1977; Forster 1985; Lai et al.1990). For invertebrates, there is only one record available that relates extracellular PO_2 to intracellular anaerobiosis; cold exposure and hypoxia in the peanut worm (*Sipunculus nudus*) result in a transition to an anaerobic mode of energy production once coelomic fluid PO_2 reaches threshold values of about 0.5–0.7 kPa (Pörtner et al. 1985; Zielinski and Pörtner 1996). Considering the much higher metabolic rate of a cephalopod, it appears reasonable that a venous PO_2 above 1 kPa is required to cover oxygen demand at rest.

Clearly, more detailed studies are needed, combining *in vivo* measurements of hemocyanin oxygen saturation, pH and PO_2 with an *in vitro* analysis of hemocyanin binding properties over a full range of temperatures to fine-resolve the intricate processes and the shift in hemocyanin functional properties finally leading to tissue oxygen limitation at extreme temperatures.

Molecular physiology of hemocyanin

The patterns of temperature-dependent functioning of hemocyanin and their likely role in whole-organism thermal tolerance led us to seek specific features of the molecular structure that may set the optimal temperature range of oxygen transport by hemocyanin. A wealth of studies exist on hemocyanin tertiary and quaternary structure (Wichertjes et al. 1986; Chignell et al. 1997; Lamy et al. 1998), yet in the past differences in protein functionality could not be allocated to (or explained by) structural changes in the amino-acid sequence, as has been shown in the case of fish lactate dehydrogenase (Fields and Somero 1997; Fields and Houseman 2004; Somero 2005). This was at least in part due to the sheer size of the protein. Only in the past

few years, have genomic data on hemocyanin sequences become available for the cephalopods *Octopus dofleini* (Miller et al. 1998), *Nautilus pompilius* (Bergmann et al. 2006), and *S. officinalis* (de Geest, Genbank accession DQ388569, DQ388570) and for some other molluscs (Markl et al. 1991; Lieb et al. 2000, 2004; Altenhein et al. 2002; Bergmann et al. 2006). These now allow structural analysis of the amino-acid sequences of these proteins. The most substantial progress was made by crystallographic studies of one functional unit (FU-g) in combination with the complete sequence of *O. dofleini* by Cuff et al. (1998) and Miller et al. (1998). These X-ray structures provided insight into the detailed tertiary structure of the smallest functional unit of this respiratory protein. In *O. dofleini*, seven of these paralogous functional units, which are named a–g, form one subunit. Each of these can be modeled according to the aforementioned 3D structures, reflecting their strong functional, structural, and evolutionary relatedness (Fig. 4). How these subunits are arranged to build the decameric "holoprotein," however, is still a matter of debate.

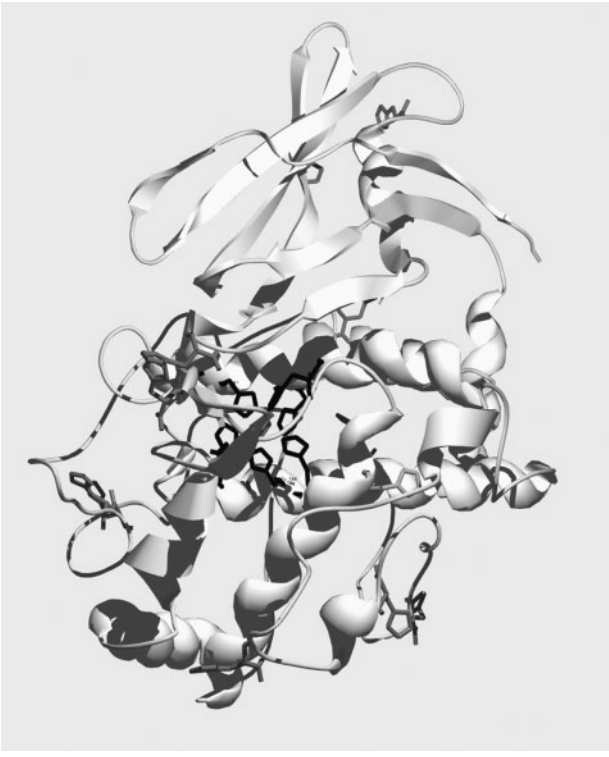

Fig. 4 Functional unit *g* of *S. officinalis* hemocyanin (Accession number DQ388569) modeled according to the X-Ray structure of *Octopus dofleini* hemocyanin [1JS8.pdb using Swiss-PdbViewer 3.7.1 (www.expasy.org), rendered with POV-Ray 3.1]. Histidyl residues are depicted in 3D, the central six His (black) are strongly conserved and involved in oxygen-binding, the remaining distal His residues are colored dark grey. Regions of prevailing helical and β-strand structures are clearly visible.

To date, it has been shown that there are two distinct hemocyanin subunits (or isoforms) expressed constitutively at least in cephalopods, yet their function remains unclear (Lang and van Holde 1991; Markl et al. 1991). Comparative study of their amino-acid sequences provides answers with respect to the biological significance and regulation of these isoforms (*O. dofleini* hemocyanin: Genbank accession numbers: AY751301, AF338426). Such approaches have been successfully applied to gastropod hemocyanins (Altenhein et al. 2002; Lieb et al. 2004; Streit et al. 2005), whilst only few studies have focussed on the comparison of the respective cephalopod hemocyanins (van Holde et al. 2001; Lieb and Markl 2004; Bergmann et al. 2006).

In *S. officinalis* and *O. dofleini* the two known hemocyanin isoforms appear to be physicochemically and physiologically distinct. The amino-acid sequence suggests different pH optima, which in turn might also reflect different thermal optima (Table 1). Sequence analysis at the nucleotide and amino-acid level *in silico* of the known two hemocyanin isoforms found in *S. officinalis* from Normandy waters (N de Geest, personal communication; Genbank accession DQ388569, DQ388570) indicate a difference in protein isoelectric points (pI) of 0.153 pH units for the holoenzyme. Differences in pI are more variable among the homologues of the functional units and are presented in Table 1. Differential expression of these two isoforms might thus be a way of maintaining constant oxygen affinities over a thermal range of about 10°C if the

Table 1 Theoretical isoelectrical points (pI) of the single subunits of the hemocyanin isoforms of *Sepia officinalis* and *Octopus dofleini* hemocyanin

Species		A	B	C	D	D′	E	F	G
Sepia officinalis	Hc1	5.86	5.46	5.35	5.66	5.94	5.86	5.31	6.14
	Hc2	6.01	5.74	5.40	5.68	5.90	6.12	5.49	6.46
	Difference	*0.15*	*0.28*	*0.05*	*0.02*	*–0.04*	*0.26*	*0.18*	*0.32*
Octopus dofleini	Hc-G	5.73	5.39	5.42	5.73		5.66	5.07	5.97
	Hc-A	6.00	5.33	5.48	5.83		5.67	5.31	5.84
	Difference	*0.27*	*–0.06*	*0.06*	*0.10*		*0.01*	*0.24*	*–0.13*

A to G represent the individual subunits of the isoforms Hc1 and Hc2 (*S. officinalis*, Sequence Accession numbers DQ388569 and DQ388570) and Hc-G and Hc-A (*O. dofleini*), respectively [sequence data retrieved from Genbank, pI calculated using MacVector 9.1 (Mac Vector Inc.)].

Differences in pI between the isoforms are given for each subunit.

pH of the blood changes with temperature according to the alpha-stat theory (–0.018 pH/°C) (Reeves 1972). Alpha-stat sensitivity requires the presence of imidazole moieties of histidine residues in the amino-acid composition of a protein. It is their pK of 6.94 that is thermally sensitive in biologically buffered systems. We present here a model of *Sepia officinal* hemocyanin functional unit g (Fig. 4, modeled according to *O. dofleini* hemocyanin functional unit g), that contains 20 such histinyl residues. Six of them are highly conserved and located in the centre of the molecule where they are involved in the binding of molecular oxygen (His residues colored black in Fig. 4). Fourteen further histidine residues are located at the outside of the molecular structure (dark grey in Fig. 4). Thus, changes in imidazole protonation and substitution of less conserved histidine residues can directly affect oxygen binding and furthermore influence cooperativity between the functional units. This likely contributes to the large pH sensitivity of hemocyanin oxygen binding as well as to its thermal sensitivity.

Hemocyanin sequence analysis at DNA level was performed for functional unit g in animals from the North Sea, the English Channel and the Bay of Biscay provided three hemocyanin subunits (Mark et al., unpublished data), two of which were identical on DNA level to the subunits known so far from Normandy coast (Genbank accession DQ388569, DQ388570). This is the first evidence for the existence of a putative third isoform of hemocyanin in the northern distributional range of *S. officinalis* (and in cephalopods in general) and might also be a result of thermal adaptation of the respiratory protein (Somero 2005). That these putative three isoforms can be found in all populations of *S. officinalis*, within the northern distributional range from the Bay of Biscay to the North Sea hints that there may be a close genetic relation among these populations, which is also corroborated by an analysis of neutral genetic markers (microsatellites) among these populations (Wolfram et al. 2006). Depending on environmental conditions, distinct allelic (iso) forms might then be differentially, or exclusively, expressed.

In the abalone *Haliotis tuberculata* the relative proportions of expressed isoforms of hemocyanin can vary considerably among individuals (Keller et al. 1999). The two isoforms might be selectively recognized and sequestered via their differential glycosylation (Lieb et al. 2000; Streit et al. 2005) according to physiological requirements. Differential glycosylation has also been reported for *S. officinalis* hemocyanin (Gielens et al. 2004), indicating that a scenario of physiological control of the expression of hemocyanin, similar to that found in *Haliotis*, might be operative in cephalopods.

As outlined above, hemocyanin can only maintain maximum cooperativity and adequate affinity within a given thermal window (Fig. 3). Beyond this thermal range, saturation of hemocyanin (in the warm) and its desaturation (in the cold) is severely impaired and this functional deficiency likely contributes to thermally-induced limitation of oxygen. It is thus conceivable that during evolution, isoforms of hemocyanin have adapted to specific environmental temperatures and that specific isoforms might be differentially expressed according to environmental thermal conditions. By thus co-defining the capacity for oxygen delivery, hemocyanin would contribute to set the limits of thermal tolerance.

In an integrative approach, further investigation of thermally-induced differential expression of hemocyanin could help bridge the gap between physiological analyses of hemocyanin functions and molecular and phylogenetic approaches that characterize isoforms of hemocyanin. Our current work addresses these relationships.

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