

Oxygen limitation of metabolism and performance in pelagic squid

H.O. Pörtner

Alfred-Wegener-Institute für Polar- und Meeresforschung, Biologie I/Ökophysiologie, Columbusstraße, D-27515 Bremerhaven, Germany

Abstract

Considering their energy requirements the highly active squid which inhabit the pelagic zone of the continental seas have been termed invertebrate athletes. Their highly concentrated haemocyanin facilitates O₂ transport. By virtue of its extreme pH dependence it is designed to load oxygen efficiently in the gills and unload it fully in the tissues. Squid regulate their extracellular pH very efficiently, thereby protecting the haemocyanin from a fatal pH change. For this reason acidification from anaerobic metabolism takes place almost entirely intracellularly. In this way squid haemocyanin helps produce the highest oxygen consumption rates measured among marine invertebrates. However, a large proportion of the oxygen requirement must be provided by uptake over the skin surface. The highly efficient use they make of the oxygen available and the extreme pH-dependence of the haemocyanin explain why these creatures require a constantly high supply of oxygen, and also why they are so sensitive to increased carbon dioxide levels in the surrounding sea. Anaerobic mechanisms set in once oxygen supply to mitochondria becomes limiting. However, the transition to anaerobic energy production is a time-limited process leading those species which dive into hypoxic environments to develop energy-saving modulations of the jet propulsion procedure, for example by oscillating between periods of high and low pressure jets, thereby minimising the net use of anaerobic resources and thus extending the period during which such resources are available.

The high performance achieved by squid inhabiting the open sea is made possible by the uniform environmental parameters. Nonetheless squid also require mechanisms which limit their energy consumption to a level in keeping with the amount of food available. Coastal species make greater use of a fin for swimming, whereas "jet-propulsion" is more economical for attaining the high velocities necessary in the open sea. Certain species have developed neutral buoyancy by accumulating ammonium salts in separate fluid filled chambers. Among them is the giant squid, which leads a life still very much shrouded by mystery in the depths of the ocean.

Keywords: pelagic squid, swimming, metabolism, oxygen consumption locomotion, pH.

Introduction

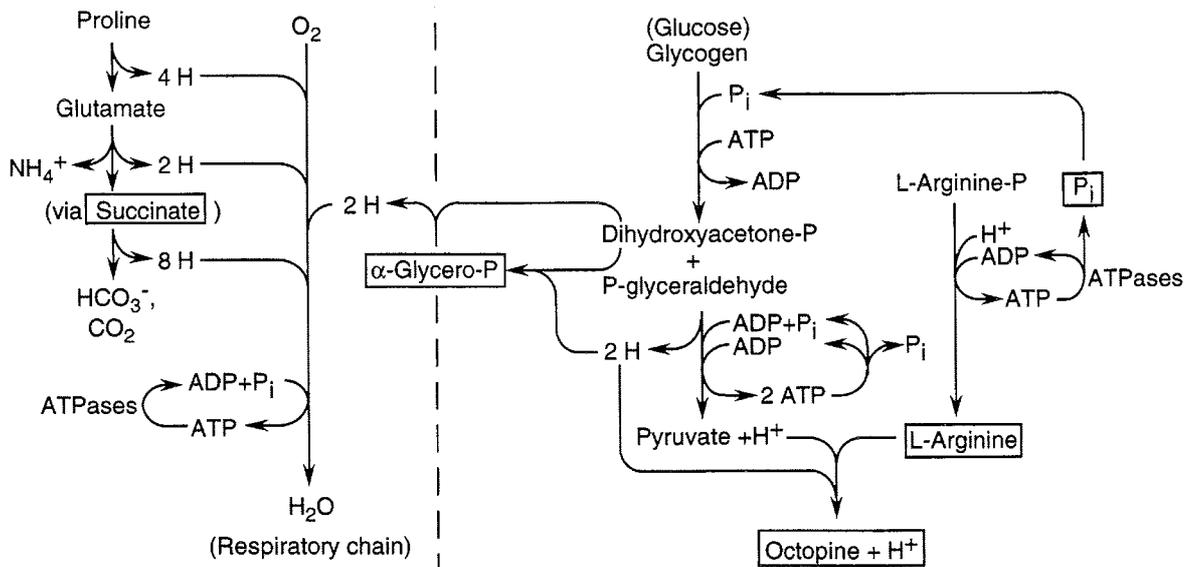
Squid are the only large invertebrates to have conquered the pelagic zone, the vast open stretches of the world's oceans. They occupy a position in the ecosystem and food web comparable to that of marine vertebrates (fish and marine mammals) and especially those squid living in shelf areas are usually extremely muscular and display a level of activity which is comparable to, or even exceeds, that of similar sized fish. They mostly swim by jet propulsion requiring high levels of energy. Accordingly, their aerobic metabolism exhibits the highest rates found in marine invertebrates, higher even than those of fish of a similar size and mode of life (O'Dor & Webber, 1986), thus ensuring their position in the pelagic ecosystem. This review deals with the physiological characteristics enabling squid inhabiting the continental seas to perform to such a high level. In addition, processes are discussed which have enabled squid living in different habitats to limit the extent of their energy consumption. Performance levels and energy metabolism have been studied in greatest detail in the ommastrephid *Illex illecebrosus* and the loliginids *Loligo pealei* and, most recently *Lolliguncula brevis*. This study is based to a large extent on these investigations and it is these species which are referred to when *Illex*, *Loligo* or *Lolliguncula* appear in the text. The majority of studies on *Illex* or *Loligo* have been carried out on specimens up to 50 cm in length and weighing between 300 and 600 g, whereas investigations in *Lolliguncula* used animals of about 10 g and 10-12 cm body length on average.

There are differences between *Illex*, *Loligo*, and *Lolliguncula* in their choice of habitat and way of life. *Loligo*, like many other loliginids, is more or less non-migratory and restricted to coastal waters; *Lolliguncula brevis* even enters very shallow and brackish waters in the inshore environment. In contrast *Illex*, like many ommastrephid squid, migrates vast distances through the open ocean as part of its life cycle. For this reason the most active cephalopods are included amongst the ommastrephids, rather than the loliginids.

Squids have developed jet-propulsion down to a fine art. They achieve particularly high speeds when swimming backwards (the maximum measured in smaller specimens was 3 m s⁻¹, and even greater speeds are attained by the larger squids). The mantle musculature is mainly (up to 90%) composed of circular musculature interspersed with narrow bands of radial muscle

fibres (running perpendicular to the mantle surface). In addition, there is a thin layer of connecting tissue on either side of the mantle (1% of the mantle tissue), which is important for the attachment of the radial muscles and the elastic collagen fibres (0.5%) which act as springs, facilitating the refilling of the mantle, after a jet of water has been expelled (Gosline & Demont, 1985). In contrast to octopods, squid have no longitudinal muscle fibres in the mantle. The circular and radial muscles are so equipped biochemically as to allow differentiation between those fibres which mainly function aerobically (muscle cells containing many mitochondria, which contract slowly and are reliant on a good oxygen supply) and those which are mainly anaerobic (muscle cells containing few mitochondria, which contract rapidly and show a great capacity for using anaerobic energy sources, such as glycogen or the phosphagen, L-arginine phosphate, Figure 1). In *Illex* with a mantle cross section of approximately 6 mm, aerobic fibres (with 47% mitochondria) comprise 14% (0.8 mm) and 5% (0.3 mm) of the circular muscle on the outer and inner mantle surfaces, respectively. The inner section (81%) is comprised of anaerobic fibres (7% mitochondria, Bone *et al.*, 1981; Mommsen *et al.*, 1981). The various types of muscle cell function in different ways during periods of ventilation or jet-propelled swimming:

Figure 1 Simplified scheme of the most important processes taking place in aerobic and anaerobic energy metabolism in squid (without strict adherence to stoichiometric relationships). Energy-rich hydrogen (2H, reducing equivalents) produced during aerobic oxidation of amino acids (mainly proline) and carbohydrates (glucose) is then oxidised using the mitochondrial respiratory chain, leading to aerobic ATP production (left). Hydrogen produced by glycolysis in the cytosol (right) is transferred to dihydroxyacetonephosphate. The resulting α -glycerophosphate is used as a means for channelling the hydrogen into the respiratory chain. The great strain put on the muscles when expelling a jet of water causes the accumulation of succinate and α -glycerophosphate, indicating that the O_2 supply is no longer sufficient. In order to produce additional ATP, L-arginine phosphate is mobilised and glycogen is broken down to give octopine, mainly in the anaerobic muscle fibres. The accumulation of ADP and protons induces the utilisation of L-arginine phosphate. Accumulation of inorganic phosphate (P_i) promotes glycogen breakdown and thus the synthesis of octopine.



The mitochondria-rich circular muscle fibres, which maintain continuous movement of the mantle for ventilation and constant swimming, function primarily at low swimming speeds (the maximum force generated by these cells is lower than that of the anaerobic fibres, since the number of power producing myofibrils is reduced due to the great numbers of mitochondria present). Whereas these cells do have a good blood supply, the capillary network in the mitochondrion-rich regions of the mantle is far less extensive than in fish: In squid (*Alloteuthis*) between five and eight muscle fibres lie between two neighbouring capillaries, but in mackerel (*Scomber*) for example, at least three capillaries surround one fibre (Bone *et al.*, 1981). This raises the question as to how squid still manage to reach a level of oxygen consumption twice that of similar sized fish.

Circular and radial muscle fibres poor in mitochondria are able to produce great power at short notice due to the rich supply of myofilaments. These anaerobic cells mainly come into play when the squid is either under attack or escaping and high speed is therefore essential. The radial cells and collagen fibres work together to speed the reinflation of the mantle and intake of water

In comparison to pure jet-propulsion alone, the use of a fin (by *Sepia* and to various degrees by squid) saves energy, since the same physical principles apply as in fish locomotion. The fin with its undulating beat bridges the inherent pauses in between jets (refilling phase) and so smooths out and stabilises movement. The more developed the fin, the more it is involved in propulsion. However, the degree of thrust attainable is much lower than for fish, since only a small mass of muscle is involved and there is no strengthening of the muscle comparable to the effect of the spinal column in fish. Thus the fin only saves energy at low speeds, particularly since, as it increases in size it increases drag by hampering the stream-lined body shape, which is an advantage at high speeds. At high velocities a large fin therefore increases energy expenditure (cf. O'Dor & Webber, 1991).

Both *Sepia* and *Loligo* use their fin at low speeds which results in a lower energy requirement than in *Illex* (see O'Dor & Webber, 1991). When it is of prime importance to reach high speeds, as is the case for *Illex*, there is only a small fin which enhances a stream-lined body shape. As a jet of water is expelled the remaining fin is flattened against the mantle, thus reducing body drag.

Growth and Reproduction

The squid's high metabolic rate, and to a limited extent that of other shell-less cephalopods, is not solely a result of the large amount of energy required for jet-propulsion. It is possible to deduce what other physiological characteristics modern day cephalopods have developed since they "lost" their shell, by comparison with *Nautilus*, which still has an external shell. Although *Nautilus* is not a direct descendant of the ancestors of modern day forms, its physiology does allow some conclusions to be drawn about certain features of the early cephalopods. The high growth rates of modern cephalopods are commensurate with their high metabolic rates. All species investigated have higher growth rates than *Nautilus* or poikilothermic vertebrates (fish, amphibians, reptiles), even reaching the same level as mammals (Calow, 1987). The subsequent high biomass production of these animals is most certainly a reason for their unique position in the food chain and also their importance to the fisheries industry. In contrast to mammals, the high metabolism and growth rates of shell-less cephalopods are connected with a short life expectancy (between less than one year and a maximum of about three years in the more common coastal forms). As a rule they die after reproducing only once, so it is obvious that rapid growth rates contribute towards speeding up sexual maturity, but also reduce life expectancy. High growth rates may be advantageous, since vulnerable juvenile stages living unprotected in the pelagic zone can escape more quickly from potential predators. A greater percentage also reach sizes which enable them to compete successfully for prey with similar sized fish, and also prey on larger fish themselves.

Energy Metabolism and Acid-Base Regulation

As molluscs, cephalopods have a special type of metabolism where proteins and in particular amino acids comprise the main substrate. In this respect, they differ from fish and other vertebrates. Aerobic metabolism in carnivorous squid is reminiscent of that in certain insects, since the amino acid proline is usually respired. Energy-rich hydrogen, obtained from glucose oxidation in the cytosol, is transported to the mitochondria with the aid of the α -glycerophosphate-shuttle. It is then oxidised as part of the respiratory chain (Figure 1).

Cephalopods, like all molluscs, crustaceans and insects have L-arginine phosphate in their muscle. This compound is found in high concentrations in the mantle and acts like phosphagen, as a reservoir of energy-rich phosphate, which maintains (buffers) ATP at a constant level. When the muscle is active and energy turnover is high, more ATP is required than can be provided (aerobically) by the mitochondria. Phosphate from this store is then coupled to ADP (Figure 1). In these circumstances glycogen degradation also plays a part in the anaerobic production of ATP. In cephalopods, pyruvate (from the Embden-Meyerhof-Parnas pathway) condenses with L-arginine (from L-arginine phosphate) and forms the opine, octopine (Grieshaber & Gäde, 1976). During periods of increased activity when the transport of oxygen to the mitochondria is no longer sufficient, high levels of α -glycerophosphate build up in the mantle tissues. This indicates that energy-rich hydrogen (i.e. reducing equivalents) is no longer

being sufficiently utilised by the respiratory chain. Anaerobic mitochondria also produce succinate, a product of anaerobic metabolism (cf. Grieshaber *et al.*, 1994).

An analysis of the use of different anaerobic pathways in *Lolliguncula brevis* led to the observation that, with increasing swimming speed, octopine, α -glycerophosphate and succinate accumulation start simultaneously. This indicates an anaerobic threshold to be reached, i.e. the critical swimming speed above which anaerobic metabolism contributes to energy production (Figure 2; Finke *et al.*, 1996). This critical threshold was found at speeds of 1.5 mantle lengths sec^{-1} , a velocity which appears low compared to pelagic fish but which may reflect the lifestyle of *L. brevis* in inshore waters. Beyond this velocity, phospho-L-arginine was progressively depleted, ATP levels fell and adenylates were degraded, while glucose-6-phosphate accumulated in the mantle muscle. The simultaneous onset of α -glycerophosphate, succinate and octopine accumulation indicates that limited oxygen supply to mitochondria is the reason for the onset of anaerobic energy production. This finding is quite opposite to the situation found in many other invertebrate and vertebrate species, where energy requirements in excess of aerobic energy production are covered by anaerobic metabolism, with the mitochondria remaining aerobic. The finding of mitochondrial hypoxia at a critical speed in squid underlines that these animals already make maximum use of available oxygen under control conditions when energy requirements are the highest among marine invertebrates.

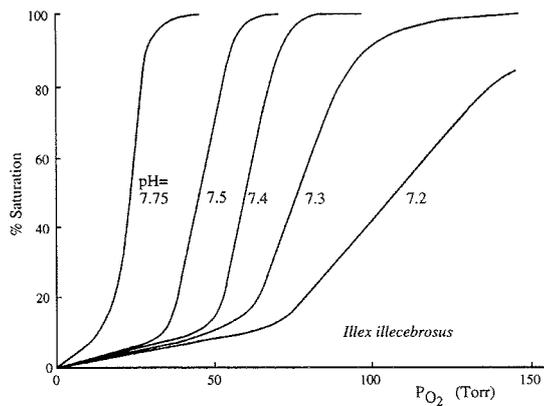
The formation of lactic acid is known to lead to acidification of the tissues and blood. However, it was long thought that, because of its chemical structure, octopine acted as a weak acid (Figure 3), permitting anaerobic metabolism (when escaping or pursuing prey), without any danger to the acid-base balance. It was suggested (Zammit, 1978) that this was advantageous to oxygen transport in the blood, which is carried out by haemocyanin. This pigment is found in extracellular solution and is therefore different to vertebrate haemoglobin in that it is not protected by containment in erythrocytes (see below).

Our research shows, however, that octopine does cause acidosis, since pyruvate formation during glycolysis is associated with the release of protons (Pörtner, 1987). L-arginine is produced by the hydrolysis of L-arginine phosphate or is taken from the extracellular pool. As expected, the build-up of octopine in *Illex* leads to marked intracellular acidosis (Figure 3). The production of octopine and protons is an advantage to the energy budget, since they supplement the use of phosphagen. At peak activity, *Loligo* produces far less octopine than *Illex*. At the same time the extensive breakdown of ATP shows that *Loligo* is less well equipped to withstand great physical demands than *Illex*. This is what would be expected from the "quieter" way of life of loliginids discussed above (Pörtner *et al.*, 1993).

Unlike lactate, octopine does not usually leave the intracellular space. A detailed study, comparing all metabolic protons with changes in the acid-base balance, verifies where the metabolically produced protons remain (Pörtner, 1994). Results show that here too squid are different from most other animals, since they primarily regulate the extracellular, not intracellular, acid-base balance. That is, virtually all the metabolic protons remain within the muscle cells. This avoids acidification of the extracellular space, even when high levels of octopine are produced. The blood is additionally protected against acidification because base equivalents (bicarbonate) are excreted from the tissues during active periods. It will become apparent that this is an adaptation to the extremely high rates of aerobic metabolism, and thus of great importance to oxygen transport in the blood of cephalopods (Figure 4).

The onset of anaerobiosis beyond the critical swimming speed appears as the key to an understanding of fatigue in squid mantle muscle. A fall in pH affects the energy status of the muscle cells which is quantified as the Gibb's free energy change of ATP hydrolysis. The term "ATP free energy change" is derived from various parameters including pH and concentrations of ATP, free ADP, inorganic phosphate, and free magnesium (Pörtner *et al.*, 1996). Assuming constant levels of all of these parameters, pH by itself causes a "drop" in standard and *in vivo* ATP free energy change values by about 2 kJ mol^{-1} with a minimum below pH 7.0 (Pörtner, 1993). In squid mantle, a minimum is reached at similar pH values, but a much larger change

Figure 4 pH dependence of oxygen binding to the haemocyanin of *Illex illecebrosus*, shown as a classical P_{O_2} /saturation diagram (Pörtner, 1994). The shift to the right in the oxygen binding curves shows clearly that oxygen affinity decreases with falling pH (Bohr effect). S-shaped binding curves, with a steep gradient around the P_{50} , result from the high degree of cooperativity between the individual haemocyanin units (cf. Figure 5).



acidosis in *I. illecebrosus* (and *Lolliguncula brevis*) may protect the ATP from major degradation (Pörtner *et al.*, 1993, 1996). With the same change and the same minimal value of ATP free energy change, more ATP was depleted in *L. pealei* and more ADP and AMP accumulated. An acidosis, although decreasing the actual value of the free energy change of ATP hydrolysis to some extent (see above), is in most cases associated with complementary ATP production, compensating for this apparent disadvantage and delaying the progressive depletion of ATP levels and the fatal decrease in ATP free energy change.

Table 2 Maximum acidification and energetic parameters limiting squid performance during functional anaerobiosis (adopted from Pörtner *et al.*, 1993, 1996)

	<i>Illex illecebrosus</i>	<i>Loligo pealei</i>	<i>Lolliguncula brevis</i>	
ΔpH_i	-0.6	>-0.1	-0.57	
Δ L-arginine phosphate	-31.2	-22.5	-18.8	mmol g ⁻¹
Δ L-arginine	14.9	28.0	0	"
Δ ATP	-2.3	-3.8	-2.5	"
Δ ADP	1.3	2.4	1.0	"
Δ AMP	0.7	2.3	0.7	"
Δ dG/dx	13.8	14.0	12	kJ mol ⁻¹
minimum dG/dx	-42	-42	-45	kJ mol ⁻¹

Certainly, this view may be too simple and other mechanisms are very likely involved in limiting the degree of muscular activity or reducing the maximum swimming velocity, a change, which we tend to interpret as fatigue. This may actually happen at higher levels of ATP free energy. For example, such a reduction in motor activity is seen in *L. brevis* at higher values of the Gibb's free energy change of ATP hydrolysis than in *I. illecebrosus* (Table 2). This may be related to the ability of *Lolligunculas* to dive into hypoxic waters, where a long term utilisation of energy stores is required at low levels of activity (Pörtner *et al.*, 1996, see below). Comparable strategies are found in animals from other oxygen deficient environments like the marine sediment (unpublished data) and may be typical for hypoxia tolerant animals.

Blue Blooded Squids

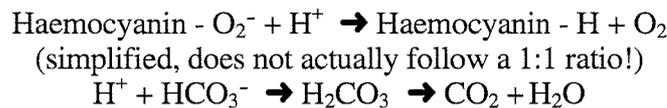
Blood oxygen transport in squid occurs by use of an extracellular pigment, haemocyanin (Mangum, 1990). The pigment concentration determines the capacity of the blood to bind and transport oxygen. However, there is a limit to the concentration of pigment, since the soluble protein binds water (colloidal osmotic pressure) and can remove it from the tissues. High protein

occurs which, though partly caused by the acidosis, tends to follow the depletion of ATP and the accumulation of ADP and inorganic phosphate. The context of the present study and our previous work (Pörtner *et al.*, 1993) demonstrate that the minimum value for ATP free energy change may indicate the onset of contractile failure or fatigue generally and independent of pH. This conclusion is supported by the observation that the respective change in energy status was similar in fatigued *I. illecebrosus* and *L. pealei* although pH fell by about 0.6 pH units in *Illex* mantle but only by less than 0.1 pH units in *Loligo*. As a corollary, the drop in ATP free energy occurs to the same extent in fatiguing muscle regardless of whether pH falls at the same time or not (cf. Table 2). However, the

concentrations also increase the viscosity of the blood. This is partly compensated for by the fact that haemocyanin is a macromolecule, consisting of ten subunits, with eight oxygen binding sites per unit. Cephalopods, and in particular squid, have the highest concentrations of haemocyanin in the animal kingdom, having over 150 mg protein ml⁻¹ blood. In spite of this the level of bound oxygen (oxygen binding capacity) at 1 to 2 mmol l⁻¹, remains below the 4 to 5 mmol l⁻¹ found in fish. Although the molecular weight per oxygen binding site is lower in haemoglobin than in haemocyanin, this comparison indicates how much more concentrated a pigment can be, contained within erythrocytes and how its level in the blood is increased by there being large numbers of erythrocytes present.

Cephalopod haemocyanins display a strong pH dependence in oxygen binding. The oxygen affinity of the pigment, measured as the O₂ -partial pressure when 50% of the pigment is saturated (P_{50}), increases as pH falls; this is indicated by a shift to the right of the oxygen-binding curve in the P_{O_2} /saturation diagram (Figure 4). The Bohr coefficient $\Delta \log P_{50} / \Delta \text{pH}$ in modern day cephalopods is less than -1 (again *Nautilus* is the exception with $\Delta \log P_{50} / \Delta \text{pH} = -0.2$, Bridges, 1994). The cooperativity gives rise to an S-shaped O₂ -binding curve with a particularly steep gradient in the range of the P_{50} . High cooperativity means that during deoxygenation the pigment is able to withstand a reduction in the P_{O_2} in the blood over a narrow P_{O_2} range. Cooperativity shifts the range of P_{O_2} in which P_{O_2} is buffered to higher values.

The reversible bonding of protons to the pigment protein underlies the pH-dependence of oxygen binding. A characteristic number of protons are released during (arterial) saturation (oxygenation), which are then taken up again during oxygen release (deoxygenation) in the tissues. The protons are involved in, or extracted from, the dissociation equilibrium of carbon dioxide:



This process leads to either the production (venous) or consumption (arterial) of bicarbonate. The Haldane coefficient $\Delta \text{HCO}_3^- / \Delta \text{HCO}_2$ (at constant pH) is numerically equivalent to the Bohr coefficient (Wyman, 1964). So, the greater the Bohr effect, the greater the number of protons which are either taken up by, or released from, the protein. This process increases the level of CO₂ (bicarbonate), which is transported in the blood from the tissues to the gills (Haldane effect). Thus, O₂ and CO₂ transport in the blood are linked through proton exchange with the pigment.

In the tissues the amount of oxygen released by the pigment and reaching the mitochondria is mainly dependent on the oxygen concentration gradient between the venous capillaries and the mitochondria themselves. The Bohr effect facilitates the oxygen supply to the mitochondria, because the pigment releases oxygen at even higher P_{O_2} values (Figure 4), increasing the concentration gradient. In order for the Bohr effect to function in this typical way, the pH of the blood must drop, not rise, en route from the gills to the tissues. In the tissues the oxygen provided by the blood is converted to CO₂, such that there are normally enough protons available to support the Bohr effect. This is, however, no longer the case where the Bohr coefficient is too high. If the pigment protein takes up too many protons during deoxygenation, too much bicarbonate is produced. This causes the pH of the blood to rise, rather than drop as it flows from the gills to the tissues. This is to be expected where the Bohr and Haldane coefficients are ≤ -1 , which is typical for shell-less cephalopods. This anomaly needs to be resolved, in order to understand more fully the way in which squid haemocyanin functions.

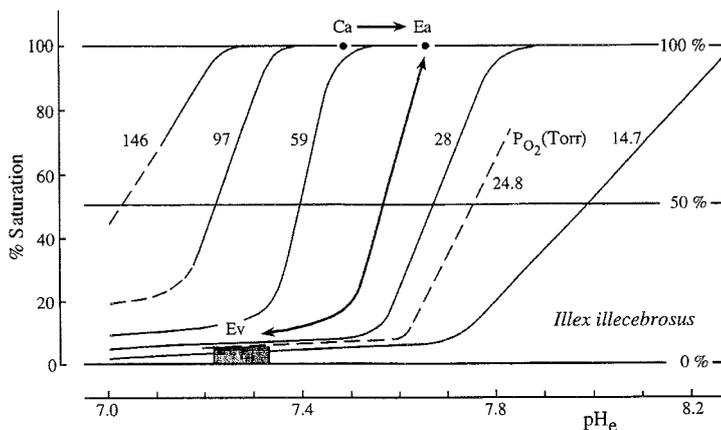
Coordination of pH Regulation, Haemocyanin Function and Gas Exchange

The processes which take place in the mantle are crucial to the squid's overall metabolism and also for understanding the function of the haemocyanin. Since the mantle is in constant motion this alone means that it has a high oxygen requirement. Unfortunately, because of the great

sensitivity of these "souped-up" animals there are to date, no measurements available of the venous blood in the mantle. In order to improve our knowledge we are therefore reliant on modelling and probability calculations. However, accurate predictions can be made about the special role of haemocyanin in squid.

The classic P_{O_2} -saturation diagram (Figure 4) can only poorly illustrate and quantify the relationship between O_2 -binding, P_{O_2} and pH. This is better achieved by pH-saturation-analysis (Figure 5, Pörtner, 1990, 1994). The oxygen isobars (lines of constant P_{O_2}) show which pH changes in the blood enable the pigment to buffer the P_{O_2} , as it unloads, and saturation drops from 100% in the artery to the venous level. The gradient is steepest where pH is close to that of *in vivo* blood (7.4 ± 0.2). This corresponds to a pH-dependent peak in cooperativity and shows that extracellular pH must be closely controlled and protected against fluctuations. A drop in arterial pH of only 0.1 to 0.15 pH units (starting from Ca) would already (with an arterial P_{O_2} of 13.3 kPa = 100 Torr) hamper the oxygen saturation of the pigment. This situation could arise in an environment where the CO_2 partial pressure was increased such as in tide pools. Squid would not be able to survive in such an environment owing to their high oxygen requirement and the high pH-dependence of oxygen binding they are reliant on a constantly high level of oxygen and a low level of carbon dioxide, in order to safeguard their oxygen transport.

Figure 5 The oxygen binding curves in the pH/saturation diagram are isobars showing how the pigment maintains constant P_{O_2} when unloading over a suitable pH range; i.e. the pigment acts as a buffer. The bold arrows show the change between *in vivo* arterial and venous levels (the precise course is unknown). Exercise causes excess base to flood the arterial blood, thereby causing pH to increase (Ea) above that of control conditions (Ca). This increases the oxygen affinity of the haemocyanin, ensuring arterial oxygen uptake. Venous pH (Ev) is reduced below that in the artery, so the classic Bohr effect takes place promoting oxygen release from the haemocyanin to supply the tissues (Pörtner, 1990, 1994).

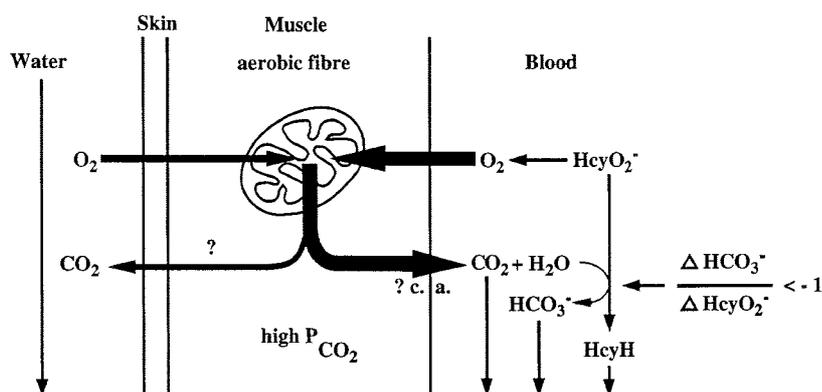


more CO_2 present than would be expected solely from the consumption of oxygen brought by the blood. This solves the above-mentioned anomaly, but at the same time poses the question as to how the production of surplus CO_2 can be explained. CO_2 production and O_2 consumption in the mantle are therefore both greater than expected from the amount of O_2 supplied by the blood alone. This is only possible if a major proportion of the oxygen enters through the skin instead (Pörtner, 1994). This is also confirmed by the fact that the circulation system would not be able to pump the necessary quantity of blood, and that the capillarisation of the mantle muscle is insufficient (see above). The mitochondrion-rich muscle cells are situated on the inner and outer mantle surfaces and are supplied with oxygen through an extremely thin skin. Continuous movement of water over the inner and outer mantle surfaces reduces the formation of oxygen-poor boundary layers. Oxygen uptake via the skin is not just dependent on diffusion, since the proportion provided from this form of respiration is too great (at times over 50% of the total oxygen uptake in the mantle). It may be enhanced by the movement of the tissue fluids, produced by the constant muscle activity (and the repeated 100% alteration in the cross section of the mantle wall). Figure 6 summarises the results of increased CO_2 production in the mantle. The binding of protons to the haemocyanin (Haldane effect) removes a large proportion of the

The danger of a drop in arterial pH during active periods is reduced by surplus bicarbonate in the blood raising pH (from Ca to Ea in Figure 5), and thereby also increasing oxygen affinity. This ensures the arterial oxygen loading of the blood. The high metabolic rate of the mantle means that the haemocyanin in the venous blood already fully releases its load (Ev) under control conditions, and not just during active periods (Pörtner, 1994). Venous pH then drops below the arterial producing the typical Bohr effect. Obviously these animals have sufficient CO_2 in their venous blood to cause acidification. In fact there is even

CO₂ from the blood, where it is bound as bicarbonate. This additional acidification causes the venous pH to drop below the arterial value, thus aiding the pigment's P_{O₂} buffer function.

Figure 6 A semi-quantitative model showing the relationship between gas exchange and haemocyanin function. The aerobic muscle fibres are supplied with oxygen from the blood and via the skin surface; the CO₂ partial pressure (P_{CO₂}) in the tissues rises due to the increased production of CO₂. Part of the additional CO₂ produced is removed in the venous blood, where it reduces pH and facilitates haemocyanin deoxygenation. CO₂ uptake by the blood and its conversion to bicarbonate (HCO₃⁻) are governed by the Haldane factor ($\Delta \text{HCO}_3^- / \Delta \text{HcyO}_2^-$) and may be assisted by the action of carbonic anhydrase (c.a.). O₂ and CO₂ transport in squid are thus linked and the typical Bohr effect takes place (see text).



It is quite remarkable that such highly developed organisms as the muscular squid inhabiting the waters of the continental seas, should be so dependent on oxygen uptake over their body surface! This suggests that these animals could only achieve such a high level of activity after their ancestors had lost their shell. However, as squid increase in size, the proportion of the oxygen supply obtained via the skin must become less owing to the short distances covered by diffusion. This seems likely, since the metabolic rate per unit weight drops and the animals can manage almost entirely on the oxygen supplied by the blood. Interestingly, the Bohr coefficient for giant squid (*Architeuthis*) with a value of -0.8 (Brix *et al.*, 1989), is in excess of -1, which means that the pigment's P_{O₂} buffer function is nonetheless guaranteed. The oxygen uptake over the skin is probably also lower in *Octopus vulgaris* and *Sepia*. These have an alternative method of venous acidification: Their haemocyanin binds CO₂ together with oxygen in the gills and transports it to the tissues (Lykkeboe *et al.*, 1980). However, nothing is known about this particular method of CO₂ binding.

Energy Saving Strategies

So far this article has focused on the outstanding performance of the squid and the physiological processes involved. How did these develop? Comparison of the way of life and physiology of today's cephalopods would suggest that certain physiological characteristics developed in the following way (Wells, 1994): The ancestors of today's shell-less species developed in an oxygen poor environment. They still had the typical molluscan-shell, which provided protection and buoyancy because of its gas-filled chambers. In fact the shell made it possible to lead a leisurely, energy-saving way of life. This is still true today of *Nautilus* and the deep sea species *Spirula* (which is, however, only found down to a depth of 1200 m).

However, other forms developed in the well lit upper layers of the sea, either in the pelagic or close to the bottom where the sea was relatively shallow. Under increasing competition and threat of attack from vertebrates after the loss of their shell, they developed the performance characteristics discussed here. The high O₂ and low CO₂ concentrations in the pelagic zone allowed for maximum energy turnover and correspondingly high growth rates. Today these types of squid are found in the pelagic waters over the continental shelves, where they compete successfully with vertebrates for food. The loss of their shell means that they are no longer neutrally buoyant; they have to remain constantly active in order to maintain their position in the

water column. On the other hand losing the shell is obviously not too great a disadvantage, since they do require a constantly high level of activity and in relatively shallow water are able to rest on the sea bed, as observed in *Illex*. Other bottom dwelling cephalopods, such as the octopus, were either no longer dependent on buoyancy mechanisms, or like *Sepia* simply retained a cuttle-bone as a gas (nitrogen) filled buoyancy organ.

It has already been shown, by comparing *Loligo* and *Illex*, that there are differences within the squid group. The theory of a maximum energy turnover applies strictly only to the most active forms, that is the ommastrephids such as *Illex*. These cover huge stretches of open ocean when migrating or in search of food, and their energy budget is better designed to cope with extreme physical demands. Squid such as *Loligo pealei* living in a "complex" coastal environment are no longer so dependent on attaining such high velocities when catching prey or escaping attack. They also do not need the stamina that *Illex* does, to quickly cover such vast stretches of ocean without resting. A well developed fin is most useful in coastal waters, for instance close to the sea bed, where there are many diverse obstacles and hiding places, when what really counts is the ability to navigate well at low speeds (this is also true for *Sepia*). Moreover, the fin is obviously an energy saving method of propulsion. In this environment squid can rest on the sea bed in shallow water or, (like *Loligo forbesi*) make use of water currents to counteract sinking to the bottom. In this case the fin takes on an additional role as a hydrofoil (O'Dor *et al.*, 1994).

An interesting strategy to save energy was recently described for *Lolliguncula brevis* (Finke *et al.*, 1996). This species lives in the brackish waters of the Gulf of Mexico and, as a coastal and inshore species, usually does not need to cover long distances during search for prey or in escape responses, a fact reflected in its low critical swimming speed (see above). The animals enter shallow coastal waters characterised by larger fluctuations of environmental parameters like temperature and salinity or is even found in hypoxic bottom waters for feeding and/or escape excursions (Vecchione, 1991). Compared to the larger squid living further offshore and on the shelf, *Lolliguncula* is characterised by a small body size, a feature possibly related to its life in shallow waters. At increased velocities measurements of pressure changes in the mantle cavity revealed that an exponential rise in performance levels, such as is observed in the other squids, was not evident (Finke *et al.*, 1996). Instead, the animals oscillate between periods of high and low muscular activity as indicated by the recording of low and high pressure jets. In the context of a small factorial rise in oxygen consumption (Table 1) this behaviour is interpreted as reducing transport cost and so as to permit a longer term net use of anaerobic resources when speed exceeds the critical value or when the squid dive into hypoxic waters.

Methods for Achieving Neutral Buoyancy

However, since the loss of their gas-filled chambers, the utilisation of fins or the development of behavioral strategies have not been the only energy-saving approaches. Squid have also mastered the deep sea, where there is no longer such close competition from similar sized vertebrates. The food density is lower than that of continental seas, and the temperature is also low, factors which make an economical way of life advisable. Since in many regions the deep sea is virtually "bottomless", there is danger of sinking into even deeper waters unless continually swimming. Pelagic species living in the deep sea and South Polar Sea have therefore developed an alternative method to achieve neutral buoyancy. In fact they have been able to conquer marine habitats which they otherwise would not have been able to with a hard external shell, since rigid, gas-filled cavities cannot indefinitely withstand the pressure which builds up as depth increases.

Some squid store fat obtained from their diet in the mid gut gland to increase their buoyancy. However, the majority of neutrally buoyant squid (and other denizens of the deep, such as certain prawns, Sanders & Childress, 1988) have altered the ionic composition of their body fluids to compensate for the high density of their tissues (Clarke *et al.*, 1979). The "heavy" cephalopods of the continental seas (in common with most other marine invertebrates) have extracellular body fluids of virtually the same density and composition as seawater (with high concentrations of sodium chloride and calcium and magnesium salts). Undiluted seawater from the open ocean has a density of between 1.023 and 1.028 kg l⁻¹. Densities for whole specimens

of 1.06 and 1.076 kg l⁻¹ have been recorded for *Loligo* and *Illex*, respectively (O'Dor & Webber, 1991). To compensate for the high specific density of their tissues, deep sea species reduce the sulphate concentration of part of their body fluids and exchange a relatively large proportion of their sodium chloride for ammonium chloride. A solution of ammonium chloride of the same osmolarity has a density of 1.01 kg l⁻¹. In order to achieve neutral buoyancy they therefore need to replace a large percentage of their body fluids with solutions containing ammonia.

At high concentrations ammonia is toxic, one reason why terrestrial animals excrete non-toxic urea or uric acid/guanine instead of ammonia, although this requires a great deal of energy. Research to date has shown that the ammonium chloride solution in cephalopods, though not in prawns, is stored in fluid filled chambers which are completely separated from the blood. Large coelomic cavities containing ammonium chloride are only found external to the tissues in certain exceptions (in the Cranchiidae). Generally, fluid filled cavities are found within the muscle of the mantle and/or tentacle, but it is not yet clear if they are intra- or extracellular. To maintain the high concentration gradients between the ammonium containing compartments (up to more than 500 mmol l⁻¹ have been measured) and the rest of the body fluids, the pH value is so low that a high percentage of NH₃ is converted to NH₄⁺. This keeps the diffusion of toxic ammonia across the surrounding membrane or epithelium to a minimum (Voight *et al.*, 1994). It is not known whether the high rate of protein degradation in cephalopods plays a part in producing the necessary concentration of ammonium.

The giant squid *Architeuthis* also has a similar approach to achieve neutral buoyancy, as shown by measurements of ammonium concentrations in tissue samples taken from stranded specimens, or on one occasion from a single tentacle caught in a net at 1000 m (Robison, 1989; this exemplifies just how difficult it is to even get hold of these animals!). The vacuolisation of the mantle tissue of the giant squid means that they have a lower proportion of active muscle when compared with their more agile relatives. For this reason, and not only due to the previously discussed size-dependence of oxygen transfer, these giant squid can never attain the same level of activity as the smaller more muscular species.

Acknowledgements

Supported by grants of the Deutsche Forschungsgemeinschaft. The skilful help of Claudia Pichler-Dieckmann in preparing the artwork is gratefully acknowledged.

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