ENVIRONMENTAL CONSTRAINTS AND THE PHYSIOLOGY
OF PERFORMANCE IN SQUIDS

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The highly active squid which inhabit the pelagic zones of continental seas are characterized by high energy requirements and have been termed invertebrate athletes. In this paper, the physiological and biochemical background of muscular performance in squid from different environments is reviewed and the physiological and environmental factors limiting performance levels are addressed. One important factor is the highly concentrated haemocyanin which, in ommastrephid squid, and by virtue of their extreme pH dependence, helps to load oxygen efficiently in the gills and unload it fully into the tissues. Squid regulate their extracellular pH more efficiently than intracellular pH, so protecting the haemocyanin from fatal pH changes. However, a large proportion of the oxygen requirement in the mantle muscle must still be provided by oxygen uptake through the skin. Anaerobic mechanisms become involved beyond critical swimming speeds once oxygen supply to mitochondria becomes limiting. Onset of anaerobiosis also characterizes the limits of long-term tolerance to progressive hypoxia at a critical P_{O_2} and to high, above-critical temperatures. In general, anaerobic energy production reflects an inability to meet oxygen demand and indicates transition to a time-limited situation. The development of energy-saving locomotion strategies therefore shifts critical thresholds and extends tolerance periods in species exposed to environmental extremes, typically in coastal areas. There, negatively buoyant squid make greater use of the fin for economical swimming, which is also advantageous because of the complexity of the environment. In *Lolliguncula brevis*, the rate at which anaerobic resources are used above the critical swimming velocity is minimized by oscillating between periods of high and low pressure jets, thereby extending the period during which the animal can dive into hypoxic or warm water. However, only jet propulsion can economically attain the high velocities necessary in the open sea. Accordingly, the highest performance levels are seen in squid inhabiting the open sea, and they are only made possible by the uniform environmental parameters. Some squid may be able to operate at their functional and environmental limits, revealing a trade-off between oxygen availability, temperature, performance level and, possibly, body size.

Squid are found in a diversity of marine environments ranging from the pelagic zones of shallow estuaries and the continental shelf and open oceans to the dark and permanently cold deep sea, including the polar oceans. They are the only large invertebrates that compete successfully with marine vertebrates (fish and marine mammals), having conquered the pelagic zone and occupying similar positions in ecosystems and foodwebs. Those squid living in shelf environments, especially in the illuminated upper water layers, are usually muscular and display a level of activity comparable to, or even exceeding, that of fish of similar size. The aerobic metabolism of muscular squid exhibits the highest rates found in marine invertebrates, so ensuring their success in the pelagic ecosystem. In contrast, deep-sea and some polar squid have developed neutral buoyancy by accumulating ammonium salts in separate fluid-filled chambers and are concluded to lead a less expensive life in deep or cold oceans (for a general review, see Wells 1994).

This review deals with the physiological characteristics enabling muscular, negatively buoyant squid to perform at such a high level. It also addresses the environmental characters limiting the performance of squid. Finally, processes are discussed which have permitted 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 paper is based to a large extent on those investigations. The majority of studies with *I. illecebrosus* or *L. pealei* have been carried out on specimens up to 50 cm long and weighing between 300 and 600 g, whereas investigations with *Lolliguncula* used animals of 6–35 g and 5–9 cm mantle length. Original comparisons of oxygen consumption rates in squid and fish of similar size and mode of life focused on salmon (O'Dor and Webber 1986). Recent data available for scombrid fish emphasize that the difference in metabolic rate between mackerel and (Atlantic) salmon is small and, therefore, confirm that the metabolic rate of squid exceeds that of comparable ectothermal fish species (Table I). Only the development of heterothermy or regional endothermy in scombrid fish such

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Table I: Comparison of basal metabolic rates with those at critical swimming velocities

Parameter	Value				
	Oncorhynchus nerka	*Scomber scombrus*	*Illex illecebrosus*	*Loligo pealei*	*Lolliguncula brevis*
Temperature (°C)	15	10	15	15	20
Mass (kg)	0.5	0.4	0.6	0.6	0.01
*Basal metabolic rate (mmol $O_2 \cdot kg^{-1} \cdot h^{-1}$)	1.8	1.5	8	4.5	22
*Active metabolic rate (mmol $O_2 \cdot kg^{-1} \cdot h^{-1}$)	21.4	8.3	48.7	38	36

* Basal metabolic rate is the standard metabolic rate for the fish species and for the negatively buoyant, but small, *Lolliguncula brevis*, as evaluated from an extrapolation of oxygen consumption to zero swimming speed. Metabolism of the larger (also negatively buoyant) squid *Illex illecebrosus* and *Loligo pealei* resting on the bottom is less than the standard metabolic rate because the standard metabolic rate includes the higher cost of maintaining position in the water column. Active metabolic rate is the rate measured at the critical swimming velocity, except for *Lolliguncula brevis*, where it is the maximum rate of oxygen consumption measured at the highest swimming speed (after Wells *et al.* 1988, O'Dor and Webber 1991, Lucas *et al.* 1993, Finke *et al.* 1996)

as tuna or in lamnid sharks may lead to metabolic expenditure greater than the rates seen in ectothermal fish and squid (Brill 1996, Korsmeyer *et al.* 1996). There are also differences between squid species, e.g. *I. illecebrosus*, *L. pealei* and *L. brevis*, in their choice of habitat and way of life. *L. pealei*, like many other loliginids, is more or less non-migratory and restricted to coastal waters. The brief squid *Lolliguncula brevis* even enters very shallow and brackish waters in the inshore environment. In contrast, *I. illecebrosus*, like many ommastrephid squid, migrates vast distances through the open ocean as part of its life cycle. For this reason, many of the fast-swimming cephalopods are included among the ommastrephids, rather than the loliginids.

PHYSIOLOGICAL CHARACTERS OF SQUID

Jet propulsion

Squid have developed jet propulsion by use of the mantle down to a fine art. The mantle mainly (up to 90%) consists 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 connective 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 and Demont 1985). In contrast to octopods, muscular 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 function mainly aerobically (muscle cells containing

many mitochondria, that contract slowly and are reliant on a good oxygen supply) and those which are mainly anaerobic (muscle cells containing few mitochondria, that contract rapidly and show a great capacity for using anaerobic energy sources, such as glycogen or the phosphagen L-arginine phosphate, see below). In *I. illecebrosus* with a mantle cross-section of approximately 6 mm, aerobic fibres (with 47% mitochondria) constitute 14% (0.8 mm) and 5% (0.3 mm) of the circular muscle on the outer and inner mantle surfaces respectively. The inner section of the squid mantle (81%) studied by Bone *et al.* (1981) and Mommsen *et al.* (1981) is composed of anaerobic fibres (7% mitochondria).

The various types of muscle cells support ventilation or jet-propelled swimming. The mitochondria-rich circular muscle fibres, which maintain continuous movement of the mantle for ventilation and constant swimming, function primarily at slow swimming speeds (the maximum force generated by these cells is less than that of the anaerobic fibres, because the number of power-producing myofibrils is reduced as a result of the great numbers of mitochondria present. The capillary network in the mitochondrion-rich regions of the mantle is far less extensive than in fish. In the squid *Alloteuthis subulata*, between five and eight muscle fibres lie between two neighbouring capillaries, but in the mackerel *Scomber* sp., for example, at least three capillaries surround one fibre (Bone *et al.* 1981). The question is how squid still manage to reach a level of oxygen consumption twice that of fish of similar size (Table I).

Circular and radial muscle fibres poor in mitochondria and rich in myofilaments mainly come into play when the squid is either under attack or escaping so that high speed is essential. The radial cells and collagen fibres work together to speed the reinflation of the mantle and intake of water after a jet has been
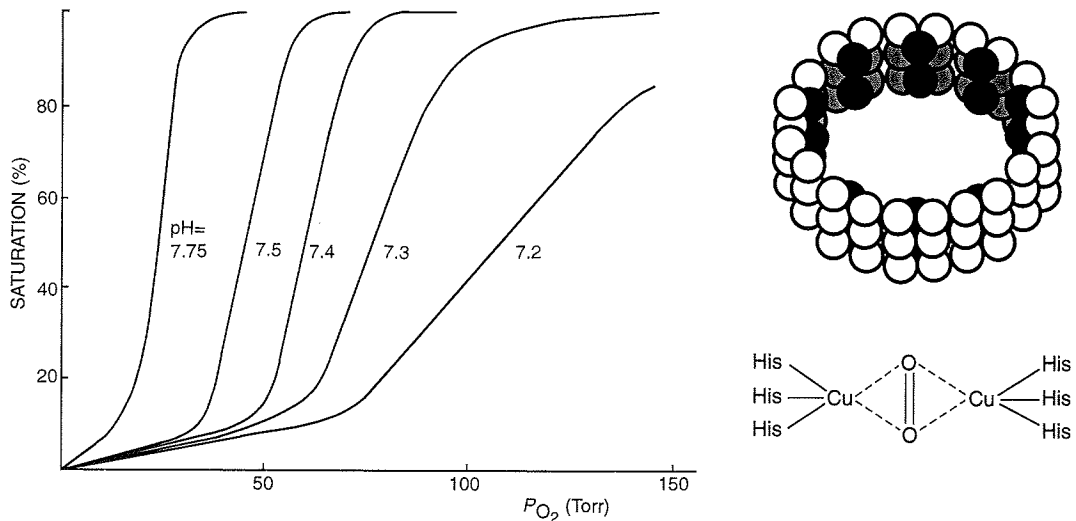
Fig. 1: The pH-dependence of oxygen-binding to the haemocyanin of *Illex illecebrosus*, shown as a classical P_{O_2} saturation diagram. The shift to the right in the oxygen binding curves shows clearly that oxygen affinity decreases with falling pH (Bohr effect). S-shaped binding curves, with a steep gradient around half saturation, result from the high degree of pH-dependent cooperativity between the 10 individual haemocyanin units (cf. Fig. 4, Pörtner 1990a; haemocyanin model with the structure of copper-containing oxygen binding sites, His = histidine, after Miller 1994)

expelled. When escaping, the mantle may even become hyperinflated, in turn producing maximum effect from the contracting circular muscle. As the circular muscle contracts, diagonal collagen fibres increase the tension and act as springs to aid the refilling of the mantle cavity. When fully contracted, the outer perimeter of the mantle is reduced by about 30%, and at the same time the mantle wall doubles in thickness (Gosline *et al.* 1983, Gosline and Demont 1985, Shadwick 1994).

In comparison with the mode of swimming in fish, oxygen consumption measurements show that jet propulsion is far more costly (Table I). Fish set in motion a large volume of water with their undulating movement. The mechanical efficiency is high, meaning that by far the greater proportion of the caudal fin movement is converted into forward thrust (Wieser 1986). Squid, on the other hand, use a great deal of energy to expel a relatively small quantity of water. Then, after each contraction, water (flowing in the opposite direction to swimming) is sucked back into the mantle cavity. This creates turbulence which increases with speed and acts as a frictional force reducing the efficiency with which muscle power is converted to propulsion (O'Dor 1988). The inefficiency of this method of propulsion is reflected in the extremely high metabolic rate (Table I).
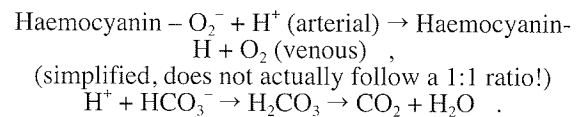
Growth rates

The high metabolic rate of squid, and to some extent that of other shell-less cephalopods, is not only attributable to the large amount of energy required for jet propulsion. The fast growth rates of modern cephalopods are commensurate with their high metabolic rates. All species investigated have faster growth rates than *Nautilus* (which has a low rate of oxygen consumption, Wells *et al.* 1992, Boutilier *et al.* 1996) or poikilothermic vertebrates (fish, amphibians, reptiles), even reaching the same level as mammals (Calow 1987). The subsequent high biomass productivity of these animals is certainly a reason for their unique position in the food chain and also their importance to fisheries worldwide. In contrast to mammals, the fast metabolic and growth rates of shell-less cephalopods are connected with a short life expectancy. As a rule cephalopods die after reproducing only once, so it is obvious that rapid growth rates contribute towards speeding up sexual maturity, but also reduce life expectancy. Fast growth rates and early maturation reduce the risk of predation, and a greater percentage of juveniles reach sizes which enable them to compete successfully for prey with fish of similar size, or even prey on larger fish themselves.

Haemocyanin function

Oxygen transport to tissues in squid occurs by use of haemocyanin (Mangum 1990). This pigment is found in extracellular solution and is therefore different from vertebrate haemoglobin in that it is not protected by being stored in erythrocytes. The pigment concentration in the blood determines its capacity to bind and transport oxygen. However, there is a limit to the concentration of pigment, because the soluble protein binds water (colloidal osmotic pressure) and can remove it from the tissues. High protein concentrations also increase the viscosity of the blood. This is partly compensated for by the fact that haemocyanin is a macromolecule, consisting of 10 subunits, with eight oxygen-binding sites per unit (Miller 1994; Fig. 1). Cephalopods, and in particular squid, have the highest concentrations of haemocyanin in the animal kingdom, with more than 150 mg protein·mℓ^{-1} blood. In spite of this, the level of bound oxygen (oxygen binding capacity), at 1 – 2 mmol·ℓ^{-1}, remains below the 4 – 5 mmol·ℓ^{-1} found in fish. Although the molecular weight per oxygen binding site is also lower in haemoglobin than in haemocyanin, this comparison primarily indicates how much more concentrated a pigment can be in the blood, when contained within erythrocytes, particularly with large numbers of erythrocytes present.

Cephalopod haemocyanins display a strong pH dependence for oxygen binding. The oxygen affinity of the pigment, measured as the O_2-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 (Fig. 1). The Bohr coefficient $\Delta \log P_{50}/\Delta pH$, an indicator of cooperativity, is less than –1 in modern-day cephalopods, but again *Nautilus* is the exception with $\Delta \log P_{50}/\Delta pH = -0.2$ (Bridges 1994). Cooperativity gives rise to an S-shaped oxygen-binding curve with a particularly steep gradient in the range of the P_{50}. High cooperativity means that, during deoxygenation, the pigment experiences a large reduction in oxygen binding in the blood over a narrow P_{O_2} range, so buffering P_{O_2} levels in the blood. Cooperativity also shifts this buffer range of P_{O_2} to higher values, supporting a high P_{O_2} gradient and, in consequence, a fast flow of oxygen between blood and mitochondria.

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 related to the dissociation equilibrium of carbon dioxide:

$$\text{Haemocyanin} - O_2^- + H^+ \text{ (arterial)} \rightarrow \text{Haemocyanin-}$$
$$H + O_2 \text{ (venous)} \quad ,$$
(simplified, does not actually follow a 1:1 ratio!)
$$H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O \quad .$$

This process leads to either the venous production or arterial consumption of bicarbonate. The Haldane coefficient $\Delta HCO_3^-/\Delta HcyO_2$ (at constant pH) is numerically equivalent to the Bohr coefficient (Wyman 1964). Therefore, the greater the Bohr effect, the more protons are either taken up by, or released from, the protein. This process increases the level of total CO_2 (as bicarbonate), which is transported in the blood from the tissues to the gills (Haldane effect). Thus, O_2 and CO_2 transport in the blood are linked through proton exchange with the pigment.

Oxygen flow to the mitochondria in the tissues 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 by causing the pigment to release oxygen even at high P_{O_2} values (Fig. 1), increasing the concentration gradient. In order for the Bohr effect to function in this typical manner, the pH of the blood must drop, not rise, on its way from the gills to the tissues. Tissue metabolism converts the oxygen provided by the blood to CO_2, such that there are normally enough protons available to support the Bohr effect. This is, however, not the case where the Bohr coefficient is large. Where the Bohr and Haldane coefficients are ≤–1, typical for shell-less cephalopods, the pigment protein may take up an excessive number of protons during deoxygenation, producing excess levels of bicarbonate. This causes the pH of the blood to rise, rather than drop, as it flows from the gills to the tissues. Resolving this anomaly is a key to understanding more fully the way in which squid haemocyanin functions and oxygen is supplied to tissues (see below).

Aerobic and anaerobic metabolism

As molluscs, cephalopods have a special type of metabolism where proteins and, in particular, amino acids constitute the main substrate. In this respect, they differ from many fish and other vertebrates. This requires many special adaptations to provide equal or even greater metabolic power outputs and assure oxygen delivery. Aerobic metabolism in carnivorous squid is reminiscent of that in certain insects, because the amino acid proline is usually oxidized. Energy-rich hydrogen, obtained from glucose oxidation in the cytosol, is transported to the mitochondria
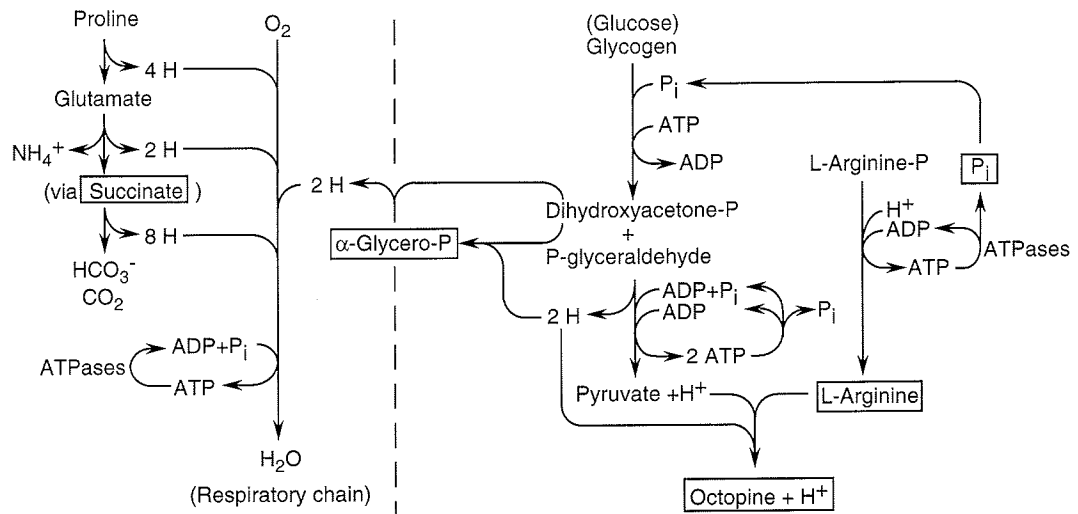
Fig. 2: Simplified scheme of the most important processes taking place in aerobic and anaerobic energy metabolism in squid (without strict adherance to stoichiometric relationships). Energy-rich hydrogen (2H, reducing equivalents) produced during aerobic oxidation of amino acids (mainly proline) and carbohydrates (glucose) is oxidized using the mitochondrial respiratory chain, leading to aerobic ATP production (left). Hydrogen produced by glycolysis in the cytosol (right) is transferred to dihydroxyace-tonephosphate. The resulting α-glycerophosphate is used as a means for channelling the hydrogen into the respiratory chain. The rapid contraction by 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 mobilized and glycogen is broken down to give octopine, mainly in the anaerobic muscle fibres. The accumulation of ADP and protons induces the utilization of L-arginine phosphate. Accumulation of inorganic phosphate (P_i) promotes glycogen breakdown and thus the synthesis of octopine

with the aid of the α-glycerophosphate-shuttle. It is then oxidized as part of the respiratory chain (Fig. 2). 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 utilized sufficiently by the respiratory chain. Anaerobic mitochondria also produce succinate, a product of anaerobic metabolism (cf. Grieshaber et al. 1994).

Cephalopods, like all molluscs, crustaceans and insects, have phospho-L-arginine in their muscle. This compound is found in high concentration in the mantle and acts like phosphagen (i.e. as a reservoir of energy-rich phosphate), maintaining ATP at a constant level when the muscle is active and the ATP demand higher than aerobic ATP provision by the mitochondria. Phosphate from this store is then coupled to ADP (Fig. 2). 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-argi-

nine (from L-arginine phosphate) and forms the opine, octopine (Grieshaber and Gäde 1976).

The formation of lactic acid in vertebrates and invertebrates is known to lead to acidification of the tissues and blood. In contrast, it was thought that, owing to its chemical structure, octopine acted as a weak acid, 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 by haemocyanin, which occurs in extracellular solution, not within erythrocytes (see above). Further work showed, however, that octopine does cause acidosis, because pyruvate formation during glycolysis is associated with the release of protons (Hochachka and Mommsen 1983, Pörtner et al. 1984, Pörtner 1987). L-arginine is either produced by hydrolysis of L-arginine phosphate or is taken from the extracellular pool (Fig. 2). Therefore, the build-up of octopine in *I. illecebrosus* and *L. brevis* leads to marked intracellular acidosis (Pörtner et al. 1991, 1993, 1996). The production of octopine and protons supports the
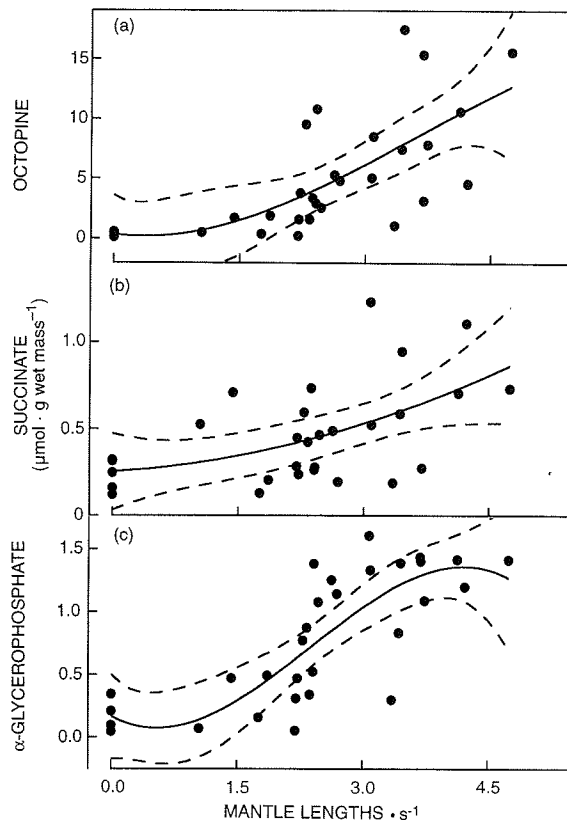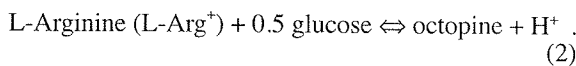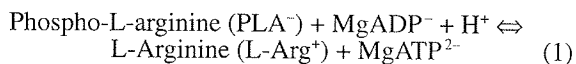
Fig. 3: Changes in (a) octopine, (b) succinate and (c) α-glycerophosphate concentrations in the mantle musculature of the brief squid *Lolliguncula brevis* sampled at different swimming speeds (after Finke *et al.* 1996). Lines represent 2nd and 3rd order polynomial fits and 95% confidence intervals

use of phosphagen via an effect on the equilibrium of the arginine kinase reaction (by providing protons and withdrawing L-arginine):

Phospho-L-arginine (PLA⁻) + MgADP⁻ + H⁺ ⇔
        L-Arginine (L-Arg⁺) + MgATP²⁻   (1)

L-Arginine (L-Arg⁺) + 0.5 glucose ⇔ octopine + H⁺ .
                                       (2)

Unlike lactate, octopine does not usually leave the intracellular space. An in-depth 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, because 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 is thus of great importance to oxygen transport in the blood of cephalopods.

FUNCTIONAL LIMITATIONS

Characterizing the anaerobic threshold

An investigation of the transition to anaerobic metabolism with increasing swimming speed (anaerobic threshold) in the loliginid *Lolliguncula brevis* led to the observation that octopine, α-glycerophosphate and succinate accumulation start simultaneously beyond a critical swimming speed (Finke *et al.* 1996). Phospho-L-arginine stores are depleted progressively, ATP levels fall and adenylates are degraded, while glucose-6-phosphate accumulates in the mantle muscle. The simultaneous onset of α-glycerophosphate, succinate and octopine accumulation is reminiscent of the metabolic situation seen in anoxic squid mantles (Hochachka *et al.* 1975) and 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 invertebrates and vertebrates, 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 the fact that these animals already make maximum use of available oxygen under control conditions when energy requirements are the highest among marine invertebrates. This critical threshold was found at speeds of 1.5 mantle lengths·s⁻¹ (Fig. 3; Finke *et al.* 1996), a velocity which appears low compared to that of active fish, where the anaerobic threshold is surpassed at a velocity of about 3–4 body lengths·s⁻¹ (Goolish 1991). To date, however, comparative data in fish and other squid species are missing, such that the meaning of these findings for squid in general cannot be evaluated.

A study of intra- and extracellular acid-base regu-

Table II: Maximum acidification and energy parameters limiting squid performance during functional anaerobiosis (adapted from Pörtner *et al.* 1993, 1996. P_i = inorganic phosphate, $dG/d\xi$ = Gibb's free energy change of ATP hydrolysis)

Parameter	Value			Unit
	Illex illecebrosus	*Loligo pealei*	*Lolliguncula brevis*	
Δ pH$_i$	−0.6	>−0.1	−0.57	
Δ L-arginine phosphate	−31.2	−22.5	−18.8	μmol·g^{-1}
Δ L-arginine	14.9	28.0	0	μmol·g^{-1}
Δ ATP	−2.3	−3.8	−2.5	μmol·g^{-1}
Δ ADP	1.3	2.4	1.0	μmol·g^{-1}
Δ AMP	0.7	2.3	0.7	μmol·g^{-1}
Δ P$_i$	33.0	36.0	17.5	μmol·g^{-1}
Δ $dG/d\xi$	13.8	14.0	12	kJ·mol^{-1}
Minimum $dG/d\xi$	−42	−42	−45	kJ·mol^{-1}

lation contributed to a deeper understanding of energetics and gas exchange in exercising squid. To obtain blood samples from unrestrained squid and to come to a quantitative picture of acid-base balance between tissues and blood, new techniques had been evolved for the anaesthesia (O'Dor et al. 1990) and cannulation of these animals (Pörtner et al. 1991), and also for the analysis of acid-base parameters. The latter methodology is suitable to measure intracellular pH and to monitor such other intracellular acid-base variables as P_{CO_2}, bicarbonate levels and buffer values (Pörtner *et al.* 1990, Pörtner 1990b). Measurements in *I. illecebrosus* swum to exhaustion in a swim tunnel demonstrated that pH is regulated with a higher priority in the extracellular than in the intracellular compartment, so maintaining haemocyanin function. Again, comparative data in other squid are missing to date. Data available for *Loligo pealei* do not yield adequate insight because anaerobic glycolytic capacity is very small in this species (Pörtner *et al.* 1991, 1993), much smaller than in *Loligo vulgaris* (Grieshaber and Gäde 1976) or *Lolliguncula brevis* (Finke *et al.* 1996).

At peak acitvity, *Loligo pealei* produces far less octopine than *Illex illecebrosus, Lolliguncula brevis* or *Loligo vulgaris*. ADP accumulation is far more expressed under these circumstances and triggers phospho-L-arginine depletion (Equation 1). The extensive breakdown of ATP to ADP illustrates that *Loligo pealei* is less well equipped to withstand great physical demands than *Illex illecebrosus* (Pörtner *et al.* 1993). This is what would be expected from the "quieter" way of life of loliginids, which use their fins extensively, as discussed below.

The onset of anaerobiosis beyond the critical swimming speed appears to be 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 Gibb's free energy change of ATP hydrolysis $(dG/d\xi)$. This parameter is seen to limit the function of cellular ATPases (Kammermeier *et al.* 1982, Pörtner 1993, Combs and Ellington 1995). The term "ATP free energy change" is derived from various parameters, including intracellular pH and cellular concentrations of ATP, free ADP, inorganic phosphate and free magnesium (Pörtner *et al.* 1996). Assuming constant levels of all of these parameters, pH alone causes a rather small "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 occurs which, although partly caused by acidosis, tends to follow the depletion of ATP and the accumulation of ADP and inorganic phosphate.

The minimum value for ATP-free energy change may indicate the onset of contractile failure or fatigue generally and may be 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 *I. illecebrosus* mantle but only by <0.1 pH units in *L. pealei* (Pörtner *et al.* 1993, 1996). As a corollary, a drop in ATP-free energy occurs to the same extent in fatiguing muscle regardless of whether or not pH falls at the same time (cf. Table II). This indicates that the drop in ATP-free energy rather than the acidosis is associated with fatigue. Intracellular acidosis as seen in *I. illecebrosus* (and *L. brevis*) may actually help to protect ATP from being degraded (Pörtner *et al.* 1993, 1996). With a similar change and the same minimal value of ATP-free energy change as in *I. illecebrosus*, 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 hy-
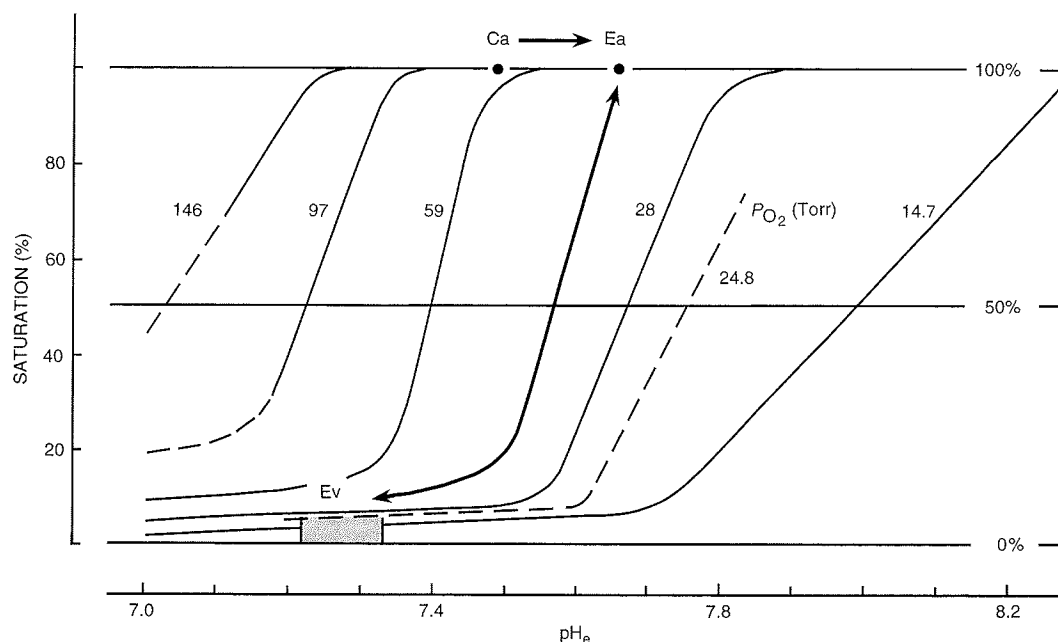
Fig. 4: Oxygen-binding curves in the pH/saturation diagram are isobars, showing how the pigment maintains constant P_{O_2} when unloading over a suitable pH range; i.e. the pigment acts as an oxygen buffer. The bold arrows suggest the change between *in vivo* arterial and venous levels (the precise course is unknown). Exercise causes excess base to flood the arterial blood, so 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 1990a, 1994)

drolysis 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.

This view may be oversimplified and such other mechanisms as the accumulation of inorganic phosphate (Table II) are very likely involved in limiting the degree of muscular activity or reducing the maximum swimming velocity, processes which tend to be interpreted as signs of fatigue (Fitts 1994). This may actually happen at higher levels of ATP-free energy. For example, fatigue or 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 II). This may be related to *Lolliguncula*'s ability to dive into hypoxic waters (Vecchione 1991), where long-term utilization of energy stores is required at low levels of activity (Pörtner *et al.* 1996, see below). Comparable strategies are found in invertebrates from other oxygen-deficient environments, such as marine sediment (unpublished data on *Sipunculus*

nudus) and may be typical for hypoxia-tolerant animals.

Role of oxygen transport

The biochemical processes which take place in the mantle are not only crucial to the squid's overall metabolism but also for understanding the function of the haemocyanin. The constant motion of the mantle alone means that it has a high oxygen requirement. Unfortunately, owing to the great sensitivity of these "souped-up" animals there are, to date, no measurements available of the venous blood in the mantle. Therefore, best knowledge relies on modelling and probability calculations, but accurate predictions can be made about the special role of haemocyanin in squid.

The classic P_{O_2} saturation diagram (Fig. 1) 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 (Fig. 4; Pörtner 1990a, 1994). The oxygen isobars (lines of constant
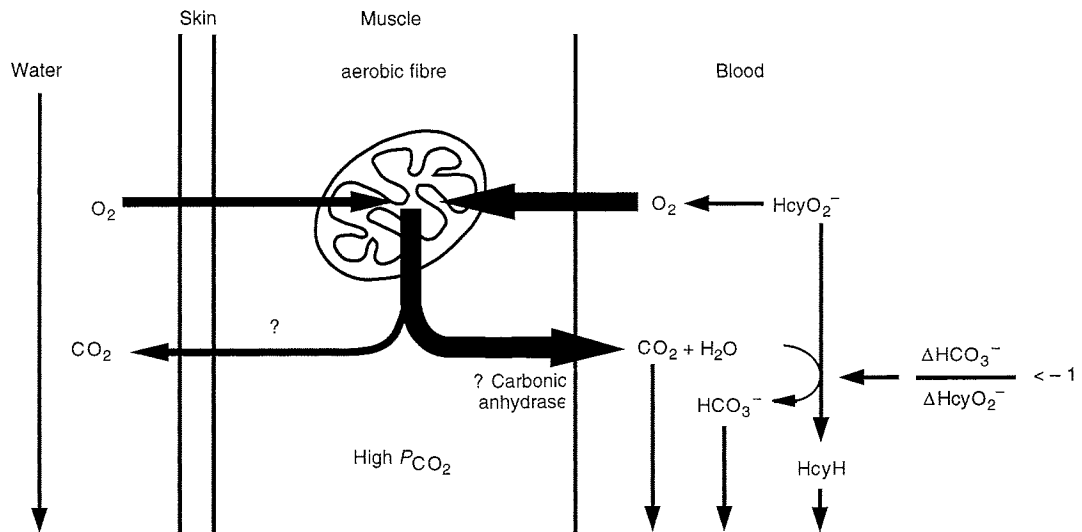
Fig. 5: Semi-quantitative model showing the relationship between gas exchange and haemocyanin function in the mantle. The aerobic muscle fibres are supplied with oxygen from the blood and via the skin surface; the CO_2 partial pressure P_{CO_2} in the tissue rises as a result of the increased production of CO_2. Part of the additional CO_2 produced is removed in the venous blood, where it reduces pH and facilitates haemocyanin deoxygenation. CO_2 uptake by the blood and its conversion to bicarbonate (HCO_3^-) are governed by the Haldane factor (Δ HCO_3^-/Δ $HcyO_2$) and may be assisted by the action of carbonic anhydrase. The O_2 and CO_2 transport in squid are thus linked and the typical Bohr effect takes place (see text)

P_{O_2} show how 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). Therefore, a pH-dependent peak in cooperativity shows that extracellular pH must be closely controlled and protected against fluctuations. A drop in arterial pH of only 0.1 – 0.15 pH units (starting from Ca) would hamper the oxygen saturation of the pigment at an arterial P_{O_2} of 100 Torr. This situation could arise in an environment, such as in tide pools at night, where the CO_2 partial pressure was increased and oxygen production by algae turns into net oxygen consumption and CO_2 production, so eliciting hypoxia and hypercapnia in the water (Morris and Taylor 1983). Ommastrephid 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 rely on a constantly high level of oxygen and a low level of carbon dioxide to safeguard their oxygen transport.

The danger of a drop in arterial pH during active periods is reduced by a surplus of bicarbonate in the blood, raising pH (from Ca to Ea in Fig. 4) and so 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 resting conditions, and not just during active periods (Pörtner 1994). Venous pH then drops below arterial pH, producing the typical Bohr effect. Obviously, these squid have sufficient CO_2 in their venous blood to cause acidification. In fact, there is even 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 must arise from O_2 consumption in the mantle, and both processes are therefore greater than expected from the quantity 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 conclusion is also confirmed by the fact that the circulatory system would not be able to pump the necessary quantity of blood, and that the capillarization of the mantle muscle is insufficient (see above). The mitochondrion-rich muscle cells are situated on the inner and outer mantle surfaces (see above) and are supplied with oxygen directly 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 passive diffusion, because the proportion provided from this form of respiration is too great (at times more than 50% of the total oxygen uptake in the mantle of *I. illecebrosus*). It may be enhanced by the movement of tissue fluids, produced by the constant muscle activity (and the repeated 100% alteration in the cross-section of the mantle wall, Gosline and Demont 1985). Figure 5 summarizes the results of increased CO_2 production in the mantle. As a result of protons binding to the haemocyanin (Haldane effect), a large proportion of the CO_2 is removed by the blood, where it is bound as bicarbonate. This additional acidification causes the venous pH to drop below the arterial value, so aiding the pigment's P_{O_2} buffer function.

It is quite remarkable that such highly developed organisms as the muscular squid inhabiting continental shelves should be so dependent on oxygen uptake over their body surface. This emphasizes the limiting role of ambient oxygen levels (see below) and 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, because the metabolic rate per unit mass drops and the animals can manage almost entirely on the oxygen supplied by the blood. Interestingly, the Bohr coefficient for giant squid *Architeuthis monachus* (−0.8, Brix *et al.* 1989), is in excess of −1, which means that the pigment's P_{O_2} buffer function is nonetheless guaranteed. Oxygen uptake over the skin is probably also lower in *Octopus vulgaris* and *Sepia*. The latter two have an alternative and unusual method of venous acidification: their haemocyanin binds CO_2 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 CO_2 binding.

ENVIRONMENTAL CONSTRAINTS: OXYGEN AVAILABILITY AND TEMPERATURE

The discussion above suggests strongly that oxygen availability limits performance levels. In some natural habitats of squid, oxygen levels may fluctuate, as reported for the brief squid *Lolliguncula brevis*. These animals not only enter shallow coastal waters characterized by larger fluctuations of environmental parameters such as temperature and salinity, but *L.*
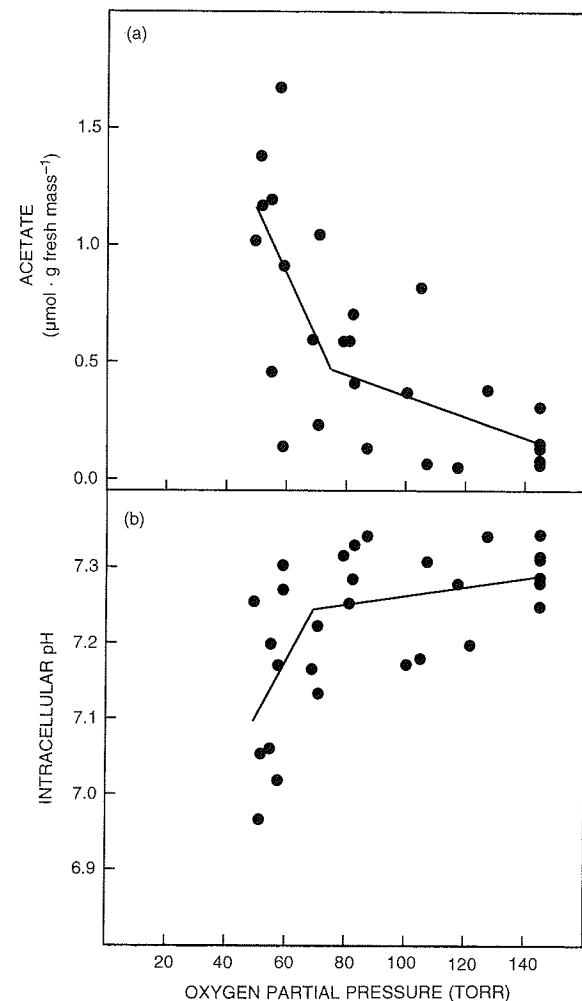

Fig. 6: During progressive hypoxia, the onset of anaerobic mitochondrial metabolism, as indicated by (a) acetate formation, quantifies the critical oxygen tension in the brief squid *Lolliguncula brevis*. Formation of anaerobic end-products leads to a progressive drop in (b) the intracellular pH of the mantle tissue (SZ and HOP, unpublished)

brevis have also been found in hypoxic bottom waters with a P_{O_2} of 2.1 kPa (Vecchione 1991). There, they have been observed to be quite active and may have entered such waters for feeding and/or escape. Furthermore, catches by trawling indicated a high abundance in waters with a P_{O_2} between 4.3 and 8.6 kPa, but animals were no longer present when oxygen tensions fell below 2 kPa at temperatures between 25 and 29°C (Vecchione 1991, pers. comm.).

Recently, the critical P_{O_2} was determined for this squid species (SZ, HOP and P.G. Lee, unpublished), the only analysis available for squid. The critical P_{O_2} is characterized by the onset of anaerobic energy metabolism and indicates oxygen limitations under resting conditions when animals exhibit standard metabolic rates (Pörtner and Grieshaber 1993). The data for *L. brevis* reveal a rather high critical oxygen level of 8–10 kPa at 20°C (Fig. 6), which apparently contradicts the findings that these animals are able to enter and stay in hypoxic water layers. However, data available on the mode of locomotion strongly suggest an energy-saving mode of jet propulsion which would allow the species to dive into hypoxic waters (Finke *et al.* 1996, see below). Although this strategy may not delay the onset of anaerobic metabolism, it will prolong the period during which anaerobic resources can be used. Nothing is known in this respect for other squid species.

Compared to other cephalopods, muscular pelagic squid, which are the cephalopods with the highest metabolic rates (Wells 1994), are supposedly the least hypoxia-tolerant, whereas benthic octopods and sepiids, some of them living in burrows, are more likely to tolerate extended periods of hypoxia (e.g. Houlihan *et al.* 1982). The maximum use of oxygen resources and the high sensitivity of blood oxygen transport suggest that *Illex illecebrosus* is intolerant to even moderate hypoxia, although the critical oxygen level (Pc = critical P_{O_2}) has not been quantified (Pörtner 1997). Short-term sensitivity of *Loligo pealei* to low oxygen and elevated CO_2 was investigated as early as 1929 (Redfield and Goodkind 1929), but true limiting oxygen levels have also not been evaluated. The key role of blood oxygen transport in the hypoxia resistance of (loliginid) squid was already recognized by Redfield and Goodkind (1929) and was confirmed by recent considerations for ommastrephid squid (Pörtner 1990a, 1994). Nonetheless, further comparative studies are needed to elaborate unifying principles and solutions in squid hypoxia-tolerance or intolerance.

The available data suggest that tolerance to fluctuating temperatures is intimately linked to the question of hypoxia-tolerance. Recent data obtained in marine ectothermal animals demonstrate that both low and high critical temperatures are characterized by the transition to an anaerobic mode of energy metabolism (Zielinski and Pörtner 1996, Sommer *et al.* 1997, Pörtner *et al.* 1998). The temperature window between low and high temperature thresholds characterizes the temperature range tolerated by a species. Insufficiency of ventilatory and circulatory mechanisms to provide sufficient amounts of oxygen to tissues are seen to set the critical temperatures (Pörtner *et al.*

1998). These critical temperatures can shift depending on the ambient temperature regime, e.g. during seasonal temperature acclimation or during evolutionary adaptation to the average temperature at a specific latitude. In two cephalopod species, the warm-water squid *Lolliguncula brevis* and the Antarctic octopod *Pareledone charcoti* (Fig. 7), upper critical temperatures were found between 26 and 30°C and between 8 and 10°C respectively. The upper limit found for *Lolliguncula brevis* is just above the range of ambient temperatures found in the species' natural environment, whereas the critical temperature is considerably above ambient in the Antarctic octopod. It should be emphasized that these critical temperatures are valid only for normoxic conditions. Critical temperatures may decrease during conditions of progressive hypoxia. Considering the environmental dataset available for *Lolliguncula brevis* (see above), these considerations emphasize that such squid operate at their functional and environmental limits. This may actually be one explanation of why the species remains rather small (see above).

Similar conclusions can be drawn for *Illex illecebrosus*. The high performance levels of that ommastrephid squid do not just take advantage of the constant environmental conditions of the pelagic, open ocean waters, but are only possible under such conditions. The analysis also suggests that adults of this species operate at their maximum body size because such size may be around the maximum possible that allows extraction of significant quantities of oxygen from the water via the skin. It is possible to speculate that the large body size of giant squid may only be possible under the cold temperatures of the deep sea, when oxygen solubility is high and metabolic energy requirements are reduced. Accordingly, long-term exposure to warm waters may cause asphyxiation and death of such animals (Brix 1983). Maximum body size in squid appears to be the result of a trade-off between maximum energy expenditure required in a certain environment, available strategies to maximize the use of ambient oxygen and the potential development and use of energy-saving strategies without compromising successful competition with pelagic vertebrates.

ENERGY-SAVING STRATEGIES

So far the focus has been on the outstanding performance of squid and the physiological processes involved. How did such processes evolve? Comparison of the way of life and physiology of today's cephalopods would suggest that certain physiological
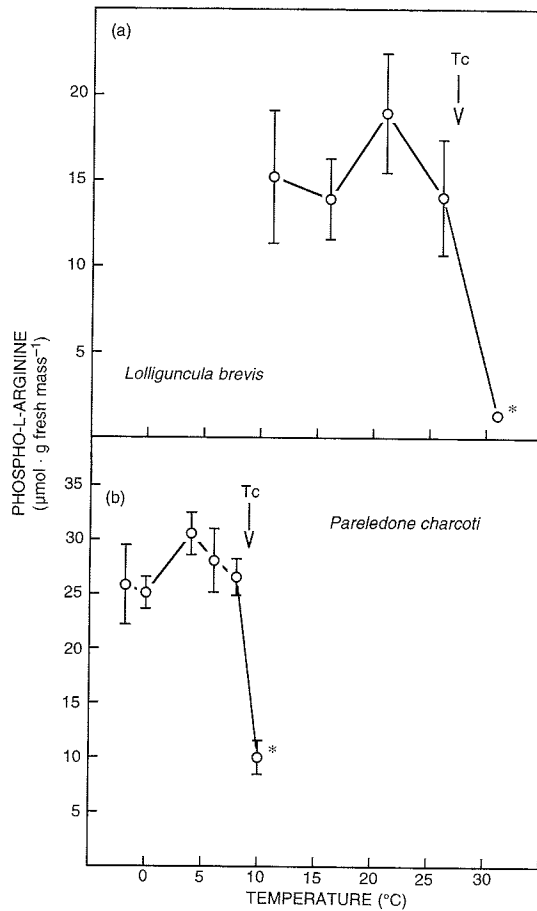
Fig. 7: High critical temperatures T_c in (a) the warm-water squid *Lolliguncula brevis* and (b) the Antarctic octopod *Pareledone charcoti* are characterized by transition to an anaerobic mode of energy production and indicate oxygen limitation of metabolism at high temperatures. The difference between the two species is attributed to the ambient temperature regime in the Gulf of Mexico or the Southern Ocean respectively. Note that the critical temperature in the squid species is much closer to ambient temperature levels than is that of the Antarctic octopod (SZ, HOP, E. Finke and B. Klein, unpublished)

lead a leisurely, energy-saving way of life. This is still true today of *Nautilus* and the open sea species *Spirula* (found to a depth of 1 200 m).

However, other forms developed in the well-lit upper layers of the sea, either in the pelagic or close to the bottom in shallow waters. Under increasing competition and threat of attack from vertebrates after the loss of their shell, they developed the performance characteristics discussed here. The high O_2 and low CO_2 concentrations in the pelagic zone allowed for a maximization of energy turnover and, correspondingly, 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 to maintain their position in the water column. On the other hand, losing the shell is obviously not too great a disadvantage, because they do require a constantly high level of activity and, in relatively shallow water, are able to rest on the sea bed, as observed even in *I. illecebrosus*. Other bottom-dwelling cephalopods either, like the octopus, were no longer dependent on buoyancy mechanisms, or like *Sepia*, simply retained a cuttlebone as a gas-(nitrogen-) filled buoyancy organ.

It has already been shown that there are differences within the squid group. The theory of maximizing energy turnover applies strictly only to the most active forms, e.g. ommastrephids such as *I. illecebrosus*. Many of these squid 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. Loliginids such as *Lolliguncula brevis* reach limits of performance at lower levels in environments characterized by larger fluctuations of abiotic parameters. 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 *I. illecebrosus* does to cover vast stretches of ocean quickly.

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 slow speeds (this is also true for *Sepia*). In such an environment, squid can rest on the sea bed or, like *Loligo forbesi*, make use of upwelling currents to counteract sinking. In that case the fin takes on an additional role as a hydrofoil (O'Dor *et al.* 1994). In comparison to pure jet propulsion, the use of a fin (by *Sepia* and to various degrees by squid) saves energy, because the same physical principles apply as in fish locomotion. The fin, with its undulating beat, bridges the inherent

characteristics developed in the following manner (Wells 1994). The ancestors of today's shell-less species were adapted to an oxygen-poor environment and still had the typical molluscan shell, which provided protection and buoyancy by means of its gas-filled chambers. In fact, the shell made it possible to

pauses in between jets (refilling phase) and smoothes and stabilizes movement. The more developed the fin, the more it is involved in propulsion. However, the degree of attainable thrust is much less than for fish, because 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. Therefore, the fin only saves energy at slow speeds. Both *Sepia officinalis* and *L. pealei* use their fins when swimming slowly, so resulting in a lower energy requirement than in *I. illecebrosus* (see O'Dor and Webber 1991). As the fin increases in size, it increases drag and energy expenditure by hampering the streamlined body shape, the latter being an advantage at high speed (cf. O'Dor and Webber 1991, Hoar *et al.* 1994). When it is of prime importance to reach high speed, as is the case for *I. illecebrosus*, there is only a small fin, which enhances a streamlined body shape. As a jet of water is expelled, the fin is flattened against the mantle, so reducing body drag.

An interesting strategy to save energy was recently described for *Lolliguncula brevis* by Finke *et al.* (1996). As this species lives as a coastal and inshore species in the brackish waters of the Gulf of Mexico, it usually does not need to cover long distances searching for prey or in escape response, a fact reflected in its slow critical swimming speed (see above). At increased velocities, measurements of pressure changes in the mantle cavity failed to reveal an exponential rise in performance levels, as in other squids (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. The small difference between resting and active oxygen consumption (Table I) suggests that this behaviour reduces transport cost and permits a longer term net use of anaerobic resources when speed exceeds the critical value.

However, since the loss of their gas-filled chambers, the utilization of fins or the development of behavioural strategies have not been the only energy-saving approaches. Squid have also mastered the deep sea, where there is not such close competition from vertebrates of similar size. The food density is lower than over continental shelves, and the temperature is also low, factors which may necessitate an economic way of life. As in many regions the deep sea is virtually "bottomless", there is danger for both shelled and continually swimming forms of sinking into deeper water. Pelagic species living in the deep sea have therefore developed an alternative method to achieve neutral buoyancy. In fact they have been able to conquer marine habitats which otherwise would have been impossible with a hard external shell, because rigid, gas-filled cavities cannot indefi-

nitely withstand the pressure of increasing depth.

Some squid store fat in the midgut gland to increase their buoyancy. However, the majority of neutrally buoyant squid (and other denizens of the deep, such as certain prawns; Sanders and 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 shelves (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). 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 *L. pealei* and *I. illecebrosus* respectively (O'Dor and 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 seawater osmolarity has a density of 1.01 kg·ℓ^{-1}. In order to achieve neutral buoyancy, deep-sea squid 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 or guanine instead of ammonia, although this sacrifices a great deal of energy. Research to date has shown that the ammonium chloride solution in cephalopods (but not in prawns) is stored in fluid-filled chambers which are completely separated from the blood. In certain exceptions (e.g. the Cranchiidae), large coelomic cavities containing ammonium chloride are found external to the tissues. Generally, fluid-filled cavities are found within the muscle of the mantle, arms and/or tentacles, although it is not completely clear if they are intra- or extracellular. To maintain the high concentration gradients between the ammonium-containing compartments (up to >500 mmol·ℓ^{-1} have been measured) and the rest of the body fluids, the pH is so low that a high percentage of NH_3 is converted to NH_4^+. 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 amino-acid catabolism in cephalopods plays a part in producing the necessary concentration of ammonium or if the capacity for bicarbonate export contributes to acidification.

The giant squid also has a similar approach to achieve neutral buoyancy, as shown by measurements of ammonium concentration in tissue samples taken from stranded specimens, or on one occasion from a single tentacle caught in a net at 1 000 m (Robison

1989). Owing to vacuolization of the mantle tissue, giant squid have a lower proportion of active muscle than their more agile shallow-water relatives. For this reason, and not only because of the previously discussed size-dependence of oxygen transfer, these giant squid can never attain the same level of activity as smaller more muscular species.

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