The Journal of Experimental Biology **199**, 911–921 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JEB0107

SQUID (*LOLLIGUNCULA BREVIS*) LIFE IN SHALLOW WATERS: OXYGEN LIMITATION OF METABOLISM AND SWIMMING PERFORMANCE

E. FINKE[1,*], H. O. PÖRTNER[1,†], P. G. LEE[2] AND D. M. WEBBER[3]

[1]*Alfred-Wegener-Institute für Polar- und Meeresforschung, Biologie I/Ökophysiologie, Columbusstraße, D-27515 Bremerhaven, Germany,* [2]*Marine Biomedical Institute, University of Texas Medical Branch, Galveston, Texas, USA* and [3]*Biology Department, Dalhousie University, Halifax, Nova Scotia, Canada*

Accepted 29 November 1995

Summary

Squid (*Lolliguncula brevis*) were exercised in a tunnel respirometer during a stepwise increase in water velocity in order to evaluate the anaerobic threshold, i.e. the critical swimming speed above which anaerobic metabolism contributes to energy production. The average anaerobic threshold was found at speeds of 1.5–2 mantle lengths s^{-1}. Above this velocity, α-glycerophosphate, succinate and octopine started to accumulate in the mantle tissue. ATP levels fell and phospho-L-arginine was progressively depleted, while the levels of glucose 6-phosphate and inorganic phosphate rose. The finding of a simultaneous onset of anaerobic metabolism in the cytosol and the mitochondria indicates that a limited oxygen supply to the mitochondria elicits anaerobic energy production. This finding is opposite to the situation found in many other vertebrate and invertebrate species, in which energy requirements in excess of aerobic energy production are covered by anaerobic metabolism, with mitochondria remaining aerobic. In *L. brevis*, swimming at higher speeds is associated with a small factorial increase in metabolic rate based on a high resting rate of oxygen consumption. Pressure recordings in the mantle cavity support this finding, indicating a high basal level of spontaneous activity at rest and a small rise in mean pressure at higher swimming velocity. Bursts of higher pressures from the jet support elevated swimming speeds and may explain the early transition to anaerobic energy production which occurs when pressure amplitudes exceed 1.2–1.5 kPa or when mean pressure rises above 0.22–0.25 kPa.

The finding of mitochondrial hypoxia at a low critical speed in these squid is interpreted to be related to their life in shallow coastal and bay waters, which limits the necessity to maintain high swimming velocities. At increased swimming velocities, the animals oscillate between periods of high and low muscular activity. This behaviour is interpreted to reduce transport cost and to permit a longer-term net use of anaerobic resources when speed exceeds the critical value or when the squid dive into hypoxic waters. The simultaneous onset of anaerobic metabolism in the cytosol and the mitochondria emphasizes that squid generally make maximal use of available oxygen under resting conditions, when their energy requirements are the highest among marine invertebrates.

Key words: anaerobic threshold, critical swimming speed, octopine, phospho-L-arginine, succinate, α-glycerophosphate, performance, pressure, power output, mantle cavity, squid, *Lolliguncula brevis.*

Introduction

Squid rely on jet propulsion for locomotion. The power of the jet depends largely upon the contraction of the obliquely striated circular mantle muscles. Squid mantle exhibits a spatial division into anaerobic and aerobic muscle layers, with differences in the degree of vascularisation, density of mitochondria and enzymatic organisation (Bone *et al.* 1981; Mommsen *et al.* 1981). On the basis of studies of the biomechanics and electrophysiology of contraction (Gosline *et al.* 1983), the current view is that the peripheral aerobic mantle layers are used during slow aerobic swimming and that the central anaerobic cells of the mantle are involved mainly in generating powerful jets to attain maximum swimming speeds during escape or during hunting of pelagic prey. Escape jets are fuelled by anaerobic metabolism. In some squid species, octopine builds up in large quantities as an end-product of anaerobic metabolism during muscular fatigue (Grieshaber and Gäde, 1976; Pörtner *et al.* 1991). Phospho-L-arginine (PLA) depletion buffers the ATP pool, but during intense muscular activity ATP is broken down, leading to the accumulation of ADP, AMP and inorganic phosphate, although *in vivo*

*Present address: Institut für Biologiedidaktik, Justus-Liebig-Universität, Karl-Glöckner-Straße 21, D-35394 Giessen, Germany.
†Author for correspondence.

degradation of the adenylates beyond AMP does not occur (Pörtner *et al.* 1993).

Cephalopods are evolutionarily constrained in that they possess a respiratory pigment which does not allow them to carry as much oxygen in their blood as does the blood of fish. A previous analysis suggests that ommastrephid squid not only make maximum use of their haemocyanin even under control conditions but also that they rely on oxygen uptake *via* the skin, especially the skin of the mantle (Pörtner, 1994). For maximal use of the available oxygen, cephalopods catabolize carbohydrates and amino acids, fuels that are more oxygen-efficient than some others, i.e. they require less oxygen per mole of generated ATP than do lipids (Hochachka, 1994). Considering these adaptations and the high rates of oxygen consumption that prevail even under control conditions, the question arises to what extent squid are able to increase their energy requirements without starting to make use of anaerobic processes. To answer this question, we investigated the activity levels and the mode of metabolism in freely swimming, unrestrained squid *Lolliguncula brevis* at different swimming speeds. This species lives in the brackish waters of the Gulf of Mexico and, as a coastal and inshore species, does not usually travel over long distances. Compared with the larger squid living further offshore and on the continental shelf (e.g. *Loligo pealei* and *Illex illecebrosus*), it is characterized by a small body size, a feature possibly related to its life in shallow waters. In loliginids, the fins are well developed for slow aerobic cruising (see Wells, 1994) and for fine locomotory control in a complex environment. These abilities should make *Lolliguncula brevis* an ideal species in which to investigate metabolism over a wide range of swimming speeds. The formation of anaerobic metabolites in the mitochondria and in the cytosol of the muscle cells would reveal whether the use of anaerobic metabolism is an early result of an increase in muscular activity.

Materials and methods

Experimental animals

Brief squid (*Lolliguncula brevis* Blainville, 6.6–34.2 g), were caught in April and May 1993 and in March 1995 in the Galveston Ship Channel and Galveston Harbour by the fishermen of the Marine Biomedical Institute of the University of Texas, Galveston, Texas, USA, and kept in aquaria with recirculating natural sea water under conditions similar to those in the natural habitat (24–26 ‰ salinity, at 20–22 °C). The squid were allowed to adjust to conditions in the aquarium for at least 24 h. The animals were fed small fish and penaeid or palaemonid shrimp, except during the last 24 h before experimentation.

Control animals

For the collection of control samples, each individual animal was placed into a darkened aquarium containing 4 l of continuously aerated sea water (25±1 ‰ salinity, 20±1 °C). After 60–100 min of recovery from handling, ethanol (120 ml) was fed into the aquarium through tubing placed close to the aeration stone to ensure rapid and full mixing with the sea water. The final maximum concentration of the anaesthetic was 3 vols%. Anaesthesia was complete after 2–4 min, as indicated by the cessation of ventilatory activity.

Muscle samples were obtained using a parallel arrangement of scalpel blades as described by Pörtner *et al.* (1991). The tissue samples were freeze-clamped immediately, weighed rapidly, wrapped in aluminium foil and stored under liquid nitrogen until analyzed. Additionally, the remaining portions of each specimen were weighed to determine the fresh mass of the whole animal, and the mantle length was measured.

Exercised squid

Squid were exercised in a Brett-type respirometer (Brett, 1964) filled with 17.76 l of normoxic sea water at 20±1 °C, the removable animal chamber (tube diameter 10.2 cm) being placed in an additional volume of fresh aerated sea water at the same temperature. After 30 min of acclimation at a water velocity of 3.0 cm s^{-1}, the respirometer was closed and the decrease in oxygen levels was monitored using an oxygen electrode (Endeco type 1128 pulsed dissolved oxygen probe; Endeco type 1125 pulsed dissolved oxygen controller; Endeco, Inc., Marion, MA, USA) integrated into the closed water circuit. After a measurement period of at least 15 min, the respirometer was opened for about 10 min so that the sea water in the tunnel system could be replaced by fully aerated sea water from the waterbath and the animal could recover from the previous level of exercise. The respirometer was then closed again and oxygen consumption was measured at a higher swimming speed. The water flow was increased up to 6.0, 9.0, 12.0, 15.0 or 18.0 cm s^{-1}. For the subsequent reoxygenation and recovery period, the velocity of the water was again decreased to 3.0 cm s^{-1}. Finally, the squid was removed from the respirometer for the determination of body mass and mantle length.

The validity of measurements and the tightness of the respirometer for gases were tested by filling the respirometer system with water low in oxygen content and verifying the maintenance of oxygen levels at different water velocities. Blanks were run without animals to correct for bacterial oxygen consumption and for the endogenous oxygen consumption of the electrode.

For metabolite analyses, squid were exercised in the open respirometer such that the water remained fully saturated with air at all times. After an acclimation period of 30 min at 3.0 cm s^{-1}, a stepwise increase in water velocity by 3.0 cm s^{-1} every 6 min was used to achieve the various final swimming speeds. At the highest velocities, chosen to be slightly submaximal but still fatiguing, the onset of fatigue became obvious when the squid repeatedly touched the downstream grid of the swimming chamber with the tip of their arms or finally collapsed from exhaustion. In order to compare non-steady-state metabolic changes at different swimming speeds, the duration of this exercise protocol was adopted as a basis for subsequent experiments and further animals were exercised for a total period

of 39±1 min at lower water velocities (see Table 1). Swimming speeds were normalized in mantle lengths s^{-1}.

At the end of the exercise period, the animal chamber was separated from the rest of the system and the experimental animals were rapidly transferred into sea water at the same temperature containing 1.5 or 3.0 vols% of ethanol. Animals responding to this procedure with vigorous jetting were excluded from the analyses. Anaesthesia was complete after 20–120 s depending on the level of exercise. Tissue samples were taken as described above.

The performance level of the animals was monitored by recording pressure changes in the mantle cavity using a cannulation technique similar to the one described by Webber and O'Dor (1986). Squid were briefly anaesthetized in a 1:1 mixture of sea water with a $MgCl_2$ solution of the same osmolarity (Messenger *et al.* 1985). 4 cm of large PE tubing (Portex tubing i.d. 0.86, o.d. 1.52 mm) was flared at one end to form a cannula and bent at 90 ° about 1 cm from the tip. A piece of silicone tubing less than 0.5 mm long was slipped over this cannula and helped to secure the flared tubing in place when fed from the cavity through the mantle musculature about 1.5 cm from the mantle edge and at an angle of 45–60 ° to the ventral mantle. The small size of the animals required that small PE tubing (i.d. 0.58, o.d. 0.96 mm) was fed into the larger tubing and stretched to increase flexibility so that the animals were completely unrestrained during swimming. The cannula was connected to a pressure transducer (UFI type 1050, Morro Bay, CA, USA) and a MacLab system (ADI Instruments, Hastings, UK; WissTech, Spechbach, Germany) for pressure recordings and subsequent data evaluation.

Analyses

Tissue analyses were carried out at the Alfred-Wegener-Institute, Bremerhaven, Germany. The muscle tissue samples were ground under liquid nitrogen using a mortar and pestle. Immediately after grinding, part of the tissue powder was used to evaluate intracellular pH and P_{CO_2} (Pörtner *et al.* 1990; H. O. Pörtner, E. Finke and P. G. Lee, in preparation). The remaining tissue powder was extracted in perchloric acid (Beis and Newsholme, 1975, modified according to Pette and Reichmann, 1982), and the concentrations of several metabolites in the pH-neutralized extracts were determined using enzymatic methods. Inorganic phosphate was estimated according to Pörtner (1990). Octopine, phospho-L-arginine and L-arginine were determined following Grieshaber *et al.* (1978). Octopine dehydrogenase was prepared and purified for these determinations from the adductor muscles of *Pecten maximus* following the procedure described by Gäde and Carlsson (1984). ATP, ADP, AMP, glucose 6-phosphate, glycerol 3-phosphate (=α-glycerophosphate) and succinate were assayed according to Bergmeyer *et al.* (1986).

The levels of free ADP and AMP were calculated on the basis of the equilibrium of arginine kinase and myokinase. Equilibrium constants for both enzymes were corrected for experimental temperature and pH-dependence as related to the changing proton and magnesium binding of the adenylates and the proton turnover of the arginine kinase reaction (Pörtner *et al.* 1993, details given by H. O. Pörtner, E. Finke and P. G. Lee, in preparation).

Up to third-order polynomial regressions were calculated for the changes in metabolite concentrations with increasing swimming speed (Sigmaplot, Jandel Scientific). Significance of changes was evaluated at the 5 % level by the determination of correlation coefficients and by using an *F*-test following an analysis of variance (ANOVA) using Statview II (Abacus Concepts). Solid lines in the graphs indicate significant effects of experimental treatment and significant correlations, with broken lines depicting the 95 % confidence interval. In addition, data for control animals and squid exercised at supracritical swimming speeds were compared using Student's *t*-test for unpaired samples. Values differing significantly from the norm (Pearson and Hartley's and Nalimov's tests) were eliminated from the data set.

Results

The polynomial fit of all oxygen consumption values shows a significant, almost exponential rise in oxygen consumption with increasing swimming speed (Fig. 1). Between 0.5 and 2.9 mantle lengths s^{-1} average oxygen consumption rose from 21 to 36 μmol O_2 h^{-1} g^{-1} body mass. Fig. 1 also quantifies the gross cost of transport in terms of ATP turnover per mantle length (as a normalized measure of distance travelled by the animals). A swimming velocity characterized by minimum cost becomes apparent only when the contribution of anaerobic ATP production is considered. This optimum speed is close, or equivalent, to the critical swimming speed (see below).

The pattern of pressure pulses generated in the mantle cavity changed with increasing swimming velocity (Figs 2–4). Fig. 2 demonstrates for one animal that, over a small range of velocities, differences in height between individual pressure peaks were small. At 0.5 mantle lengths s^{-1}, water velocity was so low that mantle contractions were almost exclusively determined by the level of spontaneous activity, with some regular oscillations occurring between lower and higher pressures. Very rarely, squid were seen in a resting ('sitting') posture, tentacles bent towards the bottom of the swimming tube. The jetting pattern became more regular when velocity increased from 0.5 to 1.1 mantle lengths s^{-1}. When velocity reached 2.1 mantle lengths s^{-1} and above, baseline pressures were maintained but jets characterized by larger pressure changes appeared at more or less regular intervals. The amplitude of these pressure changes increased as swimming velocity increased up to 4.3 mantle lengths s^{-1}, but the oscillation between low- and high-pressure jets was maintained. When the water speed was decreased to 0.5 mantle lengths s^{-1} at the end of the experiment, the respective recording indicates that the level of spontaneous activity was reduced during the initial period of recovery (Fig. 3). Overall, however, the variability in behaviour and pressure generation between animals was rather high, as shown by the data depicted in Fig. 4. Mean pressure increased in a linear fashion with increasing swimming speed. Jetting frequency increased

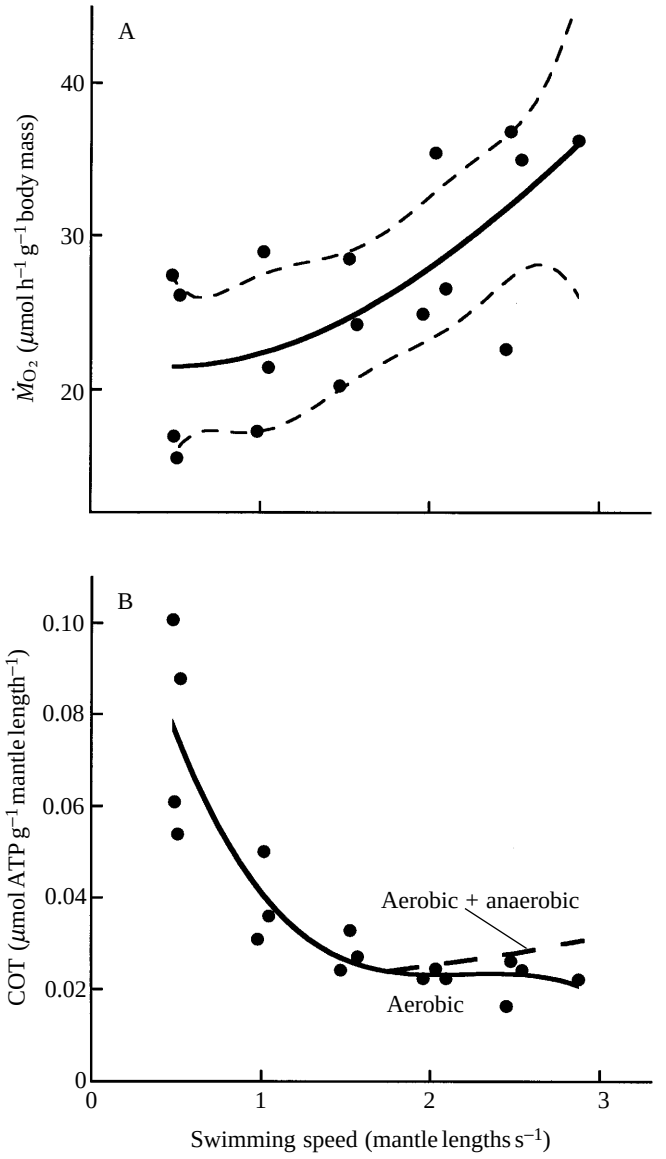

Fig. 1. (A) Rate of oxygen consumption measured in four squid *Lolliguncula brevis* (body mass 11.9–15.1 g) at different swimming speeds. Owing to the rather high variability in individual responses, data have been grouped and a third-order regression was calculated for all data points (r=0.69). The broken lines in this and all the following diagrams represent the 95 % confidence interval. (B) The effect of swimming velocity on gross cost of transport (COT, determined as the rate of aerobic + anaerobic ATP turnover, r=0.90, third-order regression). The anaerobic contribution to energy production is considered at higher swimming speeds.

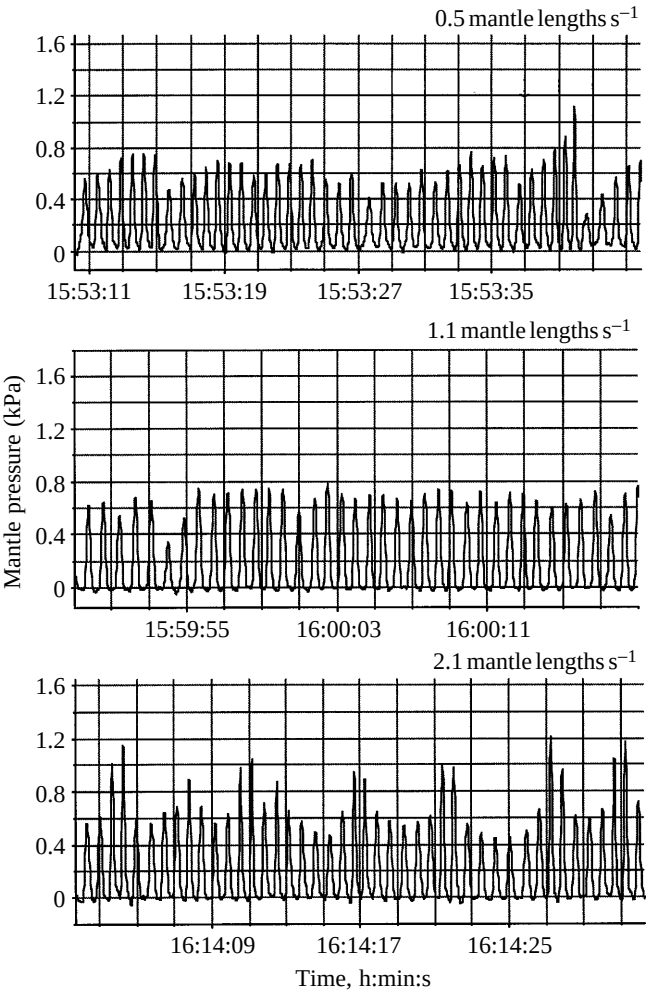

Fig. 2. Mantle pressure recordings at low swimming velocities for one squid. Pressure traces are typical examples for the respective swimming speeds given in mantle lengths s^{-1}.

only slightly and insignificantly. However, the linear increase in the amplitude of high-pressure jets with swimming speed reflects the observed rise in maximum pressure changes with rising swimming velocity (Fig. 3).

Changes in octopine, succinate and α-glycerophosphate levels indicate whether, and at what swimming speed, cytosolic (octopine formation) and mitochondrial (succinate formation) mechanisms of anaerobic energy metabolism become involved in fuelling activity. They also indicate how the transport of reducing equivalents from the cytosol into the mitochondria *via* the α-glycerophosphate shuttle was maintained (Fig. 5). All three metabolites started to accumulate simultaneously at swimming speeds above 1.5–2.0 mantle lengths s^{-1}. Octopine and succinate accumulated progressively, whereas the α-glycerophosphate concentration remained at a constant high level at swimmings speeds above 3–3.5 mantle lengths s^{-1}.

With the onset of octopine formation, the concentration of glucose 6-phosphate as an intermediate product of glycolysis rose. Phospho-L-arginine levels decreased as swimming speed increased, as can also be seen from the decline in the ratio of [phospho-L-arginine] to the sum of [phospho-L-arginine] and [L arginine] (Fig. 6). The concentration of free inorganic phosphate in the mantle tissue rose almost linearly with increasing swimming speed (by 18.6 μmol g^{-1} fresh mass between 0 and 4.7 mantle lengths s^{-1}), while the concentration of phospho-L-arginine dropped by an equivalent amount (17.7 μmol g^{-1} fresh mass) over the same range of velocity. The existence of a critical speed was not apparent from PLA or

inorganic phosphate levels because there was some change in the levels of these metabolites with the onset of activity. However, some change will occur even at low swimming speeds owing to the increased work load of the aerobic muscle. Using the ratio of [phospho-L-arginine] to the sum of [phospho-L-arginine] and [L-arginine], which corrects for some of the high variability in PLA contents (13.4–25.3 μmol PLA g^{-1} fresh mass under control conditions, mirrored by the variability in L-arginine concentrations), an early fall in phospho-L-arginine concentration is confirmed. Also, a smaller ratio of [phosphagen] to [phosphagen] plus [aphosphagen] is usually seen in aerobic tissues exhibiting a higher ATP turnover. These considerations explain why changes in the phosphagen concentrations are not suitable indicators of the onset of functional hypoxia and, thus, the critical swimming speed.

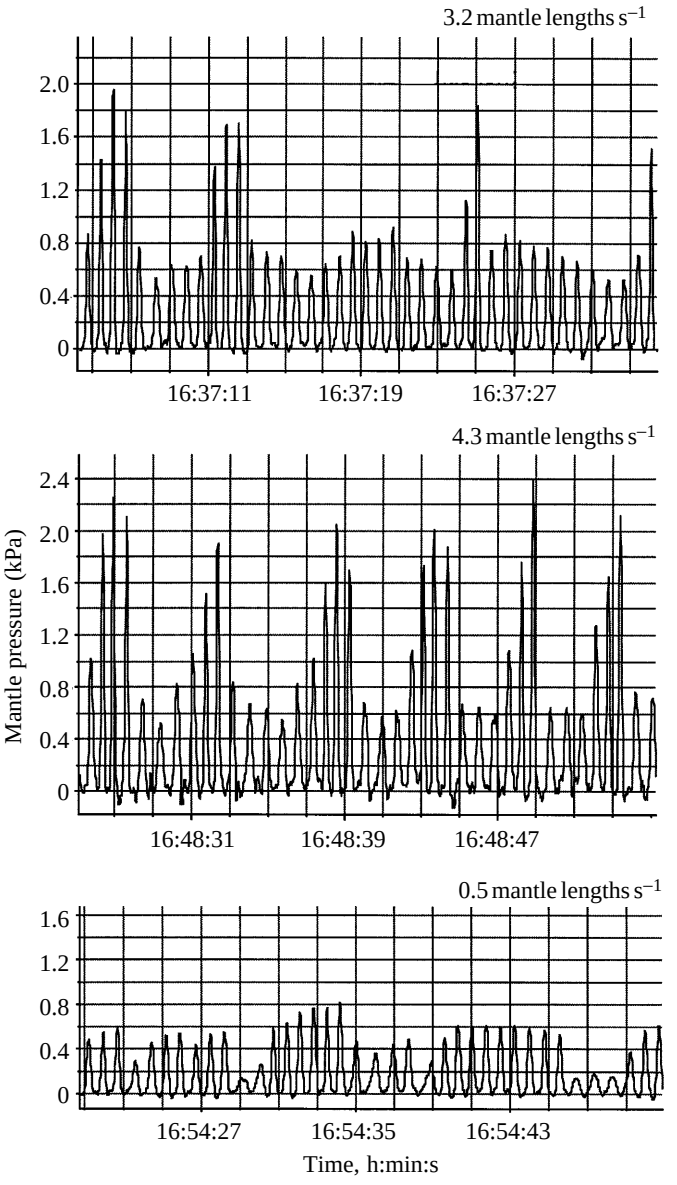

Fig. 3. Pressure recordings at higher swimming velocities (given in mantle lengths s^{-1}) for one squid (cf. Fig. 2).

ATP levels remained constant up to the critical swimming velocity and started to fall thereafter (Fig. 7). ADP concentrations increased slightly, but significantly, as swimming speed increased; however, most of the decrease in ATP concentration was reflected by a concomitant rise in total AMP levels. The changes in free ADP and free AMP levels were smaller than the mean concentration changes for the total levels. There was no significant correlation between swimming velocity and the summed concentrations of all adenylates, the sum of the concentrations of L-arginine metabolites and the sum of the

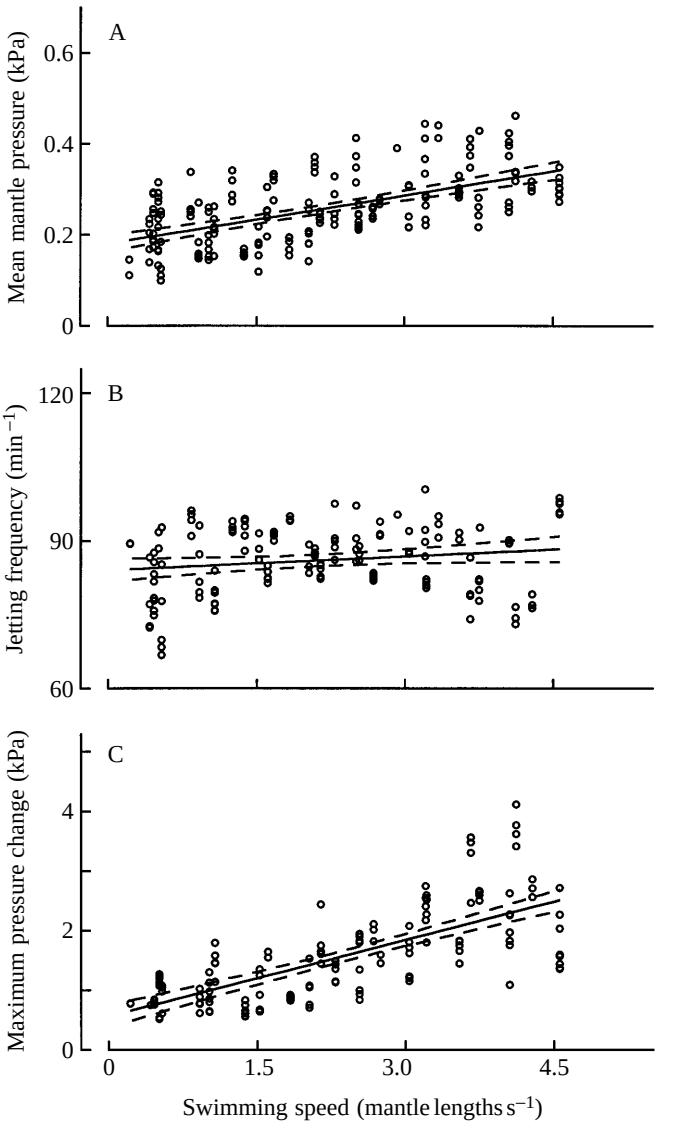

Fig. 4. Effect of swimming velocity on (A) mean mantle pressures (kPa, r=0.59, linear regression), (B) jetting frequency (min^{-1}, r=0.16, linear regression) and (C) maximum pressure changes (r=0.71, linear regression) for five squid (body mass 13.4–20.7 g) grouped together. At each swimming speed (given in mantle lengths s^{-1}), data were evaluated for several time periods indicated by stacked data points. Occasional extreme jets not related to the respective swimming velocity were omitted from the analyses. The grouped data reflect the variability in performance levels found for each individual and among individuals.

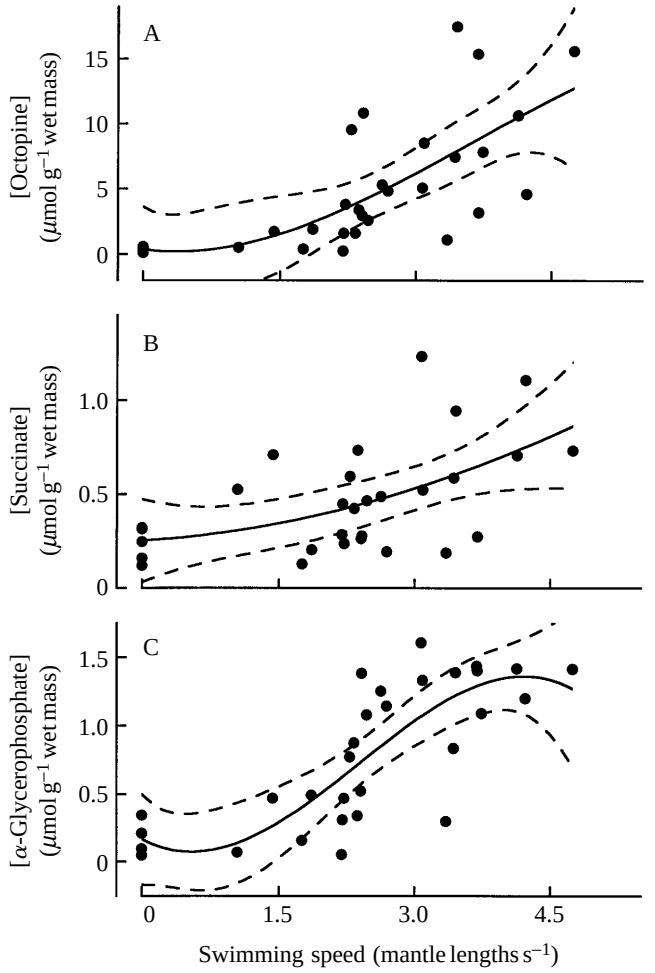

Fig. 5. Effect of swimming velocity on (A) octopine concentration (r=0.71, third-order regression), (B) succinate concentration (r=0.55, second-order regression) and (C) α-glycerophosphate concentration (r=0.80, third-order regression) in the mantle musculature of the squid *Lolliguncula brevis*.

Table 1. *Comparison of body mass, mantle length and metabolite concentrations in the mantle musculature of rested and exercised squid* Lolliguncula brevis

Variable	Control (N=5)	Exercised (N=10)
Body mass (g)	9.43±0.89	9.80±2.66
Mantle length (cm)	5.08±0.37	5.26±0.61
Maximum swimming speed (cm s^{-1})	3	18.7±1.1
Metabolite concentrations (μmol g^{-1} wet mass)		
[ATP]	4.62±0.66	2.81±0.75*
$[ADP]_{total}$	1.75±0.63	2.75±1.29
$[ADP]_{free}$	0.13±0.04	0.35±0.22
$[AMP]_{total}$	0.15±0.14	0.70±0.47*
$[AMP]_{free}$	0.005±0.004	0.06±0.06
[ATP] + [ADP] + [AMP]	6.51±0.88	6.25±1.47
[Phospho-L-arginine]	20.25±5.81	4.26±3.55*
[Inorganic phosphate]	7.07±2.27	22.24±6.03*
[PLA]/([L-Arg] + [PLA])	0.70±0.08	0.26±0.16*
[L-arginine]	8.69±4.52	11.47±4.90
[Glucose 6-phosphate]	0.08±0.05	0.17±0.09*
[Octopine]	0.43±0.18	9.59±5.04*
Σ[Arg]	29.37±8.51	25.21±4.28
[α-glycerophosphate]	0.13±0.15	1.31±0.22*
[Succinate]	0.23±0.09	1.18±0.95* (N=8)

Exercised animals are those subjected to swimming velocities above 3 mantle lengths s^{-1}, a range of swimming speeds reached after about 24 min and maintained for a further 15 min.

Values are means ± S.D.

*Significantly different from the control value, P<0.05; Student's t-test.

PLA, phospho-L-arginine; Σ[Arg], [phospho-L-arginine] + [L-arginine] + [octopine].

concentrations of inorganic and organic phosphates (Fig. 8), thus confirming previous findings (Pörtner *et al.* 1991, 1993) that all the metabolic processes investigated took place exclusively in the mantle tissue and did not involve a significant exchange between the mantle cells and other body compartments.

Tabular comparisons of values for rested and exercised squid showed that there were significant differences between the mean concentrations of ATP, total AMP, inorganic phosphate, phospho-L-arginine, glucose 6-phosphate, octopine, α-glycerophosphate, succinate and the ratio of [phospho-L-arginine] to the sum of [phospho-L-arginine] and [L-arginine] (Table 1). However, a comparison of the mean values for the two groups does not reflect the extreme changes seen in individual animals (see figures).

Discussion

Performance levels and oxygen consumption

Previous investigations of the metabolism of squid during exercise have focused on the extreme changes occurring during muscular fatigue and recovery (e.g. Grieshaber and Gäde, 1976; Pörtner *et al.* 1991, 1993). We report here the first data set on tissue metabolic status of unrestrained squid exercised at different swimming speeds. The highest swimming speed reached in the experiments was 4.7 mantle lengths s^{-1}, corresponding to 22.3 cm s^{-1}. This value is slightly above the 16 cm s^{-1} previously reported for the same species prior to rapid exhaustion (Wells *et al.* 1988). For comparison with previous studies, it is important to emphasize that the present data have been collected for a squid species with a life style distinctly different from that of the larger muscular squid living in the open pelagic conditions of coastal and shelf waters. *Lolliguncula brevis* is typically found in shallow waters, where it does not need to travel long distances during its search for prey or during escape. Life in this special environment may therefore be one reason for the swimming and jetting characteristics of *L. brevis*. Comparative data are available for *Illex illecebrosus* and some *Loligo* species (*L. opalescens*, *L.*

pealei and *L. forbesi*, O'Dor, 1982, 1988; O'Dor and Webber, 1986, 1991; O'Dor *et al.* 1994). Even if the present data are compared with those for a squid of similar size, such as *Loligo opalescens*, it becomes evident that there was a smaller increase in mean mantle pressure and jetting frequency with increased swimming speed in *L. brevis* and that the exponential increase in mean pressure with swimming speed seen in other squid species (e.g. Webber and O'Dor, 1986) was not observed (see below). In addition, *L. brevis* did not show a general rise in pressure for each individual jet. Instead, it used high-pressure jets on top of baseline low-pressure contractions in order to generate the appropriate thrust at higher swimming speeds (Fig. 3). However, since the power required for locomotion in water rises exponentially with swimming speed, an exponential increase in mean pressure would have been expected, as described by O'Dor and Webber (1986) and O'Dor (1988). Owing to the observed oscillation between low- and high-pressure jets, such an exponential rise in performance levels was not evident. Mechanisms such as changes in funnel radius may contribute to a reduction of the expected increase in mean pressure (see O'Dor, 1988).

Baseline pressure jets observed at low water velocities are fuelled completely by aerobic mechanisms and reflect the high level of spontaneous activity of the animals under what would otherwise be called resting conditions. The rates of oxygen consumption found under these conditions were similar to those measured by Segawa and Hanlon (1988) and Wells *et al.* (1988), taking into account the appropriate Q_{10} values and the body masses of the animals. Spontaneous activity in squid is present at most times and involves slow cruising activity, which helps to maintain the negatively buoyant animals in the water column. Pressure amplitudes actually fell in some animals between 0.5 and 1.1 mantle lengths s^{-1}, indicating that

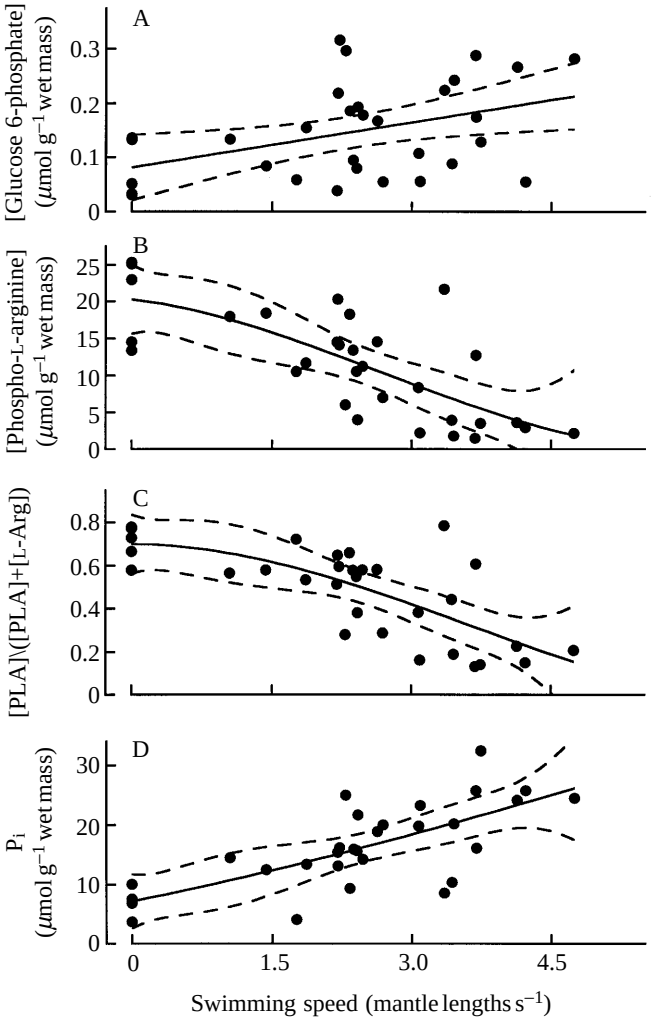

Fig. 6. (A) Glucose 6-phosphate levels (r=0.43, linear regression), (B) phospho-L-arginine content (r=0.73, third-order regression), (C) the ratio of [phospho-L-arginine] ([PLA]) to the sum of [phospho-L-arginine] and [L-arginine] ([L-Arg]) (r=0.72, third-order regression) and (D) inorganic phosphate levels (P_i) (r=0.73, third-order regression) in the mantle musculature of the squid *Lolliguncula brevis* at different swimming speeds.

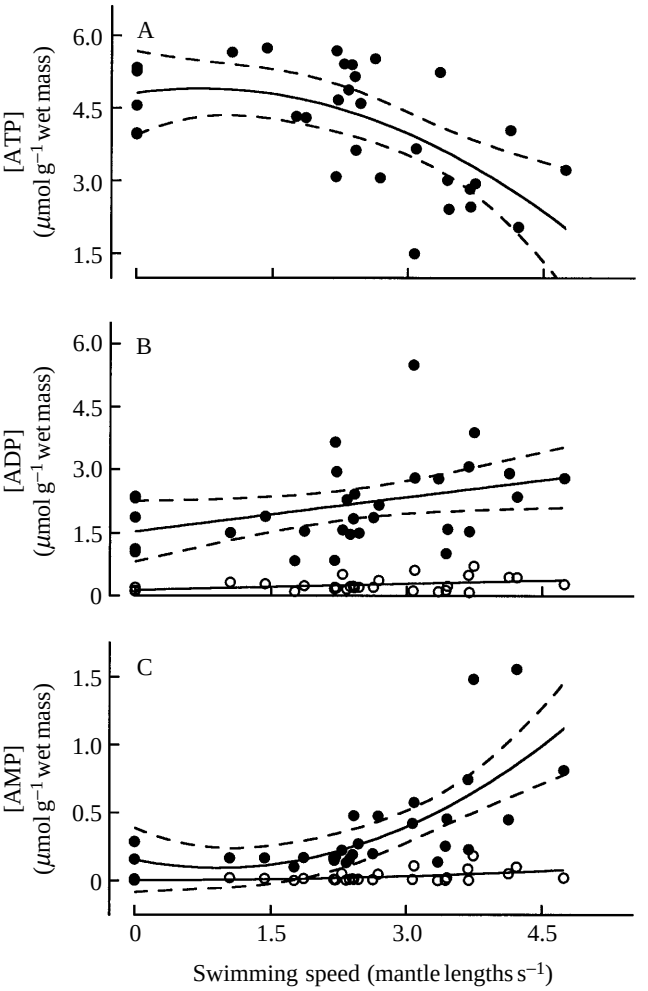

Fig. 7. Levels of (A) ATP (r=0.60, second-order regression), (B) total (●) and free (○) ADP (total, r=0.36; free, r=0.42; linear regressions) and (C) total and free AMP (total, r=0.70; free, r=0.47; second-order regressions) in the mantle musculature of the squid *Lolliguncula brevis* at different swimming speeds.

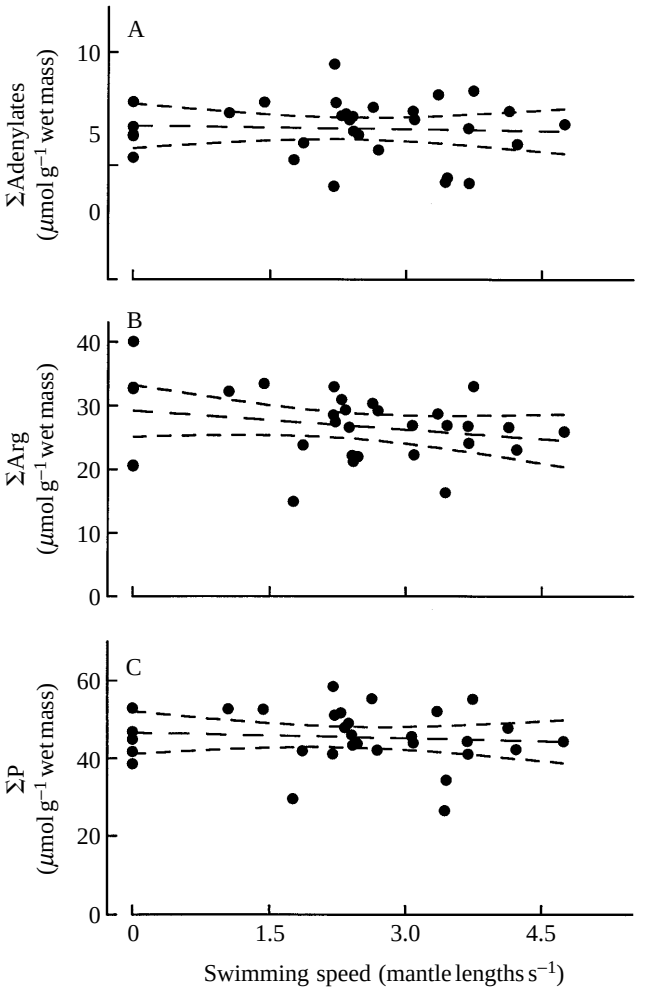

Fig. 8. Summed concentrations of (A) all adenylates (∑Adenyates = [ATP] + [ADP] + [AMP], r=0.05, linear regression), (B) L-arginine metabolites (∑Arg = [phospho-L-arginine] + [L-arginine] + [octopine], r=0.24, linear regression) and (C) inorganic and organic phosphates (∑P = [phospho-L-arginine] + 3[ATP] + 2[ADP] + [AMP] + [glucose 6-phosphate] + [α-glycerophosphate] + [P_i]; r=0.09, linear regression) in the mantle musculature of the squid *Lolliguncula brevis* at different swimming speeds.

the rising water current helped the animals to maintain their position in the water column. This is also in line with the data reviewed by O'Dor and Webber (1986), who found that the true resting metabolic rate was about 6.5 % below the standard metabolic rate (the latter was obtained by extrapolating data for swimming animals to zero activity). The factorial scope of aerobic metabolism in *L. brevis* appears to be very small (Fig. 1), even taking into account that, owing to high standard metabolic rates, this scope is generally smaller in squid than in fish (O'Dor and Webber, 1986, 1991). However, this finding can be explained for *L. brevis* in the context of a small factorial rise in mean mantle cavity pressure with faster swimming speed and the observation that faster speeds are largely achieved by oscillations between low- and high-pressure peaks. The modest rise in the rate of oxygen consumption indicates that this mode of activity may be more economical in the complex shallow-water environment where this species may oscillate between short-term acceleration and deceleration. The question arises of whether the high-pressure peaks appearing at higher speeds are still fuelled by aerobic metabolism.

Critical swimming speed

In our invasive studies of mantle metabolism, we cannot determine whether changes in metabolite concentrations occur exclusively in the central anaerobic layers or whether the peripheral aerobic layers are also involved. Nonetheless, the mean values presented here allow us to evaluate whether an average steady-state or non-steady-state mode of metabolism prevails for mantle mitochondria and cytosol when oxygen supply may change depending on the activity level of the animal.

The changes in octopine, succinate and α-glycerophosphate concentrations (Fig. 5) suggest that anaerobic metabolism becomes involved when mean mantle cavity pressures rise above a critical value of 0.22–0.25 kPa or when the maximum pressure changes increase beyond 1.2–1.5 kPa (Fig. 4). The onset and use of anaerobic metabolism is therefore associated with the increasing appearance of high-pressure jets. The variability in activity levels observed at different swimming speeds was associated with the observed variability in metabolite levels and oxygen consumption (Figs 1, 4, 5). Actually, although regression analysis suggests that the mean critical swimming velocity is at or slightly above 1.5 mantle lengths s^{-1}, the data sets for individual animals reveal that, for some specimens, the critical velocity may well be closer to 3 mantle lengths s^{-1}. A comparison of pressure recordings and metabolite analyses suggests that the individual critical swimming speed strongly depends on when that individual starts to use individual stronger jets during swimming; this may depend on the fitness of the individual (H. O. Pörtner, E. Finke, P. G. Lee, in preparation) but also to some extent on how quickly the animal adjusts to the experimental situation.

The onset of octopine formation indicates that reoxidation of NADH is occurring in the cytosol and represents glycolytic ATP production. The rising glucose 6-phosphate concentration (Fig. 6) reflects the increasing glycolytic activity with increasing swimming speed. The prevailing view of functional hypoxia is that energy requirements during exercise may exceed ATP production by mitochondria and, thus, cause anaerobic cytosolic mechanisms to produce complementary ATP, with the mitochondria remaining fully aerobic (Grieshaber *et al.* 1994). However, this is not the case in *L. brevis*. The accumulation of α-glycerophosphate indicates a reductive change in the cytosolic [NADH]/[NAD^+] ratio and either inadequate functioning of the α-glycerophosphate shuttle in the transport of reducing equivalents into the mitochondria for final oxidation or a lack of oxygen for reoxidation of accumulating NADH. Final evidence that the mitochondria become hypoxic comes from the observation that

succinate accumulates. In the context of the small factorial rise in the rate of oxygen consumption, the rise in succinate levels cannot be explained by an accumulation of tricarboxylic acid cycle intermediates. Instead, it indicates that the flux through succinate dehydrogenase becomes limiting owing to an inadequate oxygen supply. Succinate is a typical end-product of long-term anaerobic metabolism in mitochondria (Grieshaber *et al.* 1994). Therefore, in *L. brevis*, hypoxia in mitochondria triggers anaerobic energy production in both the mitochondria and the cytosol above the critical swimming speed. Since the supply of oxygen to mitochondria will then be the limiting process, this finding also supports our previous conclusions of maximal use of oxygen resources in squid even under control conditions (Pörtner, 1994).

Our analysis of a critical swimming speed differs quite substantially from the original definition by Brett (1964) in that it focuses on the biochemical events indicating a transition to a non-steady-state, time-limited mode of activity metabolism, whereas Brett focused on the actual swimming performance and its sustainability. The database for fish is too limited to allow us to conclude whether the two approaches should give similar results. However, the recent finding of different anaerobic thresholds in two fish species acting as sit-and-wait *versus* active foragers (Goolish, 1991) strongly supports our present concept that the onset of net anaerobic energy production reflects both the critical swimming speed and the beginning of fatiguing exercise. Comparative data for other squid species are not available at present. Assuming the equivalence of the two concepts, critical swimming speeds for *Illex illecebrosus* and *Loligo pealei* would be approximately 3 and 2.5 mantle lengths s^{-1} (Webber and O'Dor, 1986; O'Dor and Webber, 1991) compared with 1.5–2 mantle lengths s^{-1} in *L. brevis*, indicating that the normalized critical swimming velocity is likely to be higher in pelagic open-ocean squid than in inshore species. The optimum swimming speed at minimum cost of transport is close to the critical swimming speed and only becomes apparent when anaerobic energy production is quantified (Fig. 1). This finding confirms that the aerobic scope for activity is rather small in these animals.

Metabolic differences between squid species

Both *Illex illecebrosus* and *Lolliguncula brevis* form considerable amounts of octopine during muscular fatigue, which is not the case for the coastal squid *Loligo pealei*. The highest level observed were 15 μmol g^{-1} wet mass in the mantle tissue of *L. brevis* compared with 3 μmol g^{-1} wet mass in *L. pealei* and 25 μmol g^{-1} wet mass in *I. illecebrosus* (see Pörtner *et al.* 1993). During exercise at speeds above the critical speed in *L. brevis*, the ATP pool was progressively diminished while the total ADP content increased and the AMP content rose to even higher levels. As a consequence of insufficient glycolytic activity in *L. pealei*, ATP depletion occurred to a larger extent and total [ADP] rose to a high and similar level to total [AMP]. Although the percentage depletion of ATP in *Illex* was similar to that in *Lolliguncula* and smaller than that in *Loligo*, our previous study had shown a considerable rise in free ADP and AMP levels for both *I. illecebrosus* and *L. pealei* during exercise. In *L. brevis*, the increases in free ADP and AMP levels were comparatively small. The slopes of the regressions for free [ADP] and for free [AMP] remained below those for total [ADP] and [AMP] (Fig. 7). Obviously, free levels of ADP and AMP were buffered in *Lolliguncula brevis*. A buffering of free ADP levels would delay a drop in the energy content of the ATP system (Gibb's free energy change of ATP hydrolysis, H. O. Pörtner, E. Finke, P. G. Lee, in preparation) and indicates a better resistance to functional or environmental stresses in *L. brevis*.

Phospho-L-arginine (PLA) levels in *Lolliguncula brevis* mantle were only about half of those found in the mantle muscle of *Illex illecebrosis* or *Loligo pealei*, reflecting a lower ATP turnover rate during anaerobic swimming. As a consequence, inorganic phosphate levels only reached half of those observed in *Illex* or *Loligo* mantle during muscular fatigue, when phospho-L-arginine was used to buffer the ATP pool. This would limit the drop in ATP free energy levels and may avoid the harmful effect of inorganic phosphate on muscle function in a species which could more often rely on anaerobic resources, although at a lower rate and scope than those of *Illex*, an observation which is also reflected by the oscillation between low- and high-pressure jets (see above). For comparison between *L. pealei* and *L. brevis*, α-glycerophosphate was not seen to accumulate during fatigue in *L. pealei*, probably because the glycolytic rate remains so low in this species (Storey and Storey, 1978; Pörtner *et al.* 1993). However, succinate levels, which remained unchanged during fatigue from short-term burst swimming (Storey and Storey, 1978), rose substantially in *L. pealei* during vigorous exercise in a swim tunnel (Pörtner *et al.* 1993), indicating mitochondrial hypoxia.

In an overall balance of aerobic and anaerobic ATP production in *L. brevis*, the amount of ATP generated by anaerobic metabolism (using the relevant stoichiometries; Pörtner *et al.* 1984) can be compared with the amount of ATP resynthesized aerobically. Considering a swimming period of 33 min at a speed above the critical swimming speed and a stepwise increase in water velocity, it can be estimated that 14.4 % of the energy required for swimming originated from anaerobic mantle metabolism at 2.5 mantle lengths s^{-1} compared with 21.9 % at 3.5 mantle lengths s^{-1}. These values demonstrate that anaerobic processes significantly support swimming at higher velocities in *L. brevis*.

As a corollary, the ability of *L. brevis* to build up a large oxygen debt distinguishes this species from *L. pealei*, although both species live in coastal waters. Previously, we discussed how the limited anaerobic scope of *L. pealei* was related to its life in a complex coastal environment, thus limiting the need for high swimming velocities (Pörtner *et al.* 1991, 1993). Although the same is likely to be true for *L. brevis* (see above), a major difference is that the adult brief squid enters shallow coastal waters, characterized by larger fluctuations of environmental variables such as temperature and salinity, and may even be found in hypoxic bottom waters

during feeding and/or escape excursions (Vecchione, 1991). The features of activity metabolism discussed above may support the ability of the brief squid to tolerate these fluctuating environmental conditions, whereas fluctuations of environmental variables are likely to be limited in the natural environment of *L. pealei*.

The importance of reduced body size

Lolliguncula brevis is much smaller than most loliginids, a feature that may be related to its life in shallow waters. Virtually nothing is known about the size-dependence of the physiological characteristics of squid. In comparison with those of the larger loliginids, such as *Loligo pealei* and *Loligo forbesi*, the fins of *L. brevis* are somewhat less developed (see Wells, 1994). The fin size required for slow aerobic cruising and for fine locomotory control in a complex environment may be reduced in small compared with large squid. A reduced body size may enable the brief squid to enter shallow-water environments to collect prey. Moreover, small squid may reach a higher critical swimming speed (in body lengths s^{-1}). Within one fish species, the critical swimming speed is known to decrease with increasing body length (Webb *et al.* 1984; Kaufmann, 1990), indicating that the smaller fish is better provided with oxygen. If this also holds true for small squid, then small squid may be more tolerant of ambient hypoxia than are larger squid.

Conclusions

Oxygen supply to mitochondria limits the swimming performance of *Lolliguncula brevis* by determining a critical swimming speed above which anaerobic metabolism becomes involved. Long-term maintenance of steady swimming velocities is no longer possible since net depletion of energy stores sets in. The low critical swimming speed suggests that, in its complex and shallow-water environment, *L. brevis* usually travels short distances at lower speeds and will only occasionally and discontinuously use higher velocities. At higher velocities, *L. brevis* tends to oscillate between low- and high-pressure jets, the latter involving the use of anaerobic ATP production. Because anaerobic metabolic changes can be rapidly reversed in squid in general (Pörtner *et al.* 1993), these squid may cycle between purely aerobic and mixed aerobic and anaerobic swimming phases over short time intervals. On a longer time scale, this strategy implies a maximized use of ambient oxygen. At velocities above a critical value, the oscillation between aerobic and anaerobic jetting will allow an extended net use of anaerobic resources before fatigue sets in. This strategy may also be helpful when the animal dives into hypoxic waters. The invasive approach used in the present study does not allow us to obtain time-resolved data; however, previous time-resolved nuclear magnetic resonance experiments currently under evaluation should reveal whether these predictions really hold true (H. O. Pörtner, D. M. Webber, P. G. Lee, R. K. O'Dor and M. Quast, in preparation).

The excellent technical and logistical help from the staff of the Marine Biomedical Institute is gratefully acknowledged. This work was supported by NATO travel grants, the Deutsche Forschungsgemeinschaft (Po 278/4-7) and the National Institute of Health (DHHss RR01024 and DHHS RR04226). Alfred-Wegener-Institute Publication No. 1012.

References

BEIS, I. AND NEWSHOLME, E. A. (1975). The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. *Biochem. J.* **152**, 23–32.

BERGMEYER, H. U., BERGMEYER, J. AND GRAßL, M. (1986). (eds). *Methods of Enzymatic Analysis*. Weinheim: Verlag Chemie.

BONE, Q., PULSFORD, A. AND CHUBB, A. D. (1981). Squid mantle muscle. *J. mar. biol. Ass. U.K.* **61**, 327–342.

BRETT, J. R. (1964). The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Bd Can.* **21**, 1183–1226.

GÄDE, G. AND CARLSSON, K.-H. (1984). Purification and chracterization of octopine dehydrogenase from the marine nemertean *Cerebratulus lacteus* (Anopla: Heteronemerta): comparison with scallop octopine dehydrogenase. *Mar. Biol.* **79**, 39–45.

GOOLISH, E. M. (1991). Anaerobic swimming metabolism of fish: sit-and-wait *versus* active forager. *Physiol. Zool.* **64**, 485–501.

GOSLINE, J. M., STEEVES, J. D., HARMAN, A. D. AND DEMONT, M. E. (1983). Patterns of circular and radial muscle activity in respiration and jetting of the squid, *Loligo opalescens*. *J. exp. Biol.* **104**, 97–109.

GRIESHABER, M. AND GÄDE, G. (1976). The biological role of octopine in the squid, *Loligo vulgaris* (Lamarck). *J. comp. Physiol.* **108**, 225–232.

GRIESHABER, M. K., HARDEWIG, I., KREUTZER, U. AND PÖRTNER, H. O. (1994). Physiological and metabolic responses to hypoxia in invertebrates. *Rev. Physiol. Biochem. Pharmac.* **125**, 43–147.

GRIESHABER, M., KRONIG, E. AND KOORMANN, R. (1978). A photometric estimation of phospho-L-arginine, arginine and octopine using homogeneous octopine dehydrogenase isoenzyme 2 from the squid, *Loligo vulgaris* Lam. *Hoppe-Seyler's Z. physiol. Chem.* **359**, 133–136.

HOCHACHKA, P. W. (1994). Oxygen efficient design of cephalopod muscle metabolism. In *Physiology of Cephalopod Molluscs – Lifestyle and Performance Adaptations* (ed. H. O. Pörtner, R. K. O'Dor and D. MacMillan), pp. 61–67. Basel: Gordon and Breach Publishers.

KAUFMANN, R. (1990). Respiratory cost of swimming in larval and juvenile cyprinids. *J. exp. Biol.* **150**, 343–366.

MESSENGER, J. B., NIXON, M. AND RYAN, K. P. (1985). Magnesium chloride as an anaesthetic for cephalopods. *Comp. Biochem. Physiol.* **82**C, 203–205.

MOMMSEN, T. P., BALLANTYNE, J., MACDONALD, D., GOSLINE, J. AND HOCHACHKA, P. W. (1981). Analogues of red and white muscle in squid mantle. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3274–3278.

O'DOR, R. K. (1982). Respiratory metabolism and swimming performance of the squid, *Loligo opalescens*. *Can. J. Zool.* **39**, 580–587.

O'DOR, R. K. (1988). The forces acting on swimming squid. *J. exp. Biol.* **137**, 421–442.

O'DOR, R. K., HOAR, J. A., WEBBER, D. M., CAREY, F. G., TANAKA, S., MARTINS, H. R. AND PORTEIRO, F. M. (1994). Squid (*Loligo forbesi*) performance and metabolic rates in nature. In *Physiology of Cephalopod Molluscs – Lifestyle and Performance Adaptations* (ed. H. O. Pörtner, R. K. O'Dor and D. MacMillan), pp. 163–177. Basel: Gordon and Breach Publishers.

O'DOR, R. K. AND WEBBER, D. M. (1986). The constraints on cephalopods: why squid aren't fish. *Can. J. Zool.* **64**, 1591–1605.

O'DOR, R. K. AND WEBBER, D. M. (1991). Invertebrate athletes: trade-offs between transport efficiency and power density in cephalopod evolution. *J. exp. Biol.* **160**, 93–112.

PETTE, D. AND REICHMANN, H. (1982). A method for quantitative extraction of enzymes and metabolites from tissue samples in the milligram range. *J. Histochem. Cytochem.* **30**, 401–402.

PÖRTNER, H. O. (1990). Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* **81**, 275–288.

PÖRTNER, H. O. (1994). Coordination of metabolism, acid–base regulation and haemocyanin function in cephalopods. In *Physiology of Cephalopod Molluscs – Lifestyle and Performance Adaptations* (ed. H. O. Pörtner, R. K. O'Dor and D. MacMillan), pp. 131–148. Basel: Gordon and Breach Publishers.

PÖRTNER, H. O., BOUTILIER, R. G., TANG, Y. AND TOEWS, D. P. (1990). Determination of intracellular pH and P_{CO_2} after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* **81**, 255–274

PÖRTNER, H. O., KREUTZER, U., SIEGMUND, B., HEISLER, N. AND GRIESHABER, M. K. (1984). Metabolic adaptation of the intertidal worm *Sipunculus nudus* to functional and environmental hypoxia. *Mar. Biol.* **79**, 237–247

PÖRTNER, H. O., WEBBER, D. M., BOUTILIER, R. G. AND O'DOR, R. K. (1991). Acid–base regulation in exercising squid (*Illex illecebrosus*, *Loligo pealei*). *Am. J. Physiol.* **261**, R239–R246.

PÖRTNER, H.-O., WEBBER, D. M., O'DOR, R. K. AND BOUTILIER, R. G. (1993). Metabolism and energetics in squid (*Illex illecebrosus*, *Loligo pealei*) during muscular fatigue and recovery. *Am. J. Physiol.* **265**, R157–R165.

SEGAWA, S. AND HANLON, R. T. (1988). Oxygen consuption and ammonia excretion rates in *Octopus maya*, *Loligo forbesi* and *Lolliguncula brevis* (Mollusca: Cephalopoda). *Mar. Behav. Physiol.* **13**, 389–400.

STOREY, K. B. AND STOREY, J. M. (1978). Energy metabolism in the mantle muscle of the squid, *Loligo pealeii*. *J. comp. Physiol.* **123**, 169–175.

VECCHIONE, M. (1991). Dissolved oxygen and the distribution of the euryhaline squid *Lolliguncula brevis*. *Bull. mar. Sci.* (Abstract) **49**, 668–669.

WEBB, P. W., KOSTECKI, P. T. AND STEVENS, E. D. (1984). The effect of size and swimming speed on locomotor kinematics of rainbow trout. *J. exp. Biol.* **109**, 77–95.

WEBBER, D. M. AND O'DOR, R. K. (1986). Monitoring the metabolic rate and activity of free-swimming squid with telemetered jet pressure. *J. exp. Biol.* **126**, 205–224.

WELLS, M. J. (1994). The evolution of a racing snail. In *Physiology of Cephalopod Molluscs – Lifestyle and Performance Adaptations* (ed. H. O. Pörtner, R. K. O'Dor and D. MacMillan), pp. 1–12. Basel: Gordon and Breach Publishers.

WELLS, M. J., HANLON, R. T., LEE, P. G. AND DIMARCO, F. P. (1988). Respiratory and cardiac performance in *Lolliguncula brevis* (Cephalopoda, Myopsida): the effects of activity, temperature and hypoxia. *J. exp. Biol.* **138**, 17–36.