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Journal of Experimental Marine Biology and Ecology 342 (2007) 213–225

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

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Energy metabolism in the tropical abalone, *Haliotis asinina* Linné: Comparisons with temperate abalone species

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Received 15 March 2006; received in revised form 14 July 2006; accepted 12 September 2006

Abstract

The abalone, *Haliotis asinina*, is a large, highly active tropical abalone that feeds at night on shallow coral reefs where oxygen levels of the water may be low and the animals can be exposed to air. It is capable of more prolonged and rapid exercise than has been reported for temperate abalone. These unusual behaviours raised the question of whether *H. asinina* possesses enhanced capacities for aerobic or anaerobic metabolism. The blood oxygen transport system of *H. asinina* resembles that of temperate abalone in terms of a large hemolymph volume, similar hemocyanin concentrations, and in most hemocyanin oxygen binding properties; however, absence of a Root effect appears confined to hemocyanin from *H. asinina* and may assist oxygen uptake when hemolymph pH falls during exercise or environmental hypoxia. During exposure to air, *H. asinina* reduces oxygen uptake by at least 20-fold relative to animals at rest in aerated seawater, and there is no significant ATP production from anaerobic glycolysis or phosphagen hydrolysis in the foot or adductor muscles. This slowing of metabolism may contribute to survival at lower water oxygen levels than normally encountered by most temperate abalone. While crawling speeds of *H. asinina* in water are not exceptionally high, an aerobic expansibility of 5.5-fold at speeds less than 20% of maximum is more than 2.7-fold greater than reported for several temperate abalone. The high aerobic expansibility also supports the enhanced frequency and duration of flipping behaviour without recourse to the additional inputs from anaerobic glycolysis required by other abalone. Metabolic profiles of foot and adductor muscles of *H. asinina* are similar to those of other abalone. Common features are low activities of enzymes unique to aerobic ATP production, relatively high activities of arginine kinase, tauropine and D-lactate dehydrogenase as the predominant pyruvate reductases, and low intracellular pH buffering capacities. It is concluded that the exceptional abilities of *H. asinina* for prolonged and rapid exercise are supported by higher rates of aerobic metabolism rather than any enhanced capacity for anaerobic muscle work. It is unexpected, and instructive, that the exceptional aerobic expansibility is not apparent in obvious adjustments of the blood oxygen delivery system or muscle properties associated with aerobic ATP production. The absence of a hemocyanin Root effect, and the extent to which both aerobic and anaerobic metabolism can be reduced may be special features that assist prolonged exercise and survival of *H. asinina* when environmental oxygen becomes limiting.

Keywords: Abalone; Enzyme activity; Exercise; Hemocyanin; Respiration

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0022-0981/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jembe.2006.09.005

1. Introduction

Abalone (Gastropoda, Haliotidae) are classified as a single genus of approximately 70 marine species that are widely distributed from cool temperate to tropical regions, and from 400 m benthic to intertidal habitats (Lindberg, 1992). The ass's or donkey's ear abalone, *Haliotis asinina,* is a large tropical abalone common on coral reefs throughout the Indo Pacific and has the fastest growth rate recorded for a haliotid (Singhagraiwan and Sasaki, 1991). Morphologically, it differs markedly from other abalone species by possessing a greatly reduced and fragile shell accounting for about only 6% of total body mass instead of the usual 30% contributed by the shells of many temperate species. As the shell is too small to accommodate the massive foot musculature, it offers minimal protection against predation and wave action. (see Fig. 1).

At night on the low tide, *H. asinina* can be observed feeding in shallow, sometimes oxygen depleted water (Routley et al., 2002), and often exposed to air on top of coral outcrops. When disturbed, they crawl rapidly across the coral and retreat down into narrow crevices. Preliminary experiments in which *H. asinina* were placed shell side down in water demonstrated that they could average one righting movement per minute for periods in excess of an hour. In comparison, temperate species such as *H. iris*, *H. kamtschatkana* and *H. rubra* make fewer and slower righting movements and exhaust within 30 min (Baldwin et al., 1992; Donovan et al., 1999; Elias, 2003).

Extensive studies on temperate abalone species indicate relatively limited aerobic capacity, with dependence on anaerobic metabolism during righting behaviour and rapid crawling, and during prolonged air exposure when the gills collapse and oxygen uptake is impaired. During such exercise and environmental induced hypoxia, the foot and shell adductor muscles accumulate tauropine and D-lactate as pyruvate reductase end products of anaerobic glycolysis, and the arginine phosphate reserves decrease (Gäde, 1988; Baldwin et al., 1992; Wells and Baldwin, 1995; Donovan et al., 1999; Elias, 2003).

Fig. 1. The ass's ear abalone, *Haliotis asinina*.

The unusual morphology, feeding behaviour and exercise capabilities of *H. asinina* lead us to pose the following questions. Are the well-developed abilities for prolonged and sometimes rapid exercise, and exposure to potentially hypoxic environments, reflected in underlying biochemical and physiological specialisations that distinguish this species from previously studied temperate abalone? In particular, does *H. asinina* show enhanced aerobic capabilities, enhanced capacity for anaerobic metabolism, or some combination of both?

We addressed these questions in the following ways: 1) establishing metabolic profiles of the foot and shell adductor muscles by measuring the activities of key enzymes associated with aerobic and anaerobic pathways, and determining intracellular pH buffering capacities; 2) characterising the blood oxygen transport system in terms of blood volume, hemocyanin content, and hemocyanin oxygen binding properties; 3) measuring oxygen uptake of abalone in air and in water; and 4) measuring metabolite changes in foot and shell adductor muscles following exercise and prolonged exposure to air.

2. Materials and methods

2.1. Experimental animals

Ass's ear abalone *(Haliotis asinina*) were collected from the reef flat on Heron Island, Queensland (23°26′ S, 151°55′ E) at night when they were feeding on top of coral exposed at low tide. The animals (mean weight 151 g, range 83–200 g) were held at the Heron Island Research Station for up to 8 days without feeding in flow through seawater aquaria at the ambient water temperature of 23–26 °C.

2.2. Muscle enzymes

Samples of shell adductor and foot muscle were excised from live animals immediately after their removal from aquaria. Approximately 1 g muscle samples were finely minced on ice with scissors, and homogenised in 5 ml of ice-cold extraction buffer (75 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, 8 mmol l^{-1} $MgCl_2$, pH 7.8), using an Ultra Turrax (Janke and Kunkle, Germany). Homogenates were centrifuged at 13 000 ×*g* for 3 min at 4 °C, and supernatants were held on ice for immediate analysis.

Enzyme activities were measured with a Pharmacia LKB-Ultraspec III recording spectrophotometer in which cuvette temperature was maintained at 25 °C with a circulating water bath. Absorbance changes were followed at 340 nm, except for citrate synthase, which was assayed at 412 nm. Preliminary trials were made to determine optimal concentrations of reagents, and suitable controls lacking substrates were run to correct for non-specific activity. Assays were performed with 10 to 20 μl of muscle extract in a total volume of 1 ml. All determinations were made in triplicate on muscle samples from three animals. The compositions of the reaction mixtures were as listed:

Arginine kinase, (EC 2.7.3.3). 2 mmol l^{-1} arginine, 2 mmol l^{-1} ATP, 1 mmol l^{-1} phosphoenolpyruvate, 0.1 mmol l^{-1} NADH, 10 mmol l^{-1} $MgCl_2$, 75 mmol l^{-1} KCl, excess pyruvate kinase and lactate dehydrogenase, 50 mmol l^{-1} imidazole–HCl buffer, pH 7.5;
Citrate synthase, (EC 2.3.3.1). 0.1 mmol l^{-1} acetyl CoA, 0.5 mmol l^{-1} oxaloacetate, 0.2 mmol l^{-1} 5,5′-dithiobis-(2 nitrobenzoic acid), 50 mmol l^{-1} Tris–HCl buffer, pH 8.0;
Hexokinase, (EC 2.7.1.1). 1 mmol l^{-1} glucose, 2 mmol l^{-1} ATP, 0.4 mmol l^{-1} NADP, 10 mmol $l^{-1}$$MgCl_2$, 2 mmol l^{-1} KCL, 1 mmol l^{-1} dithiothreitol, excess glucose 6-phosphate dehydrogenase, 50 mmol l^{-1} imidazole–HCl buffer, pH 7.4;
3-Hydroxyacyl CoA dehydrogenase, (EC 1.1.1.35). 0.5 mmol l^{-1} acetoacetyl CoA, 0.1 mmol l^{-1} NADH, 50 mmol l^{-1} imidazole–HCL buffer, pH 7.0;
Phosphofructokinase, (EC 2.7.1.11). 1 mmol l^{-1} ATP, 2 mmol l^{-1} AMP, 0.15 mmol l^{-1} NADH, 3 mmol l^{-1} fructose-6-phosphate, 10 mmol l^{-1} $MgCl_2$, 250 mmol l^{-1} KCl, excess triose phosphate isomerase, glycerophosphate dehydrogenase and aldolase, 50 mmol l^{-1} Tris–HCl buffer, pH 8.2;
Phosphorylase, (EC 2.4.1.1). 2 mg glycogen, 1 mmol l^{-1} AMP, 0.4 mmol l^{-1} NADP, 25 μmol l^{-1} glucose 1,6-diphosphate, 10 mmol l^{-1} $MgCl_2$, excess phosphoglucomutase and glucose 6-phosphate dehydrogenase, 50 mmol l^{-1} sodium phosphate buffer, pH 7.4;
Pyruvate kinase, (EC 2.7.1.40). 2 mmol l^{-1} ADP, 1 mmol l^{-1} phosphoenolpyruvate, 0.1 mmol l^{-1} NADH, 10 mmol l^{-1} $MgCl_2$, 75 mmol l^{-1} KCL, excess lactate dehydrogenase, 50 mmol l^{-1} imidazole–HCl buffer, pH 7.5;
Lactate dehydrogenase, (EC 1.1.1.27). 2.5 mmol l^{-1} pyruvate, 0.15 mmol l^{-1} NADH, 50 mmol l^{-1} imidazole–HCl buffer, pH 7.0.

The following pyruvate reductase enzymes were assayed using the lactate dehydrogenase reaction mixture, but with the addition of the amino acids as listed:

tauropine dehydrogenase, (EC 1.5.1.23). 80 mmol l^{-1} taurine;
octopine dehydrogenase, (EC 1.1.1.11). 5 mmol l^{-1} arginine;
strombine dehydrogenase, (EC 1.5.1.22). 200 mmol l^{-1} glycine;
alanopine dehydrogenase, (EC 1.5.1.17). 100 mmol l^{-1} alanine;
β-alanopine dehydrogenase, (EC 1.5.1.26).
100 mmol l^{-1} β-alanine;
lysopine dehydrogenase, (EC 1.5.1.16)100 mmol l^{-1} lysine;
seropine dehydrogenase, (EC ?) 100 mmol l^{-1} serine.

2.3. Muscle pH buffering capacity

Freshly dissected muscle (0.5 g) was diced with scissors, suspended in 5 ml of 0.1 mol l^{-1} NaCl, and homogenised. Homogenates were adjusted to pH 6.0 with 1 mol l^{-1} HCL and titrated at 25 °C against 20 μl aliquots of 0.2 mol l^{-1} NaOH while the pH was recorded. Buffering capacity, β, is defined in slykes as the number of μmoles of base required to change the pH of 1 g of muscle by 1 pH unit over the range pH 6 to pH 7.

2.4. Behavioural experiments

2.4.1. Crawling behaviour of animals in the field

Abalone were collected while feeding at night on coral outcrops on the reef flat. They were placed immediately on a large coral boulder, and mean crawling rates in water were determined from the time taken to move a measured distance (20–80 cm). Two to five values were obtained for each of five animals ranging in weight from 90–120 g.

2.4.2. Behaviour of captive animals

Captive abalone held in aerated seawater aquaria for several days were divided into four groups.

2.4.2.1. Control group. Resting tank-acclimated animals ($n=6$) served as muscle metabolite controls.

2.4.2.2. Crawling group. Abalone ($n=6$), covering the size range used in the field experiments, were placed individually in aquaria of aerated seawater and crawling rates were recorded at night at a time corresponding to

low tide on the reef flat. The aquarium tanks were marked out in a 5 cm square grid pattern, and the animals were left to crawl without disturbance until a total distance of 6 m had been covered. To determine crawling speed, the time taken to cover distances from 15 to 60 cm were recorded during periods of continuous crawling for a total of 11 trials on 6 different animals. Once a total distance of 6 m had been achieved, foot and adductor muscle samples from each animal were freeze-clamped in liquid nitrogen for metabolite determinations (see below). We were unable to elicit crawling behaviour for captive animals exposed to air.

2.4.2.3. Righting group. Abalone ($n=6$) were exercised in tanks of aerated seawater by being repeatedly inverted and left to right themselves (flipping). This was repeated until they appeared exhausted and did not make righting movements within 5 min of being inverted. Muscle samples were then taken as for crawling group animals.

2.4.2.4. Air-exposed group. Abalone were placed on the bottom of shallow plastic bins on a film of water to maintain humidity at an air temperature of 24 °C, with a natural light regime. Individuals were removed after 4 h ($n=3$), 12 h ($n=3$) and 24 h ($n=3$) of air exposure, at which times muscle samples were taken as for crawling group animals.

2.4.3. Muscle metabolites in exercised and air-exposed animals

Muscle metabolite levels were determined for each of the four captive abalone behaviour groups. Thin slices cut from the dorsal surface of the shell adductor muscle and from the central area of the foot muscle were freeze-clamped in liquid nitrogen. Muscle samples of approximately 0.5 g were crushed under liquid nitrogen and homogenised in 5 ml of ice-cold 0.6 mol l^{-1} perchloric acid. The acid extracts were centrifuged at 13 000 ×*g* for 10 min at 4 °C, and the supernatants were neutralised with 5 mol l^{-1} K_2CO_3. After standing on ice for 1 h, supernatants were decanted and stored at −80 °C until assayed.

D-lactate and tauropine were assayed as described by Baldwin et al. (1992). Arginine phosphate was measured by the method of Grieshaber and Gäde (1976), using octopine dehydrogenase purified from *Strombus luhuanus* (Baldwin and England, 1982).

Differences in treatments were determined using one-way ANOVAs. Because of limitations on the number of abalone that could be collected, the same control group was used for the crawling, righting and air-exposure experiments. Therefore, statistical significance was accepted at $\alpha=0.025$.

2.5. Oxygen uptake

To measure rates of oxygen uptake in water, individual abalone ($n=10$, 88–138 g) were placed in a circular Perspex respirometry chamber (5035 ml volume) filled with stirred, fresh aerated seawater. Temperature was maintained at 25 °C and oxygen uptake was monitored continuously with a polargraphic oxygen electrode connected to a data acquisition system (Datacan, Sable Systems, Inc). Oxygen consumed by microorganisms present in the seawater was determined by measuring the decrease in oxygen levels in the respirometer with no abalone present. This was repeated twice and a mean control value was calculated. This control value was subtracted from oxygen uptake measured for each individual abalone.

For aerial oxygen uptake, individual abalone ($n=3$, 115–127 g) were placed in a circular Perspex respirometry chamber (1935 ml volume) filled with air and temperature was maintained at 25 °C. Air was pumped from the chamber into an oxygen analyser (FC-1, Sable Systems Inc.) then back into the chamber, essentially forming a closed respirometry system in which oxygen levels were monitored continuously. The air leaving the chamber was scrubbed of carbon dioxide and water before entering the oxygen analyser. The oxygen analyser was connected to a data acquisition system (Datacan, Sable Systems, Inc). Drift in the oxygen analyser was controlled by measuring respirometer oxygen levels with no abalone present. This was repeated twice and a mean control value calculated. This control value was subtracted from oxygen uptake measured for each individual abalone.

2.6. Hemocyanin concentration, and hemolymph volume and pH

Hemolymph was taken by syringe and 19 gauge needle from the large pedal sinus ($n=16$ animals), centrifuged at 4000 ×*g* for 15 min to remove cellular material, and assayed for total protein by the biuret method using bovine serum albumin standards. Analysis by Sephadex G200 gel filtration and ultracentrifugation (150,000 ×*g* for 17 h at 4 °C) showed that high molecular weight hemocyanin accounted for all the biuret protein measured in cell free hemolymph, thereby confirming the results for other abalone species (Ainslie, 1980a).

Minimum hemolymph volume was estimated by removing the abalone shell, making a deep longitudinal

incision through the foot and into the adductor muscle, and leaving the hemolymph to drain out of the animal into a beaker for several hours.

Interpretation of oxygen equilibrium curves *in vivo* was assisted by measuring the pH in hemolymph sampled from the pedal sinus of abalone (a) at rest in aerated seawater, (b) after exercising to exhaustion by repeated righting behaviour and (c) following 4 h exposure to moist air at 25 °C.

2.7. Hemocyanin oxygen binding

Pooled hemolymph, collected by syringe from the pedal sinus of several abalone, was centrifuged for 3 min at 12,000 ×*g* and 4 °C. Hemocyanin solutions with varying pH were prepared by adding to whole hemolymph stock 1 mol l^{-1} Bis–Tris or Tris–HCl buffers to a final buffer concentration of 0.1 mol l^{-1}. Oxygen binding studies were carried out at 25 °C using an optical tonometer as described by Wells and Weber (1989). The oxy-deoxy-hemocyanin ratio at 366 nm was calculated from absorbances after sequential additions of air to the evacuated tonometer. Oxygen equilibrium curves were obtained at constant pH, and full saturation (p_{100}) was defined by equilibrium in air. At least six duplicate points were obtained for each oxygen equilibrium curve and the cooperativity coefficient (n) and half-saturation pO_2 (p_{50}) were estimated from Hill Plots of the data. Continuous oxygen equilibrium curves were constructed from the following formula using TableCurve-2D software:

$Y=((1/p_{50}^n)(X^n))/(1+(((1/p_{50}^n)(X^n)))$, where Hill's n was derived from the slope of log X versus log $(Y/(1-Y))$ at $Y=0.5$, where $X=pO_2$ and $Y=$fractional saturation. Partial pressures are reported conventionally in mmHg to enable comparisons with other studies (1 mmHg = 0.133 kPa).

The effect of pH on oxygen carrying capacity (Root effect) was estimated spectrophotometrically from the decrease in the oxygenated maximum (366 nm) of buffered hemolymph equilibrated in air.

3. Results

3.1. Muscle enzyme activities and pH buffering capacity

The maximum activities of enzymes associated with pathways of aerobic and anaerobic ATP production in foot and adductor muscles of *H. asinina* are given in Table 1.

Hexokinase, 3-hydroxyacyl CoA dehydrogenase and citrate synthase, which are enzymes unique to pathways supporting aerobic respiration, are present at similar low activities in both muscle types. The two muscles also display comparable activities of the glycolytic enzymes phosphorylase, phosphofructokinase and pyruvate kinase, and of arginine kinase.

Table 1
Enzyme activities (μmol substrate min^{-1} g^{-1} wet wt muscle, 25 °C) and intracellular pH buffering capacities (β, slykes) in adductor and foot muscle of *H. asinina*

	Adductor	Foot
Arginine kinase	102.2 (81.8–113.6)	92.7 (85.8–98.2)
Hexokinase	0.2 (0.2)	0.2 (0.2–0.3)
3-Hydroxyacyl CoA dehydrogenase	<0.2	<0.2
Citrate synthase	0.2 (0.2)	0.3 (0.3–0.4)
Phosphorylase	<0.2	<0.2
Phosphofructokinase	3.3 (2.7–4.1)	2.4 (1.8–3.2)
Pyruvate kinase	47.8 (37.7–61.6)	39.7 (34.5–47.9)
Lactate dehydrogenase	4.5 (3.0–5.5)	9.1 (6.4–10.8)
Tauropine dehydrogenase	48.3 (32.3–57.7)	19.4 (11.8–26.6)
Alanopine dehydrogenase	3.1 (1.7–3.9)	0.6 (0–1.7)
Lysopine dehydrogrenase	0.9 (0–1.7)	1.7 (0–3.3)
Seropine dehydrogenase	<0.2	0.5 (0–1.6)
Octopine dehydrogenase	<0.2	<0.2
Strombine dehydrogenase	<0.2	<0.2
β-alanopine dehydrogenase	<0.2	<0.2
pH buffering (β)	32.6 (29.2–35.6)	28.5 (22.4–34.0)

Values are means, with ranges in brackets, for assays on three abalone.

Of the seven invertebrate pyruvate reductases potentially associated with anaerobic glycolysis, highest activities were found for tauropine dehydrogenase, followed by lactate dehydrogenase, with lower activities of alanopine, lysopine, and possibly seropine dehydrogenases. In other abalone species, opine dehydrogenase activities other than tauropine dehydrogenase have been attributed to the ability of tauropine dehydrogenase to catalyse pyruvate reductase reactions with amino acids other than taurine (Gäde, 1986; Sato et al., 1991). Partial purification of *H. asinina* adductor muscle tauropine dehydrogenase using ion exchange chromatography (Baldwin et al., 1992) removed lysopine and seropine dehydrogenase activities, but did not alter the activity ratios for tauropine and alanopine dehydrogenases present in the original muscle homogenate. It was concluded that while the alanopine dehydrogenase activity resided with tauropine dehydrogenase, the possibility existed that the very low lysopine and seropine activities were due to additional enzymes.

While tauropine dehydrogenase activities were higher than lactate dehydrogenase activities in both muscles, the adductor had two-fold higher activities of tauropine dehydrogenase and two-fold lower activities of lactate dehydrogenase relative to the foot muscle.

Intracellular pH buffering capacities (Table 1) were similar, with overlapping ranges, in the foot and adductor muscles.

3.2. Behavioural experiments

3.2.1. Crawling behaviour of animals in the field

When first located at night feeding on coral during low tide, the abalone were either stationary or moving very slowly. After disturbance by relocation they moved more rapidly across the coral and down into any available crevice. A total of 19 exercise trials on five different individuals gave a mean speed of 74.9± 30.1 cm min $^{-1}$(range 30.7–142.9 cm min $^{-1}$) (results are expressed as mean±S.D. unless otherwise noted) over distances ranging from 20 to 80 cm (43.6± 16.6 cm). The highest speed of 142.9 cm min^{-1} was recorded over a distance of 50 cm. The greatest distance of 80 cm was covered at a speed of 72.7 cm min^{-1}.

3.2.2. Behaviour of captive animals and effects on muscle metabolites

Captive animals placed individually into aerated seawater aquaria at a time approximating the night low tide (2000–2300 h) displayed quite uniform locomotion across the aquarium. A total of 11 trials with 6 different individuals of a size similar to the non-captive animals used above gave a mean crawling speed of 77.7± 24.3 cm min^{-1} (range 42.0–121.4 cm min^{-1}) for distances ranging from 15 to 60 cm. The highest speed of 121.4 cm min^{-1} was achieved over a distance of 38 cm.

The concentrations of metabolites associated with anaerobic metabolism in the foot and adductor muscles of these animals at the completion of the 6 m crawling trials are presented for comparison with the resting control group in Table 2. Relative to the resting controls, crawling in water significantly depleted arginine phosphate reserves ($F=14.5$, $p<0.001$) and increased tauropine concentrations ($F=78.4$, $p<0.001$) in the adductor muscle. Likewise, arginine phosphate decreased ($F=8.1$, $p=0.004$) and tauropine increased ($F=29.0$, $p<0.001$) in the foot muscle.

The behaviour of animals that were inverted in aerated seawater and left to right them selves (flipping) is summarised in Table 3. When inverted, the animals slowly elongated, rotated and extended the foot out from under the shell by relaxing the shell adductor muscle. Righting appeared to involve a more rapid contraction of the adductor to lever the body and shell into the upright position. The highest flipping rate (1.04 flips min^{-1}) was recorded for the animal that produced the greatest number of flips (70) and took the longest time to exhaust (67.5 min).

Table 3
Righting behaviour (flipping) of *H. asinina* immersed in aerated seawater

Time to exhaustion (min)	49.8±17.9 (24–67.5)
Number of flips	40±21 (15–70)
Flip min^{-1}	0.78±0.19 (0.51–1.04)

Values are presented as the mean±S.D. for 6 animals, with ranges in brackets.

Metabolite concentrations in adductor and foot muscles of these exhausted animals are presented in Table 2, for comparison with the resting controls. In marked contrast to the crawling animals, D-lactate and tauropine concentrations in adductor and foot muscles of exhausted righting animals did not differ significantly from the rested controls, and arginine phosphate concentrations decreased in the adductor ($F=14.5$, $p<0.001$), but not the foot muscle.

The air-exposed abalone showed no tendency to crawl during the course of these experiments. After several hours of exposure to humid air, the animals appeared to lose muscle tone. The foot spread out, the adductor relaxed, and the shell was displaced off to one side exposing a large surface area of pale muscle. However when handled after 4, 12 and 24 h of air exposure, the animals were still able to contract the foot and adductor muscles rapidly to recover the normal shape observed in submerged controls.

Concentrations of arginine phosphate, D-lactate and tauropine in adductor and foot muscles of the air-exposed animals are presented in Table 4, for

Table 2
Metabolite concentrations in adductor and foot muscles of captive *H. asinina* resting controls, and following crawling 6 m in seawater and righting in seawater until exhausted

Muscle metabolite	Adductor			Foot		
	Control	Crawling	Righting	Control	Crawling	Righting
Arginine phosphate	7.95±3.08	1.17±1.62[a]	2.91±1.79[a]	3.61±1.96	0.75±0.96[b]	3.38±0.92
D-lactate	0.42±0.53	0.33±0.25	1.12±1.79	0.18±0.23	0.62±0.37	0.54±0.47
Tauropine	0.09±0.09	3.67±0.73[c]	0.55±0.58	0.05±0.56	3.02±0.61[d]	0.48±0.75

Units are μmol g^{-1} wet weight muscle. Values are presented as the mean±S.D. for 6 animals in each group. Superscripts indicate differences from the control at $p<0.025$, and common superscripts denote homogenous subgroups (Tukey's test, $p<0.025$).

Table 4
Metabolite concentrations in adductor and foot muscles of *H. asinina* resting controls, and following 4 h, 12 h and 24 h air exposure

Air exposure (h)	Muscle (*N*)	Arginine phosphate	D-lactate	Tauropine
0 (control)	Adductor (6)	7.94±3.08	0.42±0.53	0.09±0.09
	Foot (6)	3.61±1.96	0.18±0.23	0.50±0.56
4	Adductor (3)	10.9±4.47	0.30±0.38	0.37±0.53
	Foot (3)	4.80±1.41	0.58±0.29	0.28±0.49
12	Adductor (3)	6.87±2.43	0.15±0.17	0.28±0.15
	Foot (3)	3.85±2.22	0.16±0.27	0.27±0.18
24	Adductor (3)	2.93±3.31	0.00±0.00	0.35±0.45
	Foot (3)	4.03±1.05	1.05±0.90	0.20±0.24

Units are μmol g^{-1} wet weight muscle. Values are presented as the mean±S.D.

comparison with the resting submerged controls. No significant differences were found between the control group and either the 4, 12 or 24 h air-exposed groups, or among the three air-exposed groups, for these metabolites in either muscle.

3.3. Oxygen uptake

The rates of oxygen uptake by *H. asinina* at rest in water, crawling in water, and at rest in air are summarised in Table 5. The six abalone that crawled in water continuously for a time long enough to measure oxygen uptake in the respirometer averaged a speed of 20.9±12.4 cm min^{-1}. Crawling in water increased the mean mass specific oxygen uptake rate by 5.5-fold above the resting value. Oxygen uptake by abalone at rest in air was below the level of detection, (about 2 μl O_2 g^{-1} h^{-1}), and animals could not be induced to crawl in the respirometer when exposed to air.

3.4. Hemocyanin concentration, and hemolymph volume and pH

Hemocyanin concentration estimated by the biuret method gave a mean value of 6.4±0.9 mg ml^{-1} hemolymph (n=16, range 5.0–7.8) for animals ranging in weight from 140–200 g. Mean minimum hemolymph volume obtained for 6 animals covering a similar size range was 51±4 ml 100 g^{-1} animal, or 55±4 when expressed as ml 100 g^{-1} wet weight animal minus the shell.

Table 5
Rates of oxygen uptake by *H. asinina* at rest in water, crawling in water and at rest in air

Experimental group (*n*)	Oxygen consumption (μl O_2 g^{-1} h^{-1}, 25 °C)
Resting in water (10)	45.4±7.5 (26.1–53.5)
Crawling in water (6)	247.6±93.4 (113.1–404.6)
Resting in air (3)	0.0±0.0 (0)

Values are presented as the mean±S.D., with ranges in brackets.

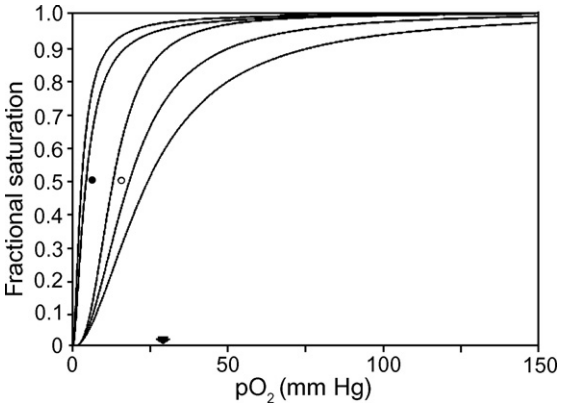

Fig. 2. Oxygen equilibrium curves for hemolymph from *H. asinina* at 25 °C in order of decreasing affinity from left to right: pH=6.61, 6.96, 7.37, 7.67, 8.00. The p_{50} values corresponding to approximate resting pH (○) and following exercise or hypoxia (●) are indicated. Arrow indicates minimum night time pO_2 on the reef flat.

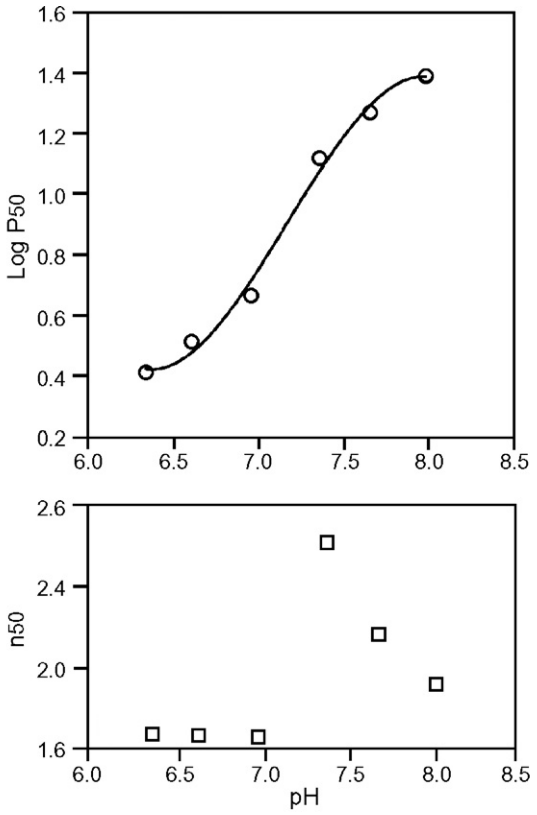

Fig. 3. Bohr plot of *H. asinina* hemolymph at 25 °C. Effect of pH on p_{50} (pO_2 mmHg at which hemolymph is half-saturated with oxygen) and n_{50} (Hill's sigmoid coefficient at p_{50}).

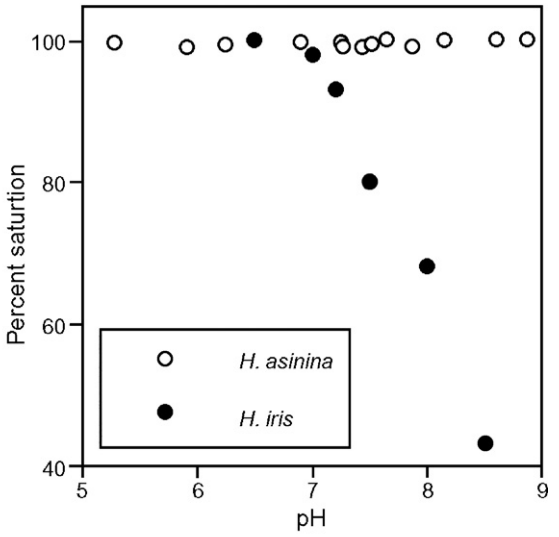

Fig. 4. Effect of pH on oxygen saturation (Root effect) of hemolymph from *H. asinina* at 25 °C. Data for the temperate species, *H. iris* at 20 °C, are from Wells et al. (1998).

The pH of hemolymph from the pedal sinus of control abalone resting in aerated seawater was 7.53± 0.09 (n=7). Values following 4 h of air exposure, and righting exercise to exhaustion, respectively, were 7.24±0.14 (n=5) and 7.28±0.01 (mean±range, n=2).

3.5. Oxygen binding properties of hemocyanin

The oxygen equilibrium curves of *H. asinina* hemolymph were sensitive to pH and showed a reversed (positive) Bohr effect, whereby hemocyanin–oxygen affinity was highest at low pH (Fig. 2). The minimum environmental pO_2 superimposed on Fig. 2 is taken from Routley et al. (2002) and corresponds to a hemolymph saturation of approximately 80% at pH 7.67. The effect of pH on the oxygen affipity coefficient, p_{50}, and Hill's cooperativity coefficient, n_{50}, are shown in Fig. 3. The maximum Bohr effect, quantified by $\Delta\log p_{50}/\Delta\mathrm{pH}=+0.93$, occurred between pH 7.0 and 7.5. Cooperativity was greatest at pH 7.4 and was reduced at the extremes of pH. There was little effect of pH on the depression of hemocyanin saturation indicating the absence of a significant reversed Root effect in *H. asinina* (Fig. 4).

4. Discussion

4.1. Muscles used for exercise

The basic question addressed in this study is whether the observed behavioural and exercise abilities of *H. asinina* are supported by enhanced capacities for energy metabolism, relative to previously studied temperate abalone species. The family Haliotidae contains some of the most active of all gastropods, probably being surpassed only by certain members of the Strombidae. Typically, abalone display relatively fast crawling locomotion on hard surfaces, dexterous feeding behaviour when dealing with drift macro algae, and rapid shell twisting used during predator avoidance and righting after dislodgement. These behaviours are brought about by contractions of the large foot muscle working in association with the smaller single shell adductor muscle. Taken together, with associated hemolymph, these muscles make up about 50–60% of the live wet weight in typical temperate abalone species (Elias, 2003). However in the case of *H. asinina,* where the shell accounts for only about 6% of total animal weight, this combined muscle mass equals about 80% of the live wet weight with the shell adductor contributing about 10% of the total muscle weight.

4.2. Hemolymph oxygen transport

Aerobic metabolism is supported by gas exchange via paired gills in the mantle coupled to an extensive circulating hemolymph system containing the oxygen binding respiratory pigment, hemocyanin. Abalone have an open, but anatomically and functionally complex, circulating system in which the cardiac output exceeds that of some cephalopods; networks of fine vessels perfuse the viscera and paired arteries supply oxygenated hemolymph to the pedal musculature (Crofts, 1929; Jorgensen et al., 1984; Bourne et al., 1990; Just, 2002).

Total hemolymph volumes for several temperate abalone species are listed for comparison with *H. asinina* in Table 6. These vary from 28% to 57% of total wet weight body tissue, placing the *H. asinina* minimum value of 55% at the top end of this range. The mean hemolymph hemocyanin concentration of 6.04 mg ml^{-1} for *H. asinina* is similar to that of the temperate *H. kamtschatkana* (Boyd and Bourne, 1995), and within the range reported for many other abalone species (2–10 mg ml^{-1}, Ainslie, 1980a; Wells et al., 1998, Elias,

Table 6
Hemolymph volumes (ml 100g^{-1} wet wt) of abalone

Species	Volume	Method	Studies cited
Haliotis asinina	55	Bleeding	This study
Haliotis fulgens	41	Inulin	Pilson (1965)
Haliotis iris	57	^{51}Cr	Taylor (1993)
	52	^{14}C Inulin	Ragg et al. (2000)
Haliotis rubra	28	Bleeding	Elias (2003)

2003). However, the variation of hemocyanin concentration observed for *H. asinina* (5.0–7.8 mg ml^{-1}, $n=16$) is rather small compared to that of other species where it appears to vary up to 900-fold (Pilson, 1965; Ainslie, 1980a).

Assuming a similar hemocyanin–oxygen binding ratio as in *H. kamtschatkana* (Boyd and Bourne, 1995), our estimation of 6.4 mg ml^{-1} hemocyanin equates to an oxygen carrying capacity of 2.75 ml oxygen 100 ml^{-1} hemolymph. Thus, the estimated 55 ml hemolymph volume provides a hemocyanin–oxygen reservoir of 1.51 ml 100 g^{-1} animal.

Marine gastropod hemocyanins appear conservative with respect to their oxygen transport properties; the oxygen equilibrium curve is generally sigmoidal and oxygen affinity is relatively low at physiological pH (Brix, 1983). Falling pH from hemolymph acidification sharply increases both oxygen affinity (positive, or reverse Bohr effect) and cooperative oxygen binding (Brix, 1983). Furthermore, a number of gastropods show depression of hemocyanin–oxygen saturation at high pH according to the reversed Root effect (Brix et al., 1979; Ainslie, 1980b; Brix, 1983).

Despite the significant Bohr effect in *H. asinina*, there was no appreciable Root effect. On occasions when *H. asinina* may be exposed to reduced environmental pO_2 on reef flats (see Routley et al., 2002), the oxygen equilibria predict an approximate hemolymph oxygen saturation of >80% for pH values at or below the resting value of 7.6 (see Fig. 2). Due to the reverse Bohr effect, metabolic or respiratory acidosis arising from air exposure or exercise is expected to maintain high saturation of oxygen in the gills.

Hemocyanin–oxygen affinity in the temperate abalone, *H. iris* is relatively higher and consistent with an oxygen-storage role for its hemolymph in support of aerobic muscle work (Wells et al., 1998). In water, oxygen transport in the tropical *H. asinina* is perfusion dependent and a relatively low affinity of the hemocyanin for oxygen favours unloading to tissues working at lower pO_2. When crawling out of water however, oxygen transport becomes diffusion dependent and accumulation of acid metabolites are expected to act in concert with the large Bohr effect to ration oxygen release through a conformational shift in the hemocyanin towards higher affinity. The rationing is further augmented by the pH-induced reduction in cooperativity whereas the more sigmoidal oxygen equilibrium curve at higher pH when water breathing provides uptake and release over a relatively narrow range of pO_2. These speculations are partly supported by measurements of hemolymph pH in the pedal sinus. pH fell approximately 0.25 units with both exhaustive righting behaviour and 4 h exposure in air. This corresponds to an increase in hemocyanin–oxygen affinity from 15 mmHg at pH 7.5 to 9 mmHg at pH 7.25 and a 25% increase in bound oxygen arising from the Bohr shift. The hemolymph does not therefore appear to facilitate oxygen flux during exercise, but conserves oxygen release to mitigate the less efficient energy production from anaerobic pathways. Further, in the absence of a large Root effect in this pH range, oxygen carrying capacity remains essentially unaltered, a feature that may also maintain oxygen loading in the gills during exercise or environmental hypoxia.

Marine gastropods do not appear to show acclimatory responses of the hemocyanin–oxygen binding in response to episodes of hypoxia (Brix, 1982). Hemocyanin in the bivalve *Nuclula sulcata,* which lives in oxygen depleted sediments, has an extremely high hemocyanin–oxygen affinity and non-cooperative oxygen binding (Taylor et al., 1995). We therefore assume that it is the intrinsic binding properties of haliotid hemocyanins that balance oxygen supply and delivery, but this remains to be investigated.

4.3. Muscle metabolic profiles

The foot and adductor muscles of temperate abalone are characterized by a limited scope for aerobic muscle work, with activities such as sustained crawling and righting behaviour requiring additional ATP input from anaerobic glycolysis and hydrolysis of the muscle phosphagen, arginine phosphate (Gäde, 1988; Donovan et al., 1999; Elias, 2003). The enzyme profile data provide useful information on the metabolic poise of these muscles in *H. asinina.* Overall, the results presented in Table 1 are very similar to comparable values in the literature obtained from temperate abalone (Gäde, 1988; Wells et al., 1998; Elias, 2003).

The low activities of the Krebs Cycle enzyme citrate synthase, 3-hydroxyacyl CoA dehydrogenase which is involved in β-oxidation of fatty acids, and hexokinase which catalyses the initial step in the use of glucose as an aerobic fuel, are in keeping with a limited aerobic capacity in both the foot and adductor muscles during exercise. Low activities of phosphorylase and phosphofructokinase, both of which are considered to catalyse flux-generating steps in anaerobic glycolysis (Newsholme and Start, 1973), also imply little scope for rapid increases in ATP production via this pathway. *H. asinina,* like other abalone, uses predominantly tauropine dehydrogenase and lactate dehydrogenase to catalyse the terminal step of anaerobic glycolysis, with

greater activities of the former enzyme in both muscle types. The activities of arginine kinase, which catalyses the hydrolysis of arginine phosphate, are consistent with the phosphagen providing a significant source of ATP in both muscles during elevated exercise.

The low intracellular pH buffering capacities of *H. asinina* foot and adductor muscles also imply limited capacity for high intensity anaerobic muscle work (Morris and Baldwin, 1984). The values fall within the range (28–37 slykes) reported for these muscles in several species of temperate abalone (Wells and Baldwin, 1995; Elias, 2003).

4.4. Energy metabolism during crawling

Mean crawling speeds of wild *H. asinina* on coral outcrops and of captive animals in glass aquaria did not differ significantly. The maximum crawling speeds of 143 cm min^{-1} and 121 cm min^{-1} respectively can be compared with a maximum value of 113 cm min^{-1} for the temperate *H. kamtschatkana* crawling in aquaria during stimulation with a live predatory starfish (Donovan and Carefoot, 1997).

Crawling at a mean speed of 20.9 cm min^{-1} in the more confined space of the water filled respirometer increased the mean mass specific oxygen uptake of *H. asinina* by 5.5-fold above resting levels. This speed was less than 30% of the mean speeds and 20% of the maximum speeds achieved in the aquarium trials and observed for wild *H. asinina* on the reef. Unfortunately it is difficult to extrapolate from the respirometer results to obtain an estimate of how much greater oxygen uptake would be at these higher speeds; however, an aerobic expansibility of 5.5-fold at even the relatively low speed of 20.9 cm min^{-1} is at least 2.7-fold greater than data available for crawling by other abalone (*H. kamtschatkana*, 1.6-fold, Donovan and Carefoot, 1998; *H. rufescens*, 2.0-fold, Donovan, unpublished data). In fact, the aerobic expansibility of *H. asinina* is greater than for most other gastropods which tend to have values between 1.2 and 2.7-fold. (Calow, 1974; Fitch, 1975; Brown, 1979; Crisp, 1979; Houlihan and Innes, 1982; Innes and Houlihan, 1985; Carefoot, 1989; Caldwell and Donovan, 2003; Thimgan, 2005). This implies that *H. asinina* has an enhanced ability to transport and utilize oxygen in the working muscles during locomotion. Unexpectedly, relative to other abalone, this ability to increase aerobic muscle work is not reflected in the activities of foot and adductor muscle enzymes unique to aerobic metabolism.

Crawling over a distance of 6 m in the aquarium trials resulted in decreased arginine phosphate and increased tauropine levels in both the foot and adductor muscles, and increased D-lactate levels in the foot muscle, relative to the resting controls. This shows that both arginine phosphate hydrolysis and anaerobic glycolysis are used to supplement aerobic ATP production during normal crawling behaviour. A similar conclusion was reached for crawling behaviour of *H. kamtschatkana* (Donovan et al., 1999).

4.5. Energy metabolism during righting behaviour

During righting behaviour experiments in which *H. asinina* were exercised to apparent exhaustion in oxygenated seawater, the total number of flips completed, the duration of righting behaviour and the flipping rate (see Table 3) were all much greater than observed during comparable trials with several species of temperate abalone: *H. lamellosa*, 12 to 15 min (Gäde, 1988); *H. iris*, 15–25 min (Baldwin et al., 1992); *H. kamtschatkana*, 25 flips at more than 1 min intervals, (Donovan et al., 1999); *H. rubra*, 9 flips in 40 min (Elias, 2003).

In the species other than *H. asinina*, this exercise always resulted in accumulation of tauropine in the adductor muscle, and more variably D-lactate in the adductor, tauropine and/or D-lactate in the foot muscle, and arginine phosphate depletion in either muscle (Gäde, 1988; Baldwin et al., 1992; Donovan et al., 1999; Elias, 2003).

The metabolite data for *H. asinina* (see Table 2) differ from this general pattern in that tauropine and D-lactate do not accumulate in either muscle, with arginine phosphate depletion in the adductor being the only change relative to the resting controls. This absence of additional ATP production from anaerobic glycolysis during righting behaviour might be explained by lower energy requirements associated with the greatly reduced shell weight, and/or a higher aerobic expansibility for *H. asinina*. The hemocyanin, in the absence of a Root effect, may help maintain environmental oxygen uptake during the observed fall in hemolymph pH that occurs during righting behaviour. Lower arginine phosphate concentrations in resting adductor and foot muscles of *H. asinina*, relative to other temperate abalone, also are consistent with reduced dependence on anaerobic muscle work (Beis and Newsholme, 1975; Gäde, 1988; Donovan et al., 1999).

4.6. Energy metabolism during exposure to air

In preliminary trials, *H. asinina* exposed to humid air for 4, 12 and 24 h recovered when returned to seawater, while animals exposed for 36 h did not revive. Initially it

was considered that the typical response of animals becoming flaccid, displacing the shell to one side and exposing a large surface area of moist upper foot muscle might aid oxygen uptake through the body surface as the gills collapsed when not supported by water; however failure to detect any oxygen uptake when *H. asinina* were exposed to air in the respirometer implies that any such contribution from additional body surfaces would be minimal. A similar conclusion was reached for *Haliotis iris* exposed to hypoxia (Taylor and Ragg, 2005). As the limit for detection of oxygen uptake in the respirometer is about 2 $\mu l\ O_2\ g^{-1}\ h^{-1}$, this indicates a reduction in oxygen uptake in air of at least 20-fold relative to animals at rest in water (see Table 5).

Aerobic metabolism may be maintained, at least in the short term, by drawing down oxygen stores held in the large volume of hemolymph. Estimates of maximum hemolymph oxygen stores based on *H. asinina* blood volume, hemocyanin concentration, and oxygen free in solution, suggest that at the resting rate of oxygen uptake measured in water, a 100 g animal would totally deplete this store in about 24 min. At the oxygen uptake rate measured during crawling in water, these stores would last about 5 min for an animal moving at 20.9 cm min^{-1} in air.

The effects of air exposure on anaerobic metabolites reported for the foot and adductor muscles of four species of temperate abalone are consistent in that both muscles reduce arginine phosphate reserves and accumulate tauropine and D-lactate (Baldwin et al., 1992; Wells and Baldwin, 1995; Donovan et al., 1999; Elias, 2003). In addition, similar metabolite changes have been observed in *H. lamellosa* maintained in anoxic seawater (Gäde, 1988). The absence of significant changes in these anaerobic metabolites in adductor and foot muscles of air exposed *H. asinina* (see Table 4) implies that arginine phosphate hydrolysis and anaerobic glycolysis are not being used as a source of ATP to support resting metabolism in air.

These results obtained for aerobic and anaerobic metabolism of *H. asinina* imply that air exposure essentially leads to a state of metabolic arrest that is reversible after 24 h, but not after 36 h. While these animals may not normally encounter prolonged air exposure, the oxygen concentration in shallow water on the Heron Island reef flats is highly variable, falling from 6.8 mg $O_2\ l^{-1}$ to as low as 1.2 mg $O_2\ l^{-1}$ at low tide on calm nights (Routley et al., 2002). Under these conditions, a reduced metabolic rate in hypoxic water, rather than during air exposure may have survival value that would not be required by temperate species inhabiting more oxygen-stable environments.

5. Conclusions

The Haliotidae are considered morphologically conservative archaeogastropods (Brown and Murray, 1992), with the greatly reduced shell of *H. asinina* representing an extreme departure from the usual body form. This conservative nature of the family also extends to the biochemical and physiological systems supporting energy metabolism. The blood oxygen transport system of *H. asinina* resembles those of temperate abalone in terms of a large hemolymph volume, similar hemocyanin concentrations, and in most hemocyanin oxygen binding properties. However absence of a Root effect appears confined to *H. asinina* hemocyanin. This property may assist oxygen uptake at the ctenidia when hemolymph pH falls during the elevated exercise of which the animal is capable, or when decreased water oxygen levels are encountered on shallow reefs.

The metabolic profiles of *H. asinina* foot and shell adductor muscles also are similar to those of other abalone. Common features are low activities of enzymes unique to aerobic ATP production, relatively high activities of arginine kinase, tauropine and D-lactate dehydrogenases as the predominant pyruvate reductases and low intracellular pH buffering capacities.

However, despite these general similarities among abalone, *H. asinina* exhibits several quite different metabolic responses to exercise and air exposure. Crawling speeds of *H. asinina* are not exceptionally high and, as in other abalone, there is some contribution from anaerobic metabolism. However the aerobic expansibility of 5.5-fold at speeds less than 20% of maximum is more than 2.7-fold greater than reported for several species of temperate abalone. This ability to elevate aerobic metabolism presumably also supports the enhanced frequency and duration of flipping behaviour without recourse to the additional inputs from anaerobic glycolysis required by other abalone.

During prolonged air exposure, *H. asinina* reduced oxygen uptake by at least 20-fold relative to animals at rest in aerated seawater. There was no evidence for significant ATP production from anaerobic glycolysis or arginine phosphate hydrolysis within the muscle mass that makes up 80% of total animal weight. Comparable data on oxygen uptake by other species of resting, air-exposed abalone could not be found in the literature. However at least four species are known to accumulate tauropine and D-lactate and to deplete arginine phosphate reserves under these conditions. This indicates that slowing down metabolism as a response to reduced environmental oxygen uptake probably is more extreme

in *H. asinina*, and may contribute to survival at water oxygen levels that are lower than normally encountered by most temperate abalone.

In answer to the original questions posed in this study, the exceptional ability of *H. asinina* for prolonged and rapid exercise has been confirmed. It is supported by a higher aerobic expansibility rather than by any enhanced capacity for anaerobic metabolism. It is unexpected, and instructive, that the greatly enhanced capacity of *H. asinina* for aerobic exercise is not more apparent in obvious adjustments of the blood oxygen delivery system or in muscle properties associated with aerobic ATP production. The absence of a hemocyanin Root effect, and the extent to which both aerobic and anaerobic metabolism can be reduced, may be special features that assist respectively aerobic exercise, and the survival of *H. asinina* when environmental oxygen is limiting.

Acknowledgements

We thank the Heron Island research Station, University of Queensland, for providing facilities used during this study, and Mr P. Domelow for constructing the respirometers. **[SS]**

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