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CAN THE END PRODUCTS OF ANAEROBIC METABOLISM, TAUROPINE AND D-LACTATE, BE USED AS METABOLIC STRESS INDICATORS DURING TRANSPORT OF LIVE SOUTH AFRICAN ABALONE *HALIOTIS MIDAE*?

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This study was undertaken to investigate whether the accumulation of end products of anaerobic metabolism can be used as an early indicator of deteriorating conditions during transport of live abalone *Haliotis midae*. A first series of experiments revealed that the enzyme tauropine dehydrogenase, responsible for the production of tauropine, is present in high activities (54 U g wet weight^{-1}) in the shell adductor muscle, but D-lactate dehydrogenase, responsible for the production of D-lactate, is the predominantly active enzyme (10 U g wet weight^{-1}) in foot muscle. The next series of experiments investigated the potential of anaerobic metabolism in the abalone by subjecting the gastropod to either functional anoxia (exercise metabolism) or 6 h of environmental anoxia (seawater gassed with nitrogen). Exercise, primarily powered by the shell adductor muscle, was mainly fueled by glycolysis resulting in the production of tauropine, whereas during 6 h of experimental anoxia, fermentation of glycogen led to the formation of mainly tauropine in the shell adductor muscle and mainly D-lactate in the foot muscle. The last experiment, investigating changes in these metabolites during simulated (abalone packed in oxygen-filled plastic bags resting on foam sponges soaked in seawater) transportation stress of up to 36 h at 7 and 10°C, clearly showed that tauropine accumulation in the shell adductor muscle and D-lactate accumulation in the foot muscle is time-dependent. Both metabolites are already produced during the first 6 h of simulated transportation (especially at 10°C), indicating that aerobic metabolism is impaired at an early stage of transportation. Hence, these metabolites can serve as indicators of the conditions abalone were subjected to during transport. Furthermore, abalone use the strategy of metabolic depression in this simulation experiment, as indicated by the decreased glycolytic flux in various tissues.

Key words: anaerobic metabolism, end products, *Haliotis midae*, live transport

Abalone *Haliotis* spp. are in considerable demand worldwide, especially in the Far East. This has encouraged industries that involve not only harvesting natural populations but also, as such populations have declined, setting up artificial cultures of appropriate species (Lo-Chai Chen 1990, Britz 1996, Knauer *et al.* 1996). Abalone farming has attracted the attention of investors as a viable commercial venture, resulting in emerging industries. Cocktail abalone, for example, fetch a high price in the world market (Gordon and Cook 2001). Abalone aquaculture has prospered during recent years in South Africa (Cook 1998), most of the production being destined for export.

For transportation of live abalone, speed of delivery is important; this is being done exclusively by air. The cost of air freight will always necessitate the use of light packing mass, excluding the possibility of transportation in tanks, as is also the case for crustaceans (Dichmont and Przybylak 1990, Dela-Cruz and Morris 1997). Abalone are therefore transported in cold containers with some humid packing material. However, one of the major problems faced by the industry is spoilage of the flesh during transport (James and Olley 1970, Baldwin *et al*. 1992). One of the reasons for spoilage may be the accumulation of metabolites when aerobic respiration is impaired during transport. It is well known that molluscs accumulate, besides lactate, a multitude of end products of anaerobic glycolysis, such as octopine, strombine, alanopine and tauropine, as a result of condensation reactions between pyruvate and the amino acids arginine, glycine, alanine and tauropine respectively, catalysed by the appropriate pyruvate dehydrogenase (reviewed by Gäde and Grieshaber 1986). These so-called opine dehydrogenases function in the same way as lactate dehydrogenase in regenerating cytoplasmic NAD^+ for the glyceraldehyde-3-phosphate dehydrogenase reaction of glycolysis.

The distribution of these pyruvate reductase enzymes and their end products is well known in some *Haliotis* species. *Haliotis discuss hannai*, *H. iris* and *H. lamellosa* exhibit both tauropine and D-lactate dehydrogenase in the foot and shell adductor muscle (Gäde 1986, Sato and Gäde 1986, Baldwin *et al.* 1992).

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Tissue-specific differences in anaerobic energy metabolism occurred in *H. lamellosa*: whereas glycolysis in the shell adductor muscle led mainly to the formation of the novel end product tauropine, D-lactate production predominantly occurred in the foot muscle during anoxia of 6 h duration (Gäde 1988). These results have been confirmed for two New Zealand abalone species, the blackfoot paua *H. iris* and the yellowfoot paua *H. australis* (Baldwin *et al.* 1992, Wells and Baldwin 1995). In the latter species, the authors speculate about using tauropine and D-lactate as metabolic stress indicators during transport after they had simulated this potential metabolic stress by keeping the abalone in moist air for up to 36 h (Baldwin *et al.* 1992; Wells and Baldwin 1995). The authors did not, however, use the exact conditions implemented by the industry for transporting these abalone species.

No data on anaerobic metabolism or transportation stress are available for the local South African abalone *H. midae*. The aim of the present study, therefore, was:

(i) to analyse the anaerobic potential of *H. midae* by determining the activities of the enzymes responsible for anaerobic glycolysis and measuring the corresponding anaerobic metabolites after extreme anaerobic conditions such as exercise to exhaustion (functional anoxia) and incubation in oxygen-free seawater for up to 6 h.
(ii) to quantify the anaerobic metabolites after incubation under simulated transport conditions that closely resemble those used by the abalone industry when air-shipping the gastropods live to the Far East.

MATERIAL AND METHODS

Experimental animals

H. midae were collected by divers from rocks at Onrus near Hermanus and from Cape Point (both locations in the Western Cape Province, South Africa). The size of the abalone was chosen to correspond to the usual size exported. Thus, the shell length was between 90 and 100 mm (measured as the distance between the posterior and anterior extremities of the shell). Specimens were removed from rock substrata using bar knives, without injuring the foot. Animals were transported to the laboratory on a flat, rigid plastic sheet in a polystyrene box ($44 \times 38 \times 26$ cm). The temperature inside the box was kept low by means of an ice block. On arrival in the laboratory, abalone were placed in glass aquaria ($90 \times 32 \times 37$ cm) in circulating, aerated seawater at an ambient temperature of 15°C. They were kept for two weeks before beginning the experiment. They were fed on kelp, their dominant food in their natural habitat (Fallu 1991, Branch *et al*. 1994); fresh kelp being provided twice a week. Abalones adhered to the glass walls of the aquarium and were difficult to remove; therefore, they were in good condition and no damage to the foot was experienced.

Sample preparation

Abalone were removed individually from the aquarium and shucked immediately by applying thumb pressure to the posterior edge of the adductor muscle where it abuts the shell. The shell adductor, foot and gill were excised for analysis of enzyme activities.

About 1–5 g wet weight of tissues were diced with scissors and homogenized (1:5 w/v) in ice-cold, 50 mM imidazole buffer, pH 7.2, containing 1 mM EDTA and 1 mM dithiothreitol (DTT), using an Ultra Turrax T-25 homogenizer. The homogenate was centrifuged at $20\,000 \times g$ for 20 minutes at 4°C. The supernatant was kept aside. The pellet was rehomogenized in the same buffer as above and, after centrifugation, the supernatants were pooled.

Enzyme assays in crude extract

The following enzyme activities were determined in the supernatant: tauropine dehydrogenase (TDH), lactate dehydrogenase (LDH), octopine dehydrogenase (ODH), strombine dehydrogenase (StrDH), alanopine dehydrogenase (AlaDH) and malate dehydrogenase (MDH). All determinations were carried out at 25°C in cuvettes with a final volume of 1 mℓ. The oxidation of NADH was monitored at 340 nm using a Vitatron Universal photometer equipped with a recorder. One unit of enzyme activity (1U) is taken as the amount of enzyme causing transformation of 1 µmol of substrate min^{-1} under standard condition. Determinations were performed at least in duplicate with different concentrations of extract to ensure linearity. The following conditions prevailed:

Tauropine dehydrogenase (*EC 1. 5. 1. X*) 2.5 mM pyruvate, 0.15 mM NADH, 50 mM imidazole-HCl buffer, pH 7.0. The reaction was started with taurine (80 mM).
Lactate dehydrogenase (*EC 1. 1. 1. 28*) 0.15 mM NADH, 50 mM imidazole-HCl buffer, pH 7.0. The reaction was started with pyruvate (2.5 mM).
Octopine dehydrogenase (*EC 1. 5. 1. 11*) 2.5 mM pyruvate, 0.15 mM NADH, 50 mM imidazole-HCl buffer, pH 7.0. The reaction was started with arginine (5 mM).
Strombine dehydrogenase (*EC 1. 5. 1. 22*) 2.5 mM pyru-

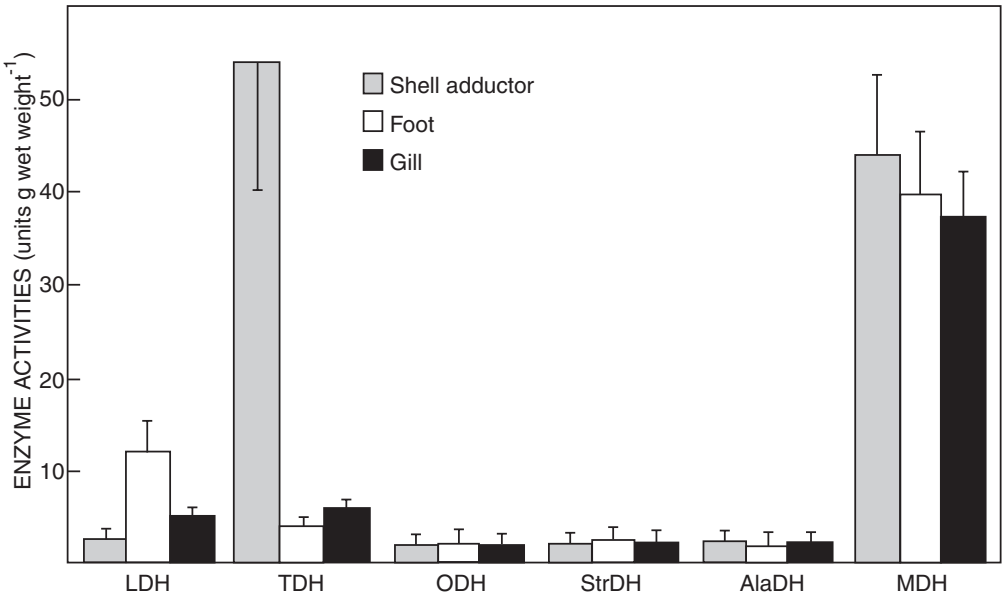

Fig. 1: Maximal activities of lactate dehydrogenase (LDH), tauropine dehydrogenase (TDH), octopine dehydrogenase (ODH), strombine dehydrogenase (StrDH), alanopine dehydrogenase (AlaDH), and malate dehydrogenase (MDH) in various tissues of *H. midae.* Bars denote 1 *SD* ($n = 7$)

vate, 0.15 mM NADH, 50 mM imidazole-HCl buffer, pH 7.0. The reaction was started with glycine (200 mM).

Alanopine dehydrogenase (*EC 1. 5. 1. 17*) 2.5 mM pyruvate, 0.15 mM NADH, 50 mM imidazole-HCl buffer, pH 7.0. The reaction was started with alanine (100 mM).

Malate dehydrogenase (*EC 1. 1. 1. 37*) 0.15 mM NADH, 50 mM imidazole-HCl buffer, pH 7.0. The reaction was started with oxaloacetate (0.5 mM).

Environmental anoxia: incubation of *H. midae* in oxygen-free seawater

Nine abalone were incubated in pairs in an aquarium ($21 \times 40 \times 17$ cm) filled with seawater (15°C) that had been gassed with pure nitrogen until PO_2 (monitored with an oxygen electrode, Syland Scientific) reached almost zero mm Hg. The oxygen electrode was calibrated with air-saturated seawater. After the animals were inserted, the aquarium surface was covered with a polystyrene sheet and the water flushed with a constant, slow stream of nitrogen gas. After 6 h of anoxic incubation, the animals were removed, and the shell adductor, foot and gill tissues excised, blotted and frozen in liquid N_2 then stored at -80°C. A further nine abalone were used as controls by placing them in well-aerated seawater at 15°C. After 6 h in normoxia, the animals were removed and their tissues treated as above.

Functional anoxia: exercise of *H. midae* in normoxic seawater

Nine individual abalone were exercised individually for 15–18 minutes in an aquarium ($25 \times 25 \times 25$ cm) at 15°C in well-aerated seawater. The animals were placed upside-down on their shells; their righting movements involved vigorous contractions of the shell adductor and foot muscles (see Gäde 1988). When the animals had regained their normal posture, they were immediately inverted again. The procedure was repeated for up to 18 minutes, when the animal could no longer make any muscular movement and appeared to be too exhausted to right itself. Animals were then killed, the shell adductor, foot and gill tissues excised, frozen in liquid N_2 and stored at -80°C.

Transport simulation studies on *Haliotis midae*

In this experiment, the simulated transportation protocol

was designed to be as similar as possible to that used in commercial operations. A clear plastic bag (74 cm long, 54 cm wide) was packed with foam sponges (2.5 cm thick) soaked in seawater. A perforated polystyrene sheet was placed on top of the sponges with an ice block underneath. Abalone were removed from seawater (7 and 10°C respectively) and packed into the plastic bag resting on the polystyrene sheet and then flushed with pure oxygen. The bag was closed and placed in a polystyrene box (44 × 38 × 26 cm). Polystyrene chips were packed into the box. The lid of the box was then closed with adhesive tape. The boxes were placed in temperature-controlled rooms at 7 and 10°C respectively and 5–6 individuals removed after 6, 12, 24 and 36 h. The adductor, foot and gill tissues were excised, frozen in liquid N_2, then stored at -80°C for further analysis.

Metabolite assays

Neutralized perchloric acid extracts were prepared from the frozen tissues of *H. midae* according to previously published methods (Gäde *et al.* 1978, Carlsson and Gäde 1986). D-lactate and tauropine concentrations were determined as outlined in Gäde (1988); the enzyme tauropine dehydrogenase was purified from the shell adductor muscle according to a method slightly modified from that of Gäde (1987). Briefly, a crude extract of shell adductor muscle was subjected to ammonium sulphate precipitation (between 45 and 80%) and subsequently processed by anion exchange chromatography on DEAE-Sephacel. During this step, tauropine dehydrogenase was purified from its main contaminant, malate dehydrogenase, which eluted at a slightly higher NaCl concentration. Glycogen was measured as glycosyl units as outlined previously (Zebe and Gäde 1993).

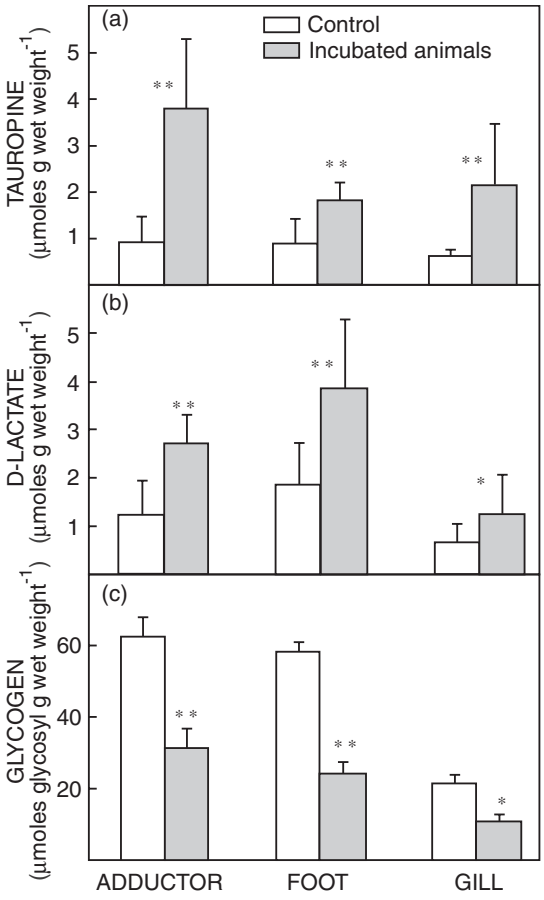

Fig. 2: Concentrations of (a) tauropine (b) D-lactate and (c) glycogen in shell adductor, foot and gill of *H. midae* in a controlled experiment and when incubated for 6 h in oxygen-free seawater. Bars denote +*SD* (n = 9). * $p \leq 0.05$, ** $p \leq 0.01$ (Student's *t*-test)

RESULTS

Activities of dehydrogenases from crude extracts of various tissues of *H. midae*

Maximum activities of pyruvate reductases, namely lactate dehydrogenase (LDH), tauropine dehydrogenase (TDH), octopine dehydrogenase (ODH), strombine dehydrogenase (StrDH) and alanopine dehydrogenase (AlaDH) in various tissues of *H. midae* were measured. As depicted in Figure 1, TDH showed the highest activity in adductor muscles, whereas LDH was highest in foot muscle. Activities of ODH, StrDH and AlaDH in adductor muscles were all very similar and very low in both muscle tissues. Activity of TDH in the foot and gill was <10% that of the adductor muscle. MDH was found in high activities in all tissues.

Environmental and functional anoxia

In order to gauge the full potential of anaerobic capacity

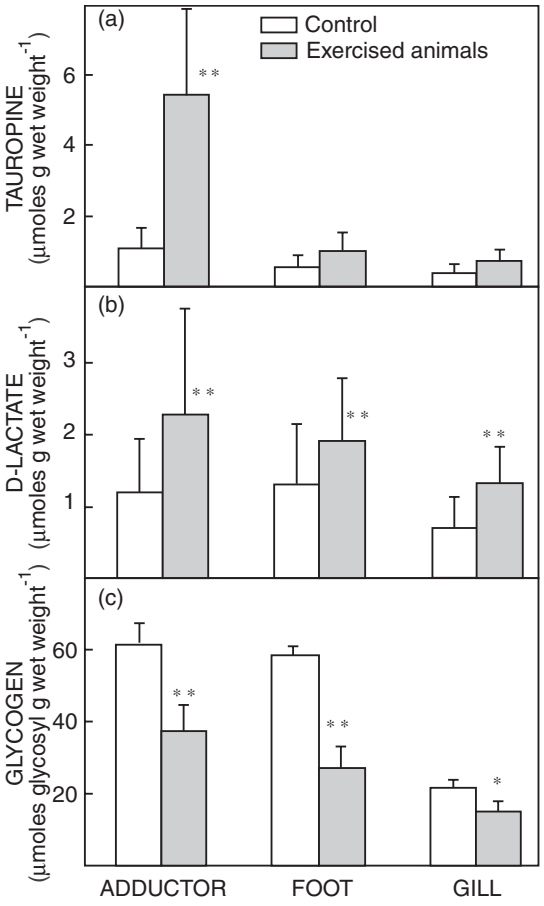

Fig. 3: Concentrations of (a) tauropine (b) D-lactate and (c) glycogen in shell adductor, foot and gill of *H. midae* in a control experiment and when exercised for 18 minutes. Bars denote +*SD* ($n = 9$). * $p \leq 0.05$, ** $p \leq 0.01$ (Student's *t*-test)

in *H. midae* and to quantify the amount of anaerobic end products accumulating during extreme conditions, the abalones were subjected to two regimes of anoxia, i.e. animals were kept in oxygen-free seawater for 6 h and animals had to perform maximal work in well-aerated seawater.

ENVIRONMENTAL ANOXIA

In the early stages of the experiment in anoxic conditions, the animals maintained strong pedal and shell adductor tonus; it was difficult to remove them from the aquarium substratum, and they responded actively to poking stimuli. After an incubation time of 6 h, foot muscles were soft with less energetic responses to poking. The animals showed excessive mucus production and kept the mantle cavity wide open. The animals appeared shrunk within the shell and the flesh was not firm. They were in an emaciated condition; the foot protruded much further from the shell margin and was flaccid. The animal was easily removed from the substratum. When held out of water, the body took the shape of an inverted mushroom. When turned upside-down, muscle movement was not strong enough to right the animal.

EXERCISE TO EXHAUSTION

During the initial stages of exercise, when the animal was turned upside-down, there was much movement of the foot and shell adductor to right itself to the normal position. After about 18 minutes of exercise, the animal appeared to be completely exhausted and stayed in the upside down position on its shell with no further sign of muscular movements to right itself.

Metabolite concentrations during environmental and functional anoxia

ENVIRONMENTAL ANOXIA

Anoxic incubation for 6 h resulted in a significant increase in the concentration in tauropine in all three tissues investigated, but the accumulation in the adductor muscle was highest by a factor of about four (Fig. 2a). D-lactate levels were also higher in all tissues; the quantitatively highest accumulation was in foot muscle (Fig. 2b). Glycogen reserves in the tissues revealed the highest concentration in the muscles (Fig. 2c). During anoxic incubations, this substrate was used extensively and all tissues showed a decrease between 40% (foot) and 50% (adductor muscle).

FUNCTIONAL ANOXIA

Exercise had a profound effect on the concentration of tauropine in the adductor muscle (five-fold increase), but this metabolite was not significantly elevated in the other tissues (Fig. 3a). In contrast, D-lactate increased significantly in all tissues, but this was quantitatively not a large increase (Fig. 3b). Although the duration of exercise was relatively short, namely 18 minutes compared to the 6 h of anoxic incubation in the previous

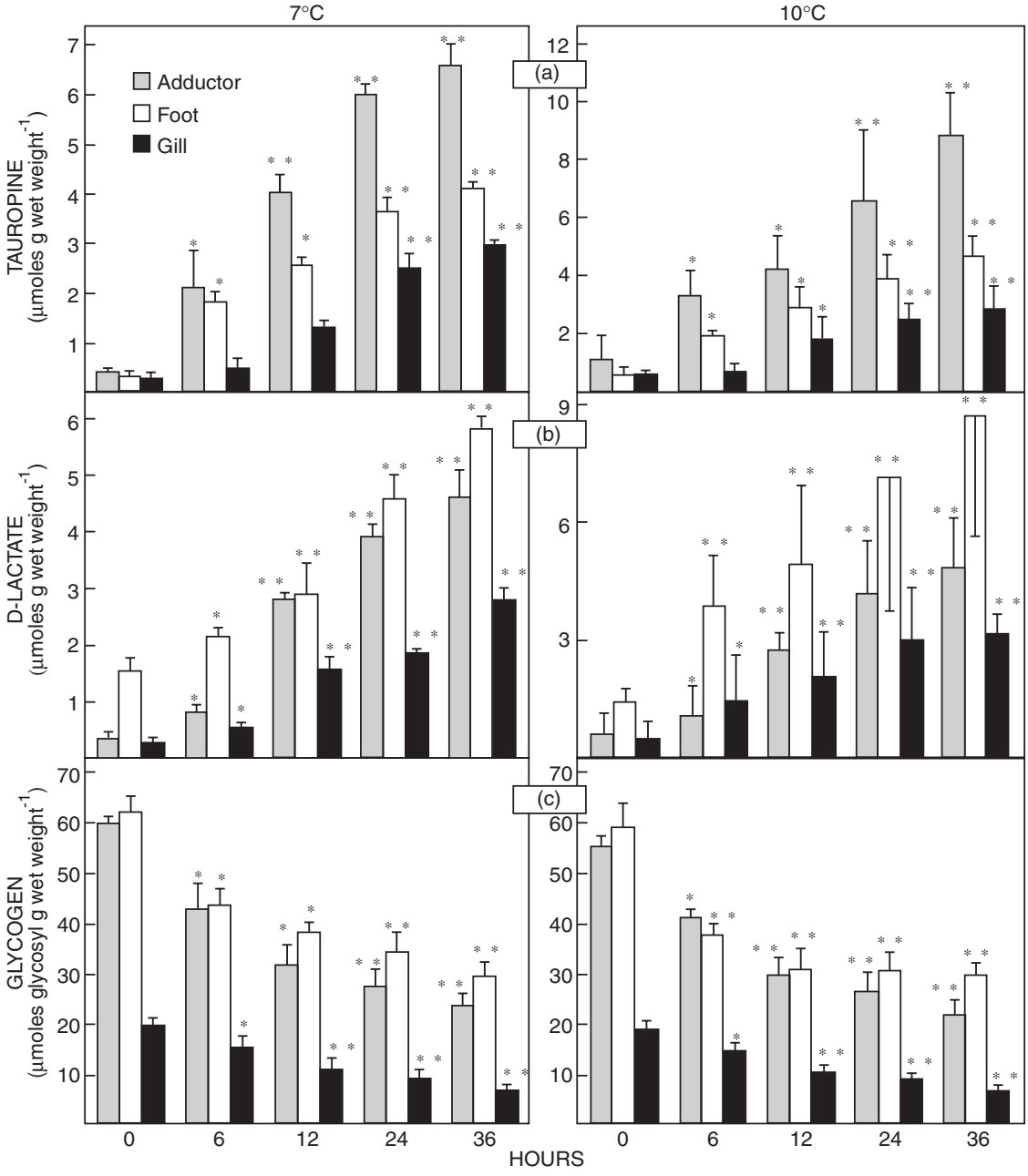

Fig. 4: Concentrations of (a) tauropine (b) D-lactate and (c) glycogen adductor, foot and gill of *H. midae* during various periods of transport simulation at 7 and 10°C. Bars denote +*SD* ($n = 5-6$). * $p \leq 0.05$, ** $p \leq 0.01$ (Student's *t*-test)

experiment, the glycogen stores were depleted significantly in all tissues after exercise (Fig. 3c). Adductor muscle tissue consumed 40% of its stored glycogen, foot muscle 50%, but gills only 33%.

Metabolic responses to transport stimulation

When live abalone were subjected to simulated transport conditions, their metabolites were analysed after various durations of stress and at two different temperatures. Because there were no qualitatively different changes between the two temperature regimes, and also because the quantitative changes in metabolites were not significantly different between the two regimes, those results are not reported separately, but the individual data are shown. It is clear from Figure 4a that tauropine increased over time in all tissues investigated and that a significant accumulation was already measured after 6 h of transportation stress. It appears that the production rate was more or less linear during the first 12 h and then slowed considerably. Whereas tauropine increased most in shell adductor muscle (from about 1.1 to 8.7 μmoles g^{-1} wet weight during the 36 h period; Fig. 4, 10°C), it accumulated only to about 30% of the adductor muscle value in gills (Fig. 4a, 10°C). Conversely, D-lactate accumulated highest in foot muscle (Fig. 4b).

Glycogen concentrations in all three tissues diminished significantly during the investigated period (Fig. 4c). For the muscle tissues (adductor and foot muscle), which started with high concentrations of glycogen, the initial decrease during the first 6–12 h of stress was much faster than during the later stage (about a seven-fold difference), whereas the rate of glycogen breakdown in gill tissue only decreased fourfold between the initial and later period (Fig. 4c).

DISCUSSION

The South African abalone, like other abalone from Europe (*H. lamellose*; Gäde 1986, 1988) and New Zealand (*H. iris* and *H. australis*; Baldwin *et al.* 1992, Wells and Baldwin 1995), contains only TDH and D-LDH in their main muscle tissues. The very low, but seemingly measurable, activities of octopine-, strombine- and alanopine dehydrogenases using the substrates arginine, glycine and alanine respectively in the cuvette are easily explained by a non-specific reaction of TDH. It has been shown numerous times for opine dehydrogenase that they exhibit low activity with other amino acid substrates (see Gäde and Grieshaber 1986 for a review). For example, TDH of *H. lamellosa* shows 5% of its maximal activity measured with taurine when the amino acid alanine was used (Gäde 1986).

As found for *H. lamellosa* (Gäde 1986), D-LDH is the predominant pyruvate dehydrogenase in the foot muscle of *H. midae* and TDH the main one in shell adductor muscle. In New Zealand abalone, however, TDH is also more active in the foot muscle (Baldwin *et al.* 1992, Wells and Baldwin 1995). One simple explanation for this may be the site from which the investigated foot tissue has been taken. In the present study and those of Gäde (1986, 1988), the site used was the anterior edge of the foot, just below the head of the abalone, because more central parts of the foot also contain muscle fibres belonging to the shell adductor muscle. If, by any chance, such tissue would be included in the enzymatic analysis, higher levels of TDH activity would result for "foot" tissue.

Both enzymes TDH and D-LDH are functionally active during lack of oxygen in *H. midae*. During such anaerobic conditions, glycogen is the only substrate and its consumption is clearly shown during the extreme conditions to what the abalone were subjected, namely 6 h incubation in oxygen-free seawater and exercise until exhaustion. During exercise, mainly the adductor muscle is involved in generating muscle output, and this is reflected in a high glycolytic flux of 149 nmole glycosyl units g wet $weight^{-1}$ min^{-1} compared to the 29 nmole glycosyl units g wet $weight^{-1}$ min^{-1} calculated for foot muscle. This high glycolytic flux is met by producing energy anaerobically, and its end product is tauropine. Thus, the function of TDH is to maintain redox balance during activity. Exercise is a very natural function for the shell adductor muscle whose task it is to right the animal when it has been detached from the rock during vigorous wave action. The foot muscle is not involved in such an action and, consequently, no tauropine accumulation is found in the foot tissue during exercise. There was, however, a slightly higher lactate concentration in the tissues of both the adductor muscle and foot, after exercise. This means that LDH was active as well to a certain extent, but much less so than TDH. Similar results have been reported after exercise in *H. lamellosa* (Gäde 1988) and *H. iris* (Baldwin *et al.* 1992). In the latter species, however, tauropine levels also increased in the foot during exercise, in keeping with the finding of a high activity of TDH in this tissue (see above).

The other extreme anaerobic situation tested was to keep abalone for 6 h in oxygen-free seawater, which appears to be a quite unnatural situation for an epifaunal gastropod in the eulittoral zone, which is most of the time covered by seawater, and maybe only occasionally exposed to air during spring tide (Tarr 1992). Occasionally, however, certain coastlines of South Africa, especially sheltered bays such as St Helena

Table I: Glycolytic flux in shell adductor, foot and gill tissue of *H. midae* during various periods of transport simulation at 7 and 10°C, calculated from production of the end products tauropine and D-lactate (see Fig. 4)

Muscle site	Temperature (°C)	Glycolytic flux (nmoles glycosyl units g wet weight^{-1} min^{-1})			
		0–6 h	6–12 h	12–24 h	24–36 h
Adductor	7	3.08	5.40	2.11	0.88
	10	3.60	3.56	2.61	1.94
Foot	7	2.92	2.02	1.88	1.19
	10	5.25	2.68	2.22	1.60
Gill	7	0.63	2.50	1.00	0.94
	10	1.46	2.27	1.11	0.28

Bay, are subject to red tide events, resulting in very low oxygen concentrations in the seawater (Pitcher and Calder 2000). The present experimental anoxia may therefore be a simulation experiment to test the ability of *H. midae* to cope with such a situation. The interpretation of the behavioural observations reveals that abalone were just able to survive 6 h of total anoxia. It can be assumed that this was about the maximum time this species can tolerate lack of oxygen. The biochemical data point to a slow anaerobic metabolism. The glycolytic flux was only 1/25 and 1/7 of the rate calculated during exercise for adductor muscle and foot respectively (i.e. 6 and 4 nmole glycosyl units g wet weight^{-1} min^{-1}), and the energy was produced anaerobically by mainly tauropine production in the adductor muscle and mainly D-lactate production in the foot, although smaller concentrations of D-lactate and tauropine were also accumulated in the adductor muscle and foot respectively. This experiment clearly demonstrates that *H. midae* can survive extreme anoxic conditions for a period. This gave hope that, during a transport simulation experiment, metabolite levels may be a good indicator of the determination of aerobic or anaerobic conditions during transport.

It became clear that aerobic metabolism in *H. midae* is impaired with time and that abalone are using two well-known biochemical adaptations to cope with such a situation. First, metabolism is depressed, which can be seen from the calculated glycolytic flux for adductor muscle and foot. The flux is higher during the first 12 h of the simulation experiment and slows down considerably with time (Table I). Second, anaerobic glycolysis is providing the energy and the two pyruvate reductase end products, tauropine and D-lactate, are produced and accumulate to an impressive concentration. It is speculated, as for other abalone (Baldwin *et al.* 1992), that during transport internal PO_2 in the tissue falls, very likely by an impairment of the ability to irrigate the gills effectively; it triggers anaerobic energy metabolism. Build-up of tauropine/D-lactate is already evident after 6 h simulation. These end products could therefore serve as an indicator of prevailing conditions during transport of *H. midae*. Because tauropine and D-lactate are very stable once the muscle tissue is frozen and stored, biopsy samples taken from animals immediately after shipment can then be used for recording shipment history. At present, it is not known whether taste/smell (and consequently marketability of abalone) is influenced by accumulation of tauropine/D-lactate. It is only clear that, during recovery from anoxic conditions in well-aerated seawater, these metabolites are oxidized again and pre-anoxic levels are found after about 12 h (Gäde 1988, Baldwin *et al.* 1992). It is therefore recommended that abalone should be kept under such oxygenated conditions after prolonged transport.

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