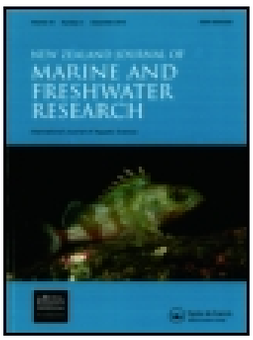

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Strategies of anaerobiosis in New Zealand infaunal bivalves: adaptations to environmental and functional hypoxia

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Abstract Under hypoxic environmental conditions, slowly burrowing bivalves such as the pipi (*Paphies australis*) and cockle (*Austrovenus stutchburyi*) support anaerobic energy production through the pyruvate reductase enzymes strombine dehydrogenase and alanopine dehydrogenase. Rapidly digging surf clams such as the tuatua (*P. subtriangulatum*) and trough shell (*Mactra discors*) do not fare as well at low oxygen levels, but perform better under the functional anaerobiosis generated by burrowing, and energy production is supported by octopine dehydrogenase. Muscle buffering capacity is higher in rapidly burrowing species and is correlated with total pyruvate reductase activity. Anaerobic pathways in *P. subtriangulatum* led to both D-lactate and octopine accumulation during environmental hypoxia, but only octopine accumulated during burrowing. Bivalves adapted to environmental hypoxia maintained high adenylate energy charge (AEC) under anaerobiosis, indicating a close matching of ATP production to consumption. AEC fell in *P. subtriangulatum* during environmental hypoxia. The significance of these findings is discussed within an ecological context, and in relation to the storage of live clams for the seafood industry.

Keywords bivalves; anaerobiosis; pyruvate reductase; buffering capacity; energy charge; lactate; octopine

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INTRODUCTION

An organism's potential for anaerobiosis may govern many aspects of its ecology and behaviour. The lack of a constant oxygen supply characterises the near-environment of many intertidal and infaunal animals and results in some spectacular tolerances (De Zwaan 1977; Hochachka & Somero 1984). Bivalves have evolved diverse and highly specialised strategies for surviving hypoxic episodes including pathways that are efficient both in terms of ATP production, and in minimising H^+ and toxic end product accumulation (Hochachka & Mommsen 1983; Livingstone 1991). Under these circumstances, glycogen is metabolised to pyruvate and the redox ratio of NADH/NAD^+ in the cytosol is balanced by the reduction of pyruvate to lactate. Alternatively, NAD^+ can be recycled more efficiently by the coupling of an amino acid to pyruvate, resulting in the formation of opines such as alanopine, tauropine, octopine, and strombine (Gäde 1983; Gäde & Grieshaber 1986; Livingstone 1991). Consequently, bivalves may accumulate one or more of these novel opine end products.

A reduction in metabolic rate seems sensible during environmental hypoxia (Storey 1988), but a different problem arises during muscle work when energy demand remains high and a brief functional hypoxia ensues. Organisms regularly experiencing environmental hypoxia are generally associated with relatively high efficiency, but low rates of energy production; this contrasts with lower efficiency, and high rates of energy production in rapidly burrowing or swimming organisms developing a functional hypoxia (Livingstone 1991). Thus, the strategy of anaerobiosis is expected to vary with both the nature of the anaerobic stress, and from species to species.

In this study, we have taken a comparative approach to investigate adaptations in selected infaunal bivalves that enable them to resist environmental and functional hypoxia. The species selected are currently harvested commercially and have the potential to support a lucrative export

market. Specific pyruvate reductase enzymes have been identified and their activities determined and related to muscle buffering capacity. Hypoxic tolerances and metabolic status have been evaluated for several intertidal bivalves. Finally, the dynamics of metabolite accumulation in the intertidal surf clam, *Paphies subtriangulatum*, have been examined under the experimental challenge of functional and environmental hypoxia.

MATERIALS AND METHODS

Collection of animals

Paphies subtriangulatum, Mactra discors, and *Dosinia anus* were collected intertidally from Orewa beach, on the north-east coast of New Zealand (36°35′S, 174°42′E), and *Austrovenus stutchburyi* and *Paphies australis* from the Orewa estuary nearby. *Paphies ventricosum* was collected from Muriwai beach (36°35′S, 174°14′E). *Spisula aequilateralis* and *Paphies donacina* were dredged subtidally from Marlborough Sounds (41°00′S,174°05′E). Bivalves were transported in autochthonous sand and aerated sea water and placed in our aquarium facility.

Enzyme extraction

Enzyme activity was determined in both the foot and adductor muscles of normoxic specimens. Muscle was excised, weighed, and 0.5–1 g of minced tissue was placed in a 10-fold dilution of ice cold 50 mmol l^{-1} sodium phosphate buffer (pH 7.0). Tissues were homogenised for 30 s at 20 500 rpm with an Ultra Turrax T25 homogeniser fitted with an 8 mm dispersing tool (Janke and Kunkel, Germany). Homogenates were centrifuged at 7500*g* for 3 min and the supernatant assayed.

Pyruvate reductase activity

Pyruvate reductase enzymes were assayed spectrophotometrically at 20°C by monitoring the reduction of NADH at 340 nm in the presence of pyruvate and amino acids as described in Baldwin et al. (1992). Enzyme activity is reported in international units (i.u.) and defined as the amount causing the conversion of 1 µmol of substrate per min.

Muscle buffering capacity

The buffering capacity due to non-bicarbonate buffers was determined as described by Castellini & Somero (1981), and modified by Morris & Baldwin (1984). Foot and adductor tissues were taken from 10 replicates of the 4 intertidal species: *M. discors, P. subtriangulatum, P. australis*, and *A. stutchburyi*. Approximately 0.5 g of tissue was weighed and homogenised at 20 500 rpm for 45 s in ice-chilled 1.2% NaCl. The homogenate was brought to approximately pH 6 with 0.2 mol HCl l^{-1} and titrated at 20°C with aliquots of 0.2 mol NaOH l^{-1} to approximately pH 7, noting the amount added and the pH change. The buffering capacity (ß) was measured in Slykes, equivalent to µmol base required to titrate the pH of 1 g wet weight of tissue by one pH unit over the range pH 6–7 (Morris & Baldwin 1984). Individual titrations were linear between pH 5.9 and pH 7.1, and buffering capacity was therefore determined from the titration slope.

Comparative resistance to environmental hypoxia

Animals were placed in oxygen-free sea water prepared by spargling with nitrogen gas and monitored with an Orion 840 dissolved-oxygen meter. Sealed 5-litre containers with autochthonous sediment were used for each species and survival estimated as the time elapsed until an animal no longer responded to a tactile stimulus to the foot or adductor muscle.

Comparative burrowing rates and functional hypoxia

Laboratory-acclimated bivalves were carefully exposed from sediment and replaced on the surface. The time was recorded from the first protrusion of the foot into the sand, and continued until the shell was no longer visible. A burrowing rate index (BRI) was calculated to correct for body mass:

$$BRI = \{\sqrt[3]{\text{mass (g)}}/\text{burrowing time (s)}\} \times 100$$

(Trueman 1983).

Analytical methods for metabolites and pH

Frozen tissue was placed in a liquid-N_2 pre-cooled stainless steel tissue crusher and fractured into a fine powder. Approximately 0.25 g tissue was extracted in 1.5 ml ice-cold 0.6 mol PCA l^{-1} and homogenised for 20 s. The supernatant of the centrifuged extract was neutralised to pH 6.8–7.0 with 2 mol KOH l^{-1}, further centrifuged, and frozen at –80°C.

Adenine nucleotides were determined by reverse-phase high-performance liquid chromatography (HPLC) modified from Ryder (1985).

Extracts were injected onto a Lichrosorb RP–18 column supported by a Waters 501 system and eluted isocratically with pH 7.1 orthophosphate buffer. Peaks were quantified from external standards using Maxima 820 software. Adenylate energy charge (AEC) was calculated according to Atkinson (1968) using the following formula:

$$AEC = ([ATP] + 0.5\,[ADP])/([ATP] + [ADP] + [AMP]).$$

Assays for D-lactate and octopine were made with a Sigma (St Louis, MO) kit no. 826-B with either D-LDH or octopine dehydrogenase substitution for the respective metabolites.

Dynamics of environmental and functional hypoxia in *P. subtriangulatum*

Clams were kept in oxygen-free sea water as described above, and in aerated sea water controls. Specimens were removed at 6, 12, 18, 36, and 48 h intervals and the foot rapidly removed and freeze-clamped in liquid nitrogen. Foot removal was greatly facilitated by cutting the whole bivalve in half with a pair of Stanley 307 bolt cutters, thus preventing the foot from contracting and performing uncontrolled muscular work. Metabolites were assayed as described above.

Foot muscle pH was determined in *P. subtriangulatum* rested, exercised for 6 digging cycles, and hypoxically exposed for 48 h. After each treatment, the foot was extracted as above, and tissue pH derived as described by Wong et al. (1991).

Statistical analysis

A two-way factorial ANOVA was used to identify differences between species and tissues, and individual differences in the slope of ß (buffer capacity). To aid in the comparison of ß for tissues and species, simultaneous 95% Bonferroni confidence intervals were used (SAS Corporation, North Carolina). Student's *t*-test was used where appropriate.

RESULTS

Enzyme activities

Our survey of pyruvate reductases is summarised in Table 1 and reveals marked differences both in activity and enzyme type. *Austrovenus stutchburyi, Spisula aequilateralis,* and *Paphies australis* have low activities of D-LDH (lactate dehydrogenase), and appreciable activities of SDH (strombine dehydrogenase) and AlnDH (alanopine dehydrogenase), whereas *Mactra discors, Paphies donacina, Paphies ventricosum,* and *Paphies subtriangulatum* have D-LDH and ODH (octopine dehydrogenase) activity. *Dosinia anus* has activity for all four enzymes. Interspecific activities varied by nearly two orders of magnitude and imply

Table 1 Pyruvate reductase activities in foot (F) and adductor (A) muscles of infaunal bivalves. D-LDH, D-lactate dehydrogenase; ODH, octopine dehydrogenase; SDH, strombine dehydrogenase; AlnDH, alanopine dehydrogenase. Mean activities ± SE (*N* observations) were recorded as mean µmol substrate min^{-1} (g wet wt)$^{-1}$ at 23°C. n.d., not detectable: < 0.1 i.u.

Species	*N*	Tissue	D-LDH	ODH	SDH	AlnDH
Veneridae						
Austrovenus stutchburyi	7	F	3.3 ± 0.19	n.d.	9.4 ± 0.4	8.0 ± 0.6
	7	A	n.d.	n.d.	8.1 ± 0.5	5.6 ± 0.2
Dosinia anus	7	F	3.7 ± 0.4	35.2 ± 4.1	71.0 ± 3.2	49.9 ± 2.7
	7	A	0.7 ± 0.4	12.2 ± 0.9	30.4 ± 1.5	22.9 ± 1.0
Mesodesmatidae						
Paphies australis	7	F	3.5 ± 0.4	n.d.	22.9 ± 2.2	13.7 ± 0.7
	7	A	0.7 ± 0.1	n.d.	18.8 ± 1.1	9.1 ± 0.6
Paphies donacina	1	F	7.0	48.6	3.5	n.d.
Paphies ventricosum	1	F	17.5	156.2	n.d.	n.d.
	1	A	7.6	73.4	n.d.	n.d.
Paphies subtriangulatum	7	F	5.4 ± 0.3	92 ± 7.0	n.d.	n.d.
	7	A	2.1 ± 0.2	60.7 ± 3.2	n.d.	n.d.
Mactridae						
Spisula aequilateralis	1	F	11.6	n.d.	15.5	16.3
Mactra discors	7	F	2.9 ± 0.3	106.0 ± 3.0	n.d.	n.d.
	7	A	0.6 ± 0.1	88.9 ± 3.7	n.d.	n.d.

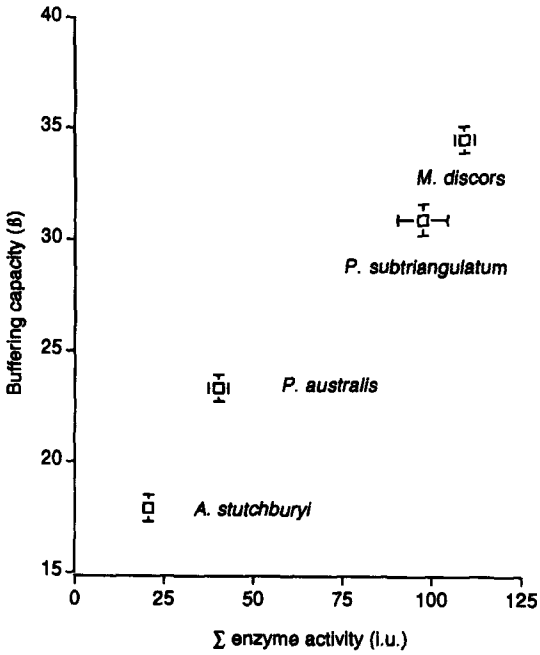

Fig. 1 Relationship between foot muscle buffering capacity and total pyruvate reductase activity in four intertidal clams (means ± SE).

considerable differences in anaerobic scope. Surprisingly, the conspecifics *P. australis* and *P. subtriangulatum* differ in reductase activity, and qualitatively in enzyme type.

Buffering capacity

Mactra discors, P. subtriangulatum, P. australis, and *A. stutchburyi* showed no intraspecific differences between foot and adductor muscles. However, a two-way factorial ANOVA indicated significant species differences ($F = 55.49$; $P < 0.05$). Buffering capacities of the foot corresponded with total pyruvate reductase activities (Fig. 1) and the observed burrowing rate of the species: *M. discors* > *P. subtriangulatum* > *P. australis* > *A. stutchburyi.*

Table 2 Species differences in range of tolerance to low oxygen levels.

Species	*N*	Anoxic survival
M. discors	15	30–35 h
P. subtriangulatum	20	36–48 h
P. australis	20	5–7 days
A. stutchburyi	20	7–7 days

Burrowing rates and anoxic tolerance

The athletic abilities of the four species is quantified by the burrowing rate index (Fig. 2A) which closely matches both the relative proportions of foot muscle (Fig. 2B). Table 2 shows the marked species differences in tolerance to low environmental oxygen levels and the order is inversely correlated with burrowing performance.

Metabolic end product accumulation and adenylate energy charge

The different pattern of lactate and octopine accumulation during hypoxic incubation and burrowing cycles for *P. subtriangulatum* is illustrated in Fig 3. Both D-lactate and octopine accumulated during environmental hypoxia, but only octopine accumulated under functional hypoxia.

Energy charge was not significantly different between exercise and rest for either *P. subtriangulatum* or the less active *P. australis*. However, from Fig. 4A it is evident that hypoxic exposure of *P. subtriangulatum* resulted in a sharp decline in muscle ATP and a corresponding rise in AMP, and hence a fall in energy charge from 0.83 to 0.40 ($P < 0.05$). By contrast, the less active *P. australis* maintained ATP and hence energy charge ($F = 1.63$: $P > 0.05$) over the longer period of exposure to hypoxia (Fig. 4B).

No difference in pH could be observed between rested (pH 6.65 ± 0.02) and hypoxic (6.67 ± 0.05) foot muscle from *P. subtriangulatum* ($P > 0.05$) suggesting that 48 h exposure does not result in significant accumulation of H^+. However, after six burrowing cycles, foot tissue pH fell to 6.37 ± 0.03 ($P < 0.05$).

DISCUSSION

Pyruvate reductase activity and buffering capacity

The distribution of pyruvate reductases showed no taxonomic pattern, but was strongly aligned with the ability to tolerate environmental hypoxia or to perform muscular work. The rapidly digging surf clam species *M. discors, P. ventricosum,* and *P. subtriangulatum* have high ODH activity, whereas *P. australis* and *A. stutchburyi* (that inhabit sheltered inlets and estuaries) have moderate activities of SDH and AlnDH. Livingstone et al. (1983) similarly noted high SDH and AlnDH, but not ODH activity in sedentary Ostreidae, whereas

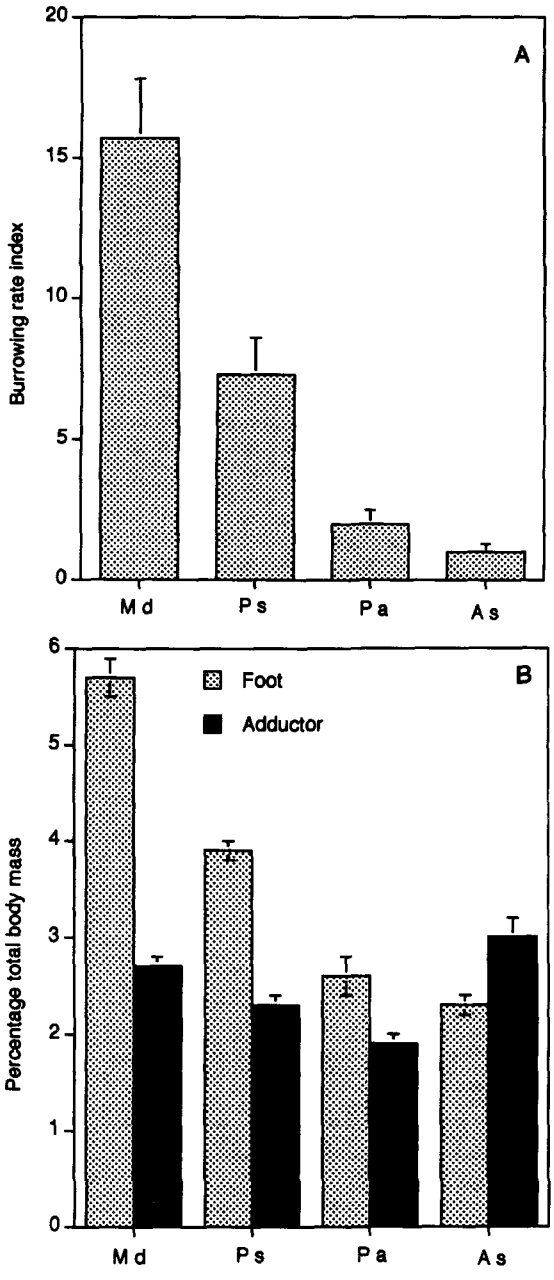

Fig. 2 Mass-specific burrowing rates (**A**) and muscle proportions (**B**) in four intertidal clams (means ± SE). Md, *M. discors*, Ps, *P. subtriangulatum*, Pa, *P. australis*, As, *A. stutchburyi*.

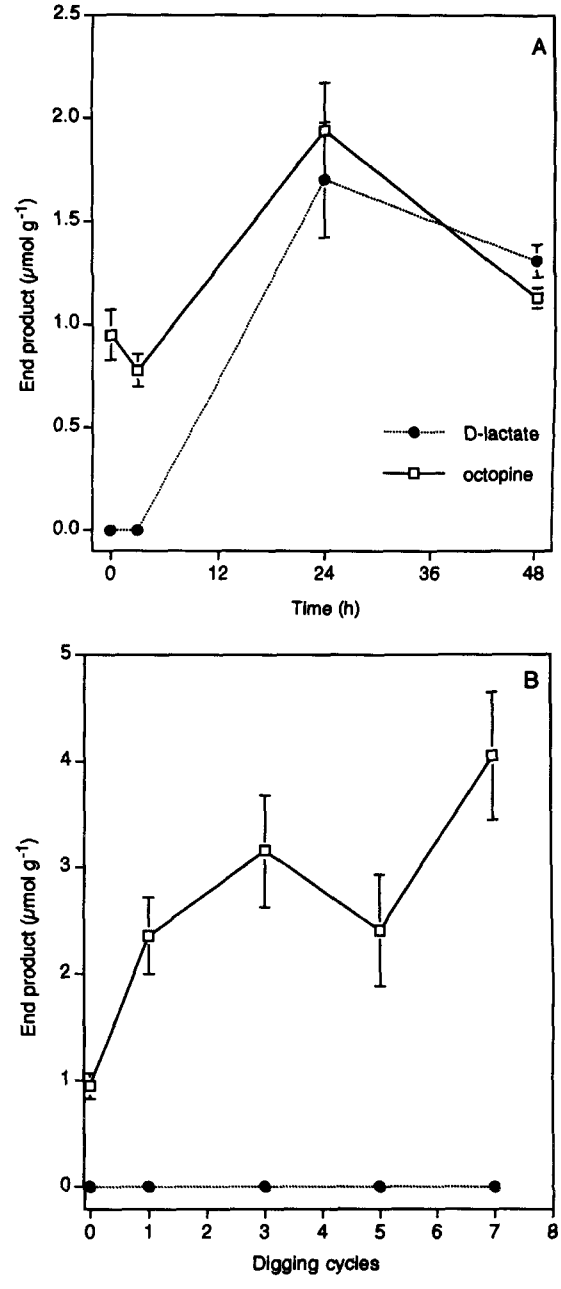

Fig. 3 Pattern of anaerobic end products accumulated in *P. subtriangulatum* under (**A**) environmental hypoxia and (**B**) functional hypoxia from digging (means ± SE).

active burrowing species of the Taxidonta, Heterodonta, and Adapedonta (Livingstone et al. 1983), and free-swimming Pectinidae exhibit high ODH activity (Baldwin & Opie 1978). Evidence from enzyme kinetics indicates that the ODH pathway is likely to realise a higher rate of energy production than either the SDH or AlnDH pathway (Gäde 1980). Thus, ODH correlates with functional

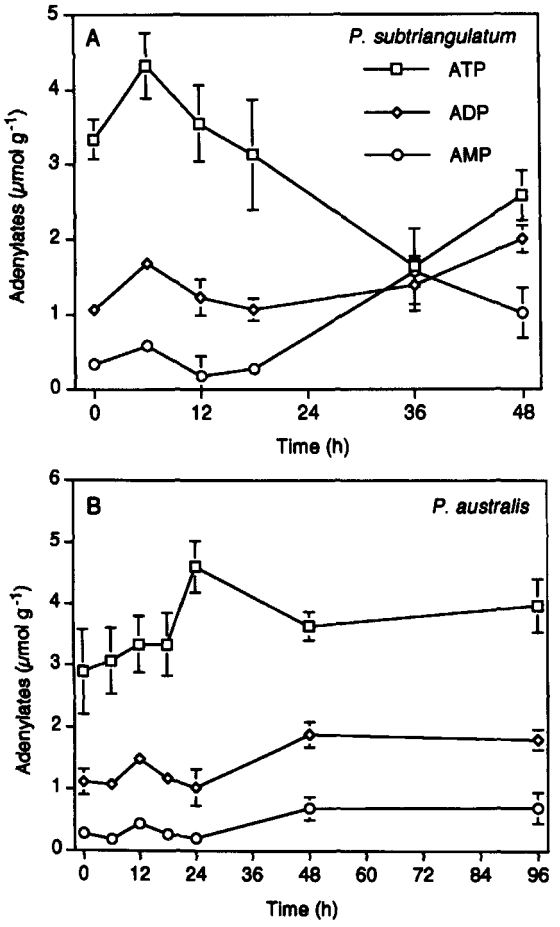

Fig. 4 Adenosine triphosphate metabolism in *P. subtriangulatum* (**A**) and *P. australis* (**B**) during environmental hypoxia (means ± SE).

hypoxia during muscular activity, whereas SDH and D-LDH correlate with periods of environmental hypoxia.

Caution is required when making assumptions about the relative importance of different pathways during anaerobiosis based on their associated enzyme activities. Dando et al. (1981) demonstrated that AlnDH from *Mytilus edulis* showed maximal activity with alanine, and slight activity with glycine, but SDH maintained high activity with both these amino acids. Further, species variation in the kinetic properties of D-LDH, AlnDH, and ODH, and the presence of tissue-specific isoenzymes have been observed (Gäde & Grieshaber 1986; Fields & Storey 1987) and in addition, changes in the activity states of key glycolytic enzymes and of LDH and ODH may occur during anaerobiosis (Storey 1988; Oeschger & Storey 1990; Ip et al. 1993).

Thus, metabolites may not accumulate in proportion to their corresponding enzyme activities. Nonetheless, Livingstone (1991) considers that the correlation between specific dehydrogenase activity and end-product formation is "by no means absolute but nevertheless reasonable". These caveats do not significantly affect the conclusions of our study, since metabolites were also measured and behavioural responses to hypoxia were observed.

Anaerobic metabolism may cause an excess of protons which leads to acidosis of intracellular fluid (Hochachka & Mommsen 1983). In bivalves this problem is exacerbated by the poor perfusion of muscles with haemolymph (De Zwaan 1977). For glycolysis to continue during periods of anaerobiosis, it is critical that the redox balance be maintained, as many principal regulatory enzymes of this process are inhibited by falling pH (Somero 1981). To counter fluctuations in pH, non-bicarbonate buffering compounds contribute to the maintenance of intracellular pH (Eberlee & Storey 1984) and allow for increased glycolytic flux over an extended period. Intracellular buffering tends to be more efficient in those muscles that have a high work output and a restricted supply of oxygen (Castellini & Somero 1981). Bivalves with the highest buffering capacity were the fastest burrowers and conversely, hypoxia-tolerant species had lower muscle-buffering capacities. Buffering capacity of bivalve muscle tissue is positively correlated with muscle work (Eberlee & Storey 1984; Morris & Baldwin 1984) and since falling pH is usually accompanied by a reduction in glycolytic rate, buffering provides stable tissue pH and energy production.

Anaerobic pathways are not all equivalent in terms of energy production and proton generation; based upon maximum rates for ATP output, lactate > octopine > alanopine = strombine (Livingstone 1991). For this reason, species utilising the octopine pathway during functional hypoxia will tend to increase energy flow more rapidly, and accumulate protons quicker than a species using the alanopine pathway. Intracellular buffering will therefore be relatively more important in those species such as *P. subtriangulatum* and *M. discors* which rapidly accumulate high concentrations of opines.

High concentrations of arginine phosphate in the foot muscles of bivalves are hydrolysed to arginine and inorganic phosphate during intense

exercise (Livingstone 1991). Hydrolysis not only consumes protons and produces energy, but also increases the availability of inorganic phosphate, which itself is an effective intracellular buffer (Portner et al. 1984). This situation might conceivably facilitate a higher glycolytic rate, both by consuming protons, and by providing a substrate (arginine) for the formation of octopine and is consistent with the observations on energy charge, and metabolite production in burrowing *P. subtriangulatum* where proton generation is a consequence of functional, but not environmental hypoxia.

Brooks et al. (1991) noted the differential survival of *Venus gallina* and *Scapharca inaequivalvis* during anoxic stress, and considered that the greater buffering capacity of the former species, which has limited anoxic tolerance, was required to counteract the greater load of end products accumulated during functional anaerobiosis. Thus, *P. australis, D. anus*, and *A. stutchburyi*—which have limited abilities for increased glycolytic flux during functional hypoxia—are not likely to require a high buffering capacity.

Anaerobiosis and energetics during environmental hypoxia

There is an obvious correlation between ecological niche and anaerobic strategy. *A. stutchburyi* and *P. australis* inhabit estuarine shores that periodically experience to low oxygen levels. By contrast, *P. subtriangulatum* and *M. discors* are characteristic of the surf zone and are seldom exposed to lethal hypoxia. Accumulation of strombine during environmental hypoxia appears a widespead strategy among intertidal invertebrates (Nicchitta & Ellington 1983; Portner et al. 1984; Meinardus & Gäde 1986; Schiedek & Zebe 1987). It therefore seems reasonable to suggest that SDH supports the hypoxic tolerance of *P. australis* and *A. stutchburyi*. Conversely, *P. subtriangulatum* and *M. discors* employ the D-lactate pathway during hypoxia, indicating greater glycolytic flux in foot muscle. Indeed, *P. subtriangulatum* and *M. discors* performed slow protruding movements of siphon and foot during hypoxic incubation, indicating that a hypometabolic state had not been established. No behaviour of this nature was observed in either *P. australis* or *A. stutchburyi*. Similarly, *Cardium tuberculatum* survived only 17 h of environmental hypoxia and accumulated D-lactate but not other opines (Gäde 1980).

Maintenance of a high adenylate energy charge (AEC) and absence of D-lactate in hypoxic *P. australis,* suggests close matching of ATP producing and consuming processes. The sharp decrease in AEC to 0.4, and accumulation of D-lactate in *P. subtriangulatum*, suggests that energy-saving mechanisms no longer compensate for the reduced energy output of anaerobic metabolism.

Anaerobiosis and energetics during functional hypoxia

The fast burrowers *M. discors* and *P. subtriangulatum* inhabit the surf zone, whereas the less athletic *P. australis* and *A. stutchburyi* burrow in sheltered waters. The dynamics of D-lactate and octopine accumulation in the foot of *P. subtriangulatum* during functional and environmental anaerobiosis confirms the independent use of these pathways, with octopine accumulating during functional, and D-lactate accumulating during environmental anaerobiosis. The primary function of ODH in bivalve muscle is to regenerate cytoplasmic NADH during short-term bursts of anaerobic muscle activity (Fields et al. 1976; Baldwin & Opie 1978; Dando et al. 1981). Gäde (1980) further proposed that the terminal dehydrogenases D-LDH and ODH in *Cardium tuberculatum* functioned under different physiological conditions.

At the completion of burrowing there was no significant fall in AEC in either *P. subtriangulatum* or *P. australis,* indicating that there is close matching of energy producing and consuming processes. An active octopine pathway is usually associated with a large arginine phosphate pool (Livingstone et al. 1983) that is used predominantly for muscle contraction, and its degradation causes an increase in free arginine and inhibition of ATP production (Meinardus & Gäde 1981). This problem is avoided in *P. subtriangulatum* by converting arginine into a less acidic octopine, an option not available to *P. australis*.

The advantages of the ODH and D-LDH system in *P. subtriangulatum* are clear. The bivalve burrows approximately 100 mm below the sediment at low tide where it remains with siphons withdrawn and unable to ventilate. When the tide begins to rise, the animal works its way to the surface and once more begins to ventilate. Clearly, there are two hypoxic states; one functional and the other environmental, together requiring more energy than can be provided by the LDH route alone.

Ecological and practical significance of results

Species living in the shifting sands of exposed beaches must be capable of accelerated rates of energy production for rapid digging, to prevent translocation from favourable habitats. Estuarine species, on the other hand, must withstand extended periods without oxygen, and require a metabolism characterised by low rates of energy production in order to survive prolonged hypoxia. Our observations confirm that differing anaerobic pathways can be designed for either high or low rates of energy production, but not both (cf. Gnaiger 1983).

The degree of species specificity offers the possibility of using pyruvate reductase enzymes as an aid to rapid identification and an alternative to electrophoresis of unidentified proteins. Applications might be useful in forensic analysis of bivalve flesh, or in separating morphologically similar species. We have recently examined what were believed to be *P. subtriangulatum/australis* hybrids but pyruvate reductase activity suggested them to be *P. subtriangulatum.*

Metabolic indicators of hypoxic stress have been proposed for successful export of live seafood products (Baldwin et al. 1992). Handling and transport of live clams using methods most likely to maintain energy charge and minimise accumulation of strombine, D-lactate, or octopine are likely to result in a premium product. Live molluscs currently presented to the consumer have difficulty ventilating when stored in moist air, and few of these animals survive when placed in aerated sea water. It is clear from Table 2 that species such as tuatua (*P. subtriangulatum*) which are widely available in local supermarkets, are less likely to survive hypoxic insults than are pipis (*P. australis*) or cockles (*A. stutchburyi*).

An ability to reduce metabolic rate and maintain energy charge during periods of environmental hypoxia could be of great ecological significance. During a toxic algal bloom in September 1992, substantial populations of *P. australis* in the Whangateau Harbour, on the north-east coast of New Zealand, were observed for approximately 5 days to be in an inactive non-feeding and non-ventilating "arrested state" (S. Hooker. pers. comm.). This behaviour has been associated with the presence of toxic algae in other bivalve species (Marsden 1993). During this period there was no reported mass mortality for *P. australis*, yet *P. subtriangulatum* suffered extensive mortality in other regions where toxic blooms occurred.

Recently there has been interest in using bivalves as bio-indicators for the detection of pollutants in the marine environment. Bivalves will exhibit stress responses often before any manifest ecological effects of pollution develop. Work by Zachariassen et al. (1991) on *Mytilus edulis* identified the accumulation of strombine as an "alarm parameter" for detecting a range of pollutants. Indeed the sensitivity of different bivalve species to hypoxia can be a valuable indicator of the potential for a population's survival in environments experiencing eutrophication (De Zwaan et al. 1991).

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