ERRATA

p 2 line 22: "and the" for "the and"

p 8 line 4: "work." for "work.."

p 10 last line: "arginine" for "Arginine"

p 15 line 21: "Russell" for Russel"

p 29 line 3: "magnesium" for "Magnesium"

p 31 line 21: "…and more detail…" for "…and a more detail…"

Chapter 2 Figure 2.2 caption: "(arterial red, venous blue, magenta mixed)" for "(arterial red, venous blue)"

p 47 line 9: "polyplacophoran" for "polyplachoporan"

p 53 line 25: "vertebrate" for "vertebarte"

p 69 line 7: "obtained" for "obatined"

p 76 line 26: "like" for "unlike"

p 91 line 1: "It" for "lit"

p 104 line 29: "performed" for "preformed"

p 108 line 7: "min" for "mins"

p 134 line 2: "Russell" for "Russel"

p 165 line 15: "dodecamer" for "didecamer"

p 175 line 13: "(…53,000 Da)" for "(…53,000 Da Mwt)"

p 175 line 17: "Data were transformed" for "Data was transformed"

Chapter 6 Figure 6.8 caption: "…Bis-Tris buffer…" for "…Tris-HCl buffer…"

p 214 line 39: "ed. K.M.Wilbur and C.M.Yonge" for "ed. K.M.W. a C.M.Yonge"

p 218 line 39: "haemoglobin" for "hemoglobin"

ADDENDA

p 10 line 17: add "(*Buccinum undatum* and *Busycon canaliculatum*)" and read "Whelk (*Buccinum undatum* and *Busycon canaliculatum*) radula muscle…"

p 15 line 16: add "…the red shell, and is distinguished by…" to read "…derives its name from the red shell, and is distinguished by its black foot wall …"

p 51: Add at the end of para 2:

"Tissue sampling was completed within 5-6 min of shucking."

p 65 line 11: add "approximately" and read "…observed in the region of approximately pH 7.0 to 7.4."

p 68 line 8: add "(ANOVA; $P<0.01$ for both adductor and foot muscle)" and read "…wet wt, respectively (ANOVA; $P<0.01$ for both adductor and foot muscle))"

Chapter 3 Figure 3.1: Standard Error Bars inserted on graph

p 99 line 24: insert "Mangum and van Winkle 1973" before "Newell 1973" to read "(Mangum and van Winkle 1973; Newell 1973; Truchot and Duhamel-Jouve 1980)"

p 112 line 20: add "…± 0.02" and read "…from 0.09 ± 0.02 μmol mL^{-1} to…"

p 201 line 10: delete "r" and read "… context directly applicable …"

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ECOPHYSIOLOGY OF THE BLACKLIP ABALONE *HALIOTIS RUBRA* LEACH: METABOLIC ASPECTS OF MUSCLE FUNCTION AND BLOOD OXYGEN DELIVERY IN A COMMERCIALLY IMPORTANT SPECIES

John Peter Elias (Bsc Hons)

A thesis submitted to the School of Biological Sciences, Monash University, Australia, in fulfilment of the requirements for the Degree of Doctor of Philosophy

September 2003

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September 2003

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ABSTRACT

This study is concerned with the ecophysiology of the commercially important Blacklip abalone *Haliotis rubra*. This study is believed to be the first of its nature on an Australian abalone species. Despite contributing a large and important economic resource, little was known about the biochemistry and metabolism of *H. rubra*. Information was especially lacking for the radula muscles which, although constitute only around 1% of tissue weight, importantly sustain feeding in abalone.

The aims of this study were to investigate and describe the metabolic aspects of muscle function and oxygen delivery in *H. rubra*. This was considered important for a number of reasons: primarily, to fill the large gap in knowledge of the metabolism and biochemistry of this species; and to integrate aspects of abalone physiology and bicchemistry, especially those aspects important to the ecology and commercial integrity of this species.

Indices of metabolic poise were determined for *H. rubra* muscles. The large and economically important pedal muscles are predominantly facultatively anaerobic and show similarities to other abalone species by exhibiting high activities of the pyruvate reductase enzyme tauropine dehydrogenase (TDH), high pH buffering capacities and high concentrations of glycogen and the high-energy phosphagen arginine phosphate. In contrast, the smaller radula muscles are predominantly aerobic, as suggested by higher activities of enzymes unique to aerobic metabolism, high levels of the respiratory pigment myoglobin, and low pyruvate reductase enzyme activities and pH buffering capacity. This is the first metabolic profile of abalone radula muscles. Interestingly, D-lactate dehydrogenase (D-LDH) from the foot and radula muscles of *H. rubra* show kinetic and electrophoretic similarity, indicating tissue specific isozymes of D-LDH may not be present in these muscles of *H. rubra*.

The different metabolic poise of *H. rubra* muscles translate into different responses when subjected to muscle work and lowered environmental oxygen. As with other abalone species, the pedal musculature of *H. rubra* employs facultative anaerobic metabolism to supplement energy demand, with D-lactate and tauropine accumulating. It was also found that during hypoxia, D-lactate appears in the haemolymph and the haemolymph glucose titre increases in *H. rubra*. The radula muscles however maintain aerobic metabolism during lowered environmental oxygen and during natural *in vivo* muscle work associated with feeding on macro- and microalgae. The metabolic poise of these different muscle masses, and

their responses to hypoxia and work, may relate partly to their position within the circulatory system of *H. rubra*.

The amount of haemocyanin in the haemolymph of *H. rubra* varies among individual animals, even when estimated using multiple methods. Compared to previous estimates, this study suggests *H. rubra* haemocyanin has a relatively high oxygen-affinity and a low oxygen-carrying capacity. Whilst the effects of a haemolymph pH change associated with hypoxia manifest themselves with a left shift of the oxygen-binding curve (reverse Bohr effect), D-lactate in the haemolymph shows no significant modulatory effect on haemocyanin. Factors such as the reverse Bohr effect and increases in peripheral circulatory resistance during hypoxia and activity in abalone do not appear to severely affect the delivery of oxygen to the radula muscles.

In abalone to . ate, anaerobiosis has been a rather 'tissue-centric' view. The results of this study suggest that different muscles in abalone possess different metabolic poise, and these are exhibited in distinct strategies to muscle work and lowered oxygen. These responses, and their effects on muscle and haemolymph biochemistry, may be important for the aquacultural and commercial health of *H. rubra*.

CHAPTER ONE

ENERGY METABOLISM AND PHYSIOLOGY OF ABALONE: AN OVERVIEW

1.1 ENERGY METABOLISM OF MOLLUSCS

The phylum Mollusca encompasses a very large number of highly diverse animals inhabiting a wide range of aquatic and terrestrial environments. Molluscs vary in size from the microscopic and planktonic to the massive tridacnid clams and giant squid *Archaeotuthis*, the largest extant invertebrate. Lifestyles and behaviours range from the cryptic and attached sessile, to the highly active pelagic predators found amongst the Cephalopoda.

This great diversity of habitat, anatomy and locomotory behaviour is underlain by marked differences and specialisations of the biochemical and physiological systems associated with energy metabolism. For example, at one extreme the active cephalopods are able to support the highest rates of aerobic metabolism with a closed double-pump blood circulatory system analogous in function to that of birds and mammals. In contrast, molluscs other than cephalopods generally display low aerobic scopes, and low-pressure open blood systems often with shared hydraulic and transport functions for the blood (Bourne *et al.* 1990). Sessile intertidal bivalves require few, if any, locomotory specialisations, but must be able to withstand periods of emersion and associated stresses of tidal flux.

Possibly as a consequence of these limitations to aerobic capabilities, many species have well developed capacities for anaerobic energy production to support both periods of elevated muscle work associated with activities such as locomotion involved with prey capture and predator evasion, and periods of environmental hypoxia.

The biochemical pathways of aerobic metabolism are essentially similar across the animal phyla, in respect to the reactions involved with the catabolism of fuels via glycolysis, β oxidation of fatty acids, protein deamination, the Krebs cycle and the electron transport chain. However, major differences exist among species in the patterns of anaerobic metabolism, with numerous strategies available.

Aerobic specialisations may involve both intracellular components, and the delivery of oxygen and fuels to the tissues via circulatory and perfusion adaptations and modifications to respiratory pigments. Among the Mollusca, the predominant respiratory pigment is circulatory haemocyanin, although a number of species also exhibit blood haemoglobins and

tissue myoglobins (Mbs). In contrast, enhancement of anaerobic capacity involves principally only intracellular modifications.

Molluscs are favoured organisms for studying metabolic biochemistry, due in part to the interest maintained in the ability of intertidal representatives to withstand the harsh littoral environment (Newell 1973; McMahon and Russell-Hunter 1978; McMahon 1988a, 1988b). While the success of these organisms may require respiratory adjustments to lowered O_2, it is the physiology and biochemistry of the numerous pathways involved in facultative anaerobic energy provision that has received particular attention. As a consequence, the involvement of these pathways during periods of internal hypoxia in molluscs and invertebrates in general, is well studied.

For metabolism to continue in the absence of O_2, whether environmentally or physiologically induced, both an alternative electron acceptor and a mechanism of balancing the cellular redox ratio are required. Among molluscs it is evident that the reduction of pyruvate to L-lactate, as is typical for vertebrates and arthropods, is not the only, or even major, means for the re-oxidation of NADH and the maintenance of the cytoplasmic redox ratio (Gäde and Grieshaber 1986; Gäde and Meinhardus-Hager 1986; Grieshaber and Kreutzer 1986). Numerous additional metabolic options are available to molluscs experiencing hypoxia, which may explain their impressive locomotory capabilities, and successful colonisation of demanding environments such as the intertidal zone (Grieshaber *et al.* 1994). Specialisations are found in the use of high-energy phosphates, the production of alternate pyruvate reductase end products, the utilisation of ATP producing pathways in addition to glycolysis, the and ability to withstand short term increases in proton load. Details of these options have been the subject of numerous reviews (*e.g.* Fields 1983; De Zwaan and Dando 1984; De Zwaan and Putzer 1985; Gäde and Grieshaber 1986; Gäde and Meinhardus-Hager 1986; Grieshaber and Kreutzer 1986; Grieshaber *et al.* 1994; Lutz and Storey 1997).

Energy metabolism in most molluscs primarily is carbohydrate based and predominantly aerobic in the presence of plentiful O_2 (Hammen 1980; De Zwaan 1983; Livingstone and De Zwaan 1983). This especially holds true for low intertidal and subtidal molluscs, which infrequently need to rely on facultative anaerobic metabolism during periods of reduced O_2 associated with emersion. In molluscan muscles, the sole high-energy phosphagen, arginine phosphate, serves as an immediate ATP source during periods of high metabolic demand (Gäde 1983; Ellington 1989), particularly muscle work. Its depletion during such activity is often accompanied by activation of anaerobic glycolysis and the accumulation of pyruvate reductase end products other than L-lactate.

The catabolism of carbohydrate in further pathways, which may require additional substrates such as aspartate, or the involvement of the mitochondria, results in the accumulation of succinate, alanine and volatile acids such as acetate or propionate (De Zwaan and van den Thillart 1985). In these instances, pyruvate may be coupled with aspartate degradation to yield malate, oxaloacetate or succinate. Alternatively, these pathways by-pass pyruvate altogether. Such extended pathways, which yield more ATP per glucosyl unit metabolised than glycolysis, are typical of molluscs such as intertidal bivalves enduring extended periods of air exposure or metabolic depression (Hochachka *et al.* 1973; De Zwaan 1983; De Zwaan and Putzer 1985). The patterns of end product accumulation are influenced by the rate of ATP turnover; intense or sustained muscular activity relies on arginine phosphate hydrolysis and anaerobic glycolysis leading to pyruvate reductase end products, whereas the lower metabolic flux during hypoxia associated with extended air exposure is often sustained by multiple pathways (Hochachka *et al.* 1983; De Zwaan and van den Thillart 1985; Lutz and Storey 1997).

Fluctuations in pH that traditionally accompany anaerobic metabolism arise from the uncoupled hydrolysis of ATP and the production and accumulation of acidic end products (Hochachka and Mommsen 1983; Pörtner *et al.* 1984; Pörtner 1987). The ability of molluscs to buffer these changes in intracellular pH is often correlated with the frequency or intensity of the anaerobic episode (Eberlee and Storey 1984; Morris and Baldwin 1984; Kinsey and Ellington 1995).

1.2 GENERAL PHYSIOLOGY AND METABOLISM OF ABALONE

The largest and most ubiquitous class of molluscs is the Gastropoda, of which the prosobranchs are the most numerous (Hughes 1986; Edgar 2000). Together with the highly active Strombidae, the Haliotidae or abalone are considered amongst the most active of gastropods. Their familiar sessile appearance belies their activity, as abalone display fast gliding rates during locomotion (Donovan and Carefoot 1997; Donovan *et al.* 1999), dexterous feeding movements, and rapid and impressive shell twisting behaviour utilised during predator avoidance and righting after dislodgment.

Of indigenous North American origin, the common name Abalone is given to members of the genus *Haliotis* in the family Haliotidae (Class Gastropoda; Subclass Prosobranchia; Order Archaeogastropoda; Suborder Zygobranchia; Superfamily Pleurotomariacea). Abalone are a morphologically conservative and distinct group of gastropods inhabiting both sheltered and exposed coastal marine habitats across a wide range

of latitudes. All members of the genus are identified by the characteristic flat auriform shell with a row of respiratory tremata, low spire, and an enlarged last whorl, which accommodates the body. The large, primitive bipectinate gills and flow of water through the mantle cavity are typical of many Archaeogastropods.

Taxonomists have noted some 130 species and sub-species of abalone, although the demarcation of species and sub-species is somewhat equivocal (Brown 1991a; Lindberg 1992). There may be up to 20 generally recognised species of abalone in Australian waters, with some species thought to be synonomous or conspecific (Shepherd 1973a; Lindberg 1992). *Haliotis coccoradiata*, *H. scalaris* (*H. emmae*), *H. cyclobates*, *H. elegans* and *H. semiplicata* are small non-commercial species (Wilson 1993; Edgar 2000). *Haliotis conicopora* (Brownlip abalone), *H. roei* (Roe's abalone), *H. laevigata* (Greenlip abalone) and *H. rubra* (Blacklip abalone) all support commercial fisheries in the southern states (McShane 1999; Shepherd 1999). The tropical asses ear abalone *H. asinina* is becoming an important commercial species in warmer Indo-Pacific waters of northern Australia.

Due to their importance as a fisheries resource, abalone are the subject of a growing number of studies. The increasing epicurean and economic value of the large pedal muscle mass of the animal has precipitated a severe depletion of a number of the world's wild populations (Gordon and Cook 2001), including several Australian *H. laevigata* stocks (Shepherd and Rodda 2001; Shepherd *et al.* 2001). As a result, there is an escalating focus on *Haliotis* aquaculture. Although the significance of abalone as an economic, cultural and recreational resource has encouraged much investigation, many aspects of the biology of these gastropods remain unresolved.

1.2.1 Cardiovascular physiology of *Haliotis*

Abalone are generally regarded as possessing an open circulatory system. Conventionally, an open circulatory system lacks capillaries (endothelially lined vessels), with blood accessing and bathing the tissues through a system of sinuses and lacunae (Prosser 1973; Bourne *et al.* 1990), and is considered low pressure and inefficient. Moreover, a possible hydraulic function of the gastropod circulatory system further distinguishes it from the closed cephalopod system, which is restricted to blood delivery (Bourne *et al.* 1990).

However, the open systems of gastropods share many features with those of cephalopods, including basic similarities in heart structure and function (Kling and Schipp 1987). Also, the haemolymph of abalone plays very little role in assisting movement, as the muscle system of *Haliotis* is a muscular, rather than fluid-hydrostat, system (Voltzow 1986). Furthermore, the viscera of abalone, where no hydraulic function is required, are perfused by

a fine network of small vessels, and the abalone *H. cracherodii* and *H. iris* display cardiac output and stroke volumes above those of a number of cephalopods (Jorgensen *et al.* 1984; Bourne *et al.* 1990; Just 2002). Present work on the circulation of *H. iris* suggests that the term "open" should not necessarily imply inadequacy (N. Ragg University of Canterbury, pers. comm), and that the circulatory system of abalone is not so easily classified.

The following brief outline of the cardiovascular system of abalone is based on the excellent descriptions of Crofts (1929) for *H. tuberculata*. Russell and Evans (1989) provide a fine study on the cardiovascular anatomy of *H. rubra*, a more detailed description of which is given in the following chapter.

Abalone possess paired ctenidia which lie within the mantle cavity, situated beneath the row of shell respiratory tremata on the left of the animal. The heart, consisting of paired atria and a single ventricle, lies posterior to the mantle cavity and receives oxygenated haemolymph from the ctenidia. The oxygenated haemolymph is pumped anteriorly via the aorta, branching to supply most of the viscera on the way, until the aorta reaches the head of the animal. Here, the aorta widens to form the cephalic arterial sinus (CAS), which bathes the muscular and neural structures of the head, including the feeding apparatus. From the CAS, the haemolymph travels anteriorly to supply the head structures, and posteriorly along paired arteries to supply the massive pedal musculature and epipodium.

The haemolymph from the pedal musculature and head then collects in venous pedal and cephalic sinuses, before making its way via the kidneys to the ctenidia to be oxygenated, collecting visceral venous blood and mantle-oxygenated blood on the way.

Compared to the paired pedal and the single shell adductor arteries supplying the whole of the pedal musculature, abalone radula muscles are exposed to a more direct haemolymph supply by their position close to the CAS. Given that the CAS is an extension of the aorta, the blood bathing the head and radula muscles of abalone presumably is the most highly oxygenated, providing these muscles with a relatively high O_2 and blood-borne fuel supply. In addition, the volume of haemolymph supplied to various abalone tissues is not weight-specific, with tissues such as the radula muscles, gills and kidneys receiving proportionately more blood than the large foot and adductor muscles (Jorgensen *et al.* 1984).

As with other molluscs, the haemolymph of abalone performs varied functions. The blood volume of abalone is surprisingly high, and has been estimated for *H. iris* at around 50% wet weight (Taylor 1993; Ragg *et al.* 2000). The haemolymph of abalone circulates the respiratory protein haemocyanin. Haemocyanins (Hcs) are copper containing proteins of high

molecular weight (around 9×10^6 Da, Wood *et al.* 1971; Ellerton *et al.* 1983), and their main function is to transport O_2.

The respiratory function and properties of molluscan Hcs, like those from the Phylum Arthropoda, are controlled by a number of allosteric mechanisms that bring about changes in conformation, O_2-binding capacity and affinity. Positive binding-site interactions, referred to as cooperativity, confer upon the Hc a sigmoidal binding curve over a narrow O_2 partial pressure range. Effector molecules such as H^+, CO_2, anions and cations may change reactivity and affinity for O_2 at the active site of the protein (van Holde *et al.* 1992; van Holde and Miller 1995).

Unlike vertebrate globins and arthropod Hcs, for a great number of marine gastropods a decrease in the haemolymph pH results in an increase in Hc O_2-affinity (Mangum 1980, 1992, 1997). This negative or reverse Bohr shift has been noted for the Hc of the Australian abalone *H. laevigata*, *H. roei* and *H. rubra* (Ainslie 1977, 1980b) and for *H. iris* and *H. australis* (Wells *et al.* 1998a; Behrens *et al.* 2002). It is debated as to whether this reverse Bohr effect imparts any respiratory advantage to abalone (Mangum 1998). The function of Hc in abalone, regardless of any O_2-binding peculiarities, is principally to bind and transport O_2. The amount of Hc required for the animal to maintain an effective O_2 supply is difficult to ascertain, as the Hc concentration of haemolymph in wild abalone varies considerably, up to 900-fold in some species (Pilson 1965).

There has been little work investigating the role organic effectors (as opposed to inorganic ions, pH or temperature) play in modulating abalone Hc. To date, two major anaerobic end products have been identified in abalone: D-lactate and tauropine. The effect of these metabolites on abalone Hc is unknown, although abalone Hc may share the general insensitivity to organic effectors exhibited by other molluscan Hcs (Mangum 1983a, 1983b, 1992, 1997).

1.2.2 Muscle physiology of *Haliotis*

The foot or pedal muscle of abalone, which enamours the animal to the gourmet, is a large dense muscle mass, and constitutes a muscle-muscle antagonistic, or muscular-hydrostat system (Trueman 1983; Kier and Smith 1985; Trueman and Brown 1985; Voltzow 1986). This 'foot' consists of two morphologically distinct regions, the central columellar or shell adductor muscle, and the ventral and peripheral tarsos. The author has highlighted the term foot to convey that the term does not necessarily relate to the pedal sole of the animal. Often, the whole muscle mass is referred to as the pedal musculature. The adductor muscle is attached to the shell on its dorsal surface and functions to bring about major body movements

and changes in shape such as protraction and retraction of both shell and body, twisting, clamping onto the substrate and righting the animal when dislodged. The tarsos or foot is involved in fine movements of locomotion (gliding, twisting) and food manipulation (Voltzow 1986, 1990; Gäde 1988). The pedal musculature may account for around 55% of wet tissue weight in *H. iris* (Just 2002), and up to 66% of the wet tissue weight in *H. cracherodii* (Jorgensen *et al.* 1984).

The adductor muscle of abalone consists of a central core of dorsoventral or retractor bundles associated with connective tissue (Frescura and Hodgson 1992), surrounded by transverse fibres and cut across by radial fibres. Contraction of these fibres effects shortening or extension of the adductor muscle, respectively. The adductor muscle fibres originate from the dorsal aspect where they attach the shell, and may insert into the epithelium of the sole. In addition, some of the circular and transverse fibres appear to be helical, which may bring about the twisting action of this muscle (Voltzow 1990). Clamping is achieved by powerful contractions of the adductor muscle with minimal changes in muscle length (Frescura 1991, as cited in Frescura and Hodgson 1992).

The tarsos also consists of bundles of muscle fibres wrapped in connective tissue sheaths, which divide and ramify as they approach the sole and sides of the foot, resulting in a network of small fibres embedded in a dense matrix (Voltzow 1990). The tarsic region of Haliotid foot muscle completely surrounds the adductor muscle core (Voltzow 1990; Frescura and Hodgson 1992).

Measurements of pressure waves during locomotion in abalone indicates higher intramuscular force is generated when abalone clamp down, twist and increase crawling speed (Bourne and Redmond 1977a; Trueman and Brown 1985; Voltzow 1986). Likewise, contraction of the shell adductor raises blood pressure in the cephalic and pedal venous sinuses for up to 25 seconds, and also raises blood pressure in the aorta and ctenidial veins (Bourne and Redmond 1977a; Trueman and Brown 1985). Elevated muscular activity in abalone therefore has the potential to disrupt the flow and distribution of haemolymph.

In contrast to the pedal musculature, the structure of abalone buccal and radula muscle more closely resembles vertebrate skeletal muscle, with more discernible fibres and striations, and a reputedly higher mitochondrial content (Bevelander 1988). The buccal and radula muscles of abalone are situated in the buccal cavity and are responsible for the repetitive protrusion and retraction of the odontophore cartilages and radula during feeding and for maintaining the apparatus under tension. The cephalic arterial sinus (CAS) bathes the radula muscles and, unlike the foot and adductor muscles, radula muscles do not contribute significantly to abalone weight (Jorgensen *et al.* 1984).

The colour of abalone radula muscles differs markedly from the foot and adductor, being a deep red both *in vivo* and *in vitro*. This is attributed to the presence of the respiratory pigment myoglobin (Mb). As a general rule, intracellular respiratory proteins such as Mb are found in muscles that experience regular and sustained periods of work.. In these situations, Mb plays a primarily respiratory role, assisting the diffusion of O_2 to the mitochondria (Wittenberg 1970; Wittenberg *et al.* 1975).

It has long been known that Mb is concentrated in the radula and buccal muscles of some gastropods (Lankaster 1872), and may also be associated with cardiac and gastro-intestinal smooth muscles (Manwell 1960a). Australian abalone, like many other molluscs, possesses Hc in the haemolymph, but the presence of a muscle respiratory protein has not been quantified.

It is assumed that, owing to the colour of the radula muscles, most abalone possess Mb in these muscles. This assumption was first verified by Terwilliger and Read (1970b) with work on *H. kamtschatkana*. Recently however, it has become apparent that abalone possess a structurally unusual Mb in the radula muscles. Although displaying similar absorption spectra to more traditional Mbs, these proteins are approximately twice the size of globin-based Mbs, and are thought to have evolved from the tryptophan-degrading enzyme, 2,3-IDO (Suzuki *et al.* 1998a). As such, IDO-type Mbs show a rather different structure to traditional Mb. To date, this Mb appears to be restricted to the Haliotidae, and some other members of the Archaeogastropoda.

1.2.3 Energy metabolism of *Haliotis*

Normal aerobic metabolism of abalone at rest is supported by gas exchange through gills located in the mantle cavity. Abalone are believed to be O_2-regulators, such that O_2-consumption remains constant until a critically low PO_2 is reached (Ainslie 1977; Jan and Chang 1983; Boyd and Bourne 1995). Most species of haliotids prefer a subtidal niche while others successfully exploit the intertidal zone and can survive for days emerged, as long as desiccation is prevented (Bowen 1987).

Hypoxic conditions may ensue in the respiratory/mantle cavity of abalone when the animals are clamped tightly to the substrate, or exposed to air during tidal flux or during commercial handling and transport (Ryder *et al.* 1994). The mode of gas exchange in abalone precludes making use of aerial O_2, as the ctenidia collapse in air, rendering gas uptake and transfer difficult (Houlihan *et al.* 1981). During periods of clamping, a primary predator and disturbance avoidance response in abalone, irrigation of the gills is difficult and ventilation is inhibited. Emersed *H. cracherodii* show a significant decrease in heart rate and blood stroke

volume (Jorgensen *et al.* 1978). During periods of emersion, the major source of O_2 for abalone is believed to be that dissolved in the mantle cavity fluid. Under these conditions, a mature abalone could metabolise aerobically, at typical immersion rates, only for about 5 min without replacing O_2 (Pilson 1963, as cited in Bowen 1984).

In addition to the above, periods of prolonged or intense muscular work typical of forced clamping, exercise and locomotion, result in increased metabolic costs and structural stresses on both the animal as a whole, and especially on the locomotory muscles. Accordingly, the capacity for aerobic energy production is surpassed, and anaerobic metabolism assumes increasing importance. Such anaerobic metabolism in haliotid muscle is supported by high activities of the pyruvate reductase enzymes D-lactate dehydrogenase (D-LDH) and tauropine dehydrogenase (TDH), and an elevated pH buffering capacity. Activities of the phosphagen-hydrolysing enzyme Arginine kinase and levels of arginine phosphate are also substantial (Wells *et al.* 1998a).

Under these exercise induced hypoxic conditions, the large adductor and pedal muscles retain considerable potential for ATP production. Arginine phosphate reserves are hydrolysed, and the pyruvate reductase end products D-lactate and tauropine (D-rhodoic acid [*N*-(D-1-carboxyethyl)-taurine]) accumulate in both the adductor and foot muscles as a result of the anaerobic glycolysis of carbohydrate (Gäde 1988; Sato *et al.* 1991; Baldwin *et al.* 1992; Wells and Baldwin 1995; Donovan *et al.* 1999). In conjunction with the production of D-lactate and tauropine, tissue and blood pH of abalone decrease (Tjeerdema *et al.* 1991a, 1991b; Baldwin *et al.* 1992; Wells *et al.* 1998a). These patterns, especially the tissue-specific production of the pyruvate reductase end products, have become a hallmark of facultative anaerobic metabolism in abalone. As such, the presence of these metabolites in abalone muscles has been utilised as an indication of animal and muscle condition during commercial transport (Ryder *et al.* 1994).

Abalone pedal musculature is regarded as possessing a limited aerobic capacity. Recent evidence suggests that even relatively slow gliding locomotion in a number of species may require additional input from anaerobic sources (Donovan and Carefoot 1997; Donovan *et al.* 1999; Baldwin *et al.* in prep).

While the origin of pyruvate reductase end products is known in a number of abalone species, information on the fate or transport of these metabolites is limited. It is often presumed that these metabolites are retained and re-metabolised *in situ*, as little convincing data exists on the presence of D-lactate or tauropine in the haemolymph of any abalone, or any prosobranch gastropod for that matter.

1.2.4 Aerobic and anaerobic muscles in *Haliotis*

Emphasis in abalone muscle biochemistry studies has so far been on the large and commercially important foot and shell adductor muscles, and for good reason. Understanding the responses of these tissues to the types of stress most likely to be encountered during production, harvesting, and transport is deemed important, as are the biochemical characteristics of the muscles influencing texture, taste and therefore market value (James and Olley 1970, 1971a; Olley and Thrower 1977). However, little attention has been paid to other, more aerobic muscles, or to the potential fates of anaerobic products typically produced by the large anaerobic muscles.

Abalone, and most gastropods in general, display a convenient internal juxtaposition of muscle function. Whereas the foot and adductor may be considered to represent facultatively anaerobic tissues, as the situation demands, tissues such as the heart, kidneys, ctenidia and radula muscles may occupy a more aerobic position. Of these, the buccal and radula muscles engaged in feeding provide a perfect contrast for an inter-tissue investigation, due to the evidence from the literature on other gastropods that these muscles may be largely aerobic in function.

The metabolic capabilities of gastropod radula muscles may be quite high. Whelk radula muscle for example contains high glycolytic enzyme and cytochrome levels, large numbers of mitochondria, and displays a high O_2-consumption *in vitro*, all of which are indicators of O_2-utilisation by normal aerobic pathways (Ball and Meyerhoff 1940; Fänge and Matisson 1958; Zammit and Newsholme 1976a). High activities of the Krebs cycle enzymes isocitrate dehydrogenase and citrate synthase are found in the radula retractor muscles of several other gastropods (Alp *et al.* 1976), again indicating that such muscles, which tend to be mechanically active over long periods of time, probably operate aerobically.

These studies suggest that the both the scope (the maximum rate of ATP production, as gauged by enzyme activity) and capacity (factors ensuring continued aerobic energy production such as levels of fuels and O_2-delivery) for aerobic metabolism are high. However, this simple classification is confused by the high activities of pyruvate reductase enzymes in some radula muscles (Ellington and Foreman 1981; Ellington 1982), and the presence of anaerobic metabolites such as D-lactate and other pyruvate reductase end products in these muscles after artificial stimulation *in vitro* (Wiseman and Ellington 1987) or environmental anoxia *in vivo* (Eberlee and Storey 1988). Additionally, the radula muscles of intertidal limpets and littorinids display extremely high activities of Arginine kinase (Zammit

and Newsholme 1976a; Livingstone *et al.* 1983). In this respect, the muscles may also be considered to possess a high scope and capacity for anaerobic metabolism.

The metabolic poise or capacity of abalone radula muscles is unknown, and the author is unaware of any work on these muscles other than that concerning the identification and properties of Mb. It even appears that the amount of Mb has yet to be properly quantified for Haliotids. Whilst the biomechanics of these muscles during their primary function of feeding is known, their physiology and biochemistry associated with energy metabolism remains largely unresolved.

The many and varied radula and buccal muscles of gastropods function to bring about the movements of the radula and odontophore cartilages associated with feeding, especially the protrusion and retraction of these structures. The number of times such movements are made during a feeding episode in abalone can be quantified by encouraging them to graze on a glass aquarium through which observations can be made. No estimate is available on the frequency or duration of feeding in abalone, although it is generally agreed that they, as is typical for most gastropods, display extended feeding capabilities (Shepherd 1973a).

Despite the levels of anaerobic enzymes in the radula muscles of some gastropod species, it is likely that radula work in abalone primarily is an aerobic operation. How does one determine this, given that artificially stimulated contractions of isolated radula muscle result in accumulations of anaerobic end products? It should be possible in feeding trials to measure any anaerobic component of muscle metabolism. Utilising the natural phenomenon of feeding itself has advantages, and is more relevant, than subjecting isolated radula muscle preparations to artificial *in vitro* conditions. Indeed, this approach is probably more natural than the techniques that are used routinely to subject the foot and adductor to muscle work.

1.3 OBJECTIVES AND OUTLINE OF THE STUDY

Work on the physiology and biochemistry of Australian abalone species is progressive. The effects of physical and chemical aquaculture conditions on the health, growth and viability of *H. rubra* and *H. laevigata* have been relatively well investigated (see Burke *et al.* 2001, and references therein). However, apart from the early cursory work by James and Olley (1970, 1974) and Olley and Thrower (1977), very few studies have examined the effects of exercise or the unnatural conditions experienced by Australian abalone during harvest, commercial shipping and handling. This is surprising considering the importance of the Blacklip abalone *H. rubra* as an economic resource.

The physiology of the massive pedal musculature of abalone, amenable to both study and cuisine, is familiar. What is unknown is the physiology of other abalone muscles, among the most important of which are the radula muscles powering energy acquisition in abalone. No information is available on important aspects such as the levels of Mb and the activities of aerobic and anaerobic enzymes, nor on the presence or amounts of metabolic fuel available to these radula muscles.

The radula and buccal muscles occupy an extremely important position both in the anatomy and ecology of *H. rubra*. The feeding efficiency, growth, health and survival of abalone, and ultimately their commercial value, relies upon these muscles, which may account for only up to 1% of body weight. Ultimately, the function of the foot and adductor muscles and all other tissues in abalone are reliant upon the capacity of the radula muscles to provide them with substrates for growth and work. When approached in this manner, the importance of the radula muscles is paramount, and it is clear that no study to date has attempted to characterise the metabolic capacity and function of abalone radula muscles, or muscles of *H. rubra* as a whole. This thesis aims to address these shortcomings.

It is apparent from the literature that more information is required on the physiology and biochemistry of Australian abalone species, especially those that constitute both an ecological and commercial importance. The importance of *H. rubra* as a study animal is obvious when considering the role it plays in the marine ecology and economy of southern Australia. *Haliotis rubra* support important commercial fisheries in all the southern states, with just under 5,000 tonne of wild *H. rubra* landed each year in Australia. Of this total, 2,510 tonne is harvested from Tasmania alone (McShane 1999). Across the quota year 1997/1998, 1,437 tonne of abalone were caught in Victoria (Anon 1998). In the year 2000, the total allowable catch of *H. rubra* was worth about $AUD 80 million at the first point of sale (Officer *et al.* 2001a).

The persistence of a lucrative commercial fishery in Victoria since the 1960s attests to the apparent resilience of *H. rubra* to prolonged and intense fishing. The total Australian harvest of Blacklip abalone represents about 40% of the total world catch, making *H. rubra* the most important of the world's commercial species (McShane 1999). In comparison, the total commercial harvest of *H. laevigata* is currently a little over 1000 tonne (Shepherd 1999). The meat of *H. laevigata* is regarded as the sweetest, which may account for it being the most commonly farmed abalone in Australia (Harris *et al.* 1999b).

With the genus being rather morphologically and ecologically conservative, much can be estimated about *H. rubra* from other abalone species. We are familiar with the

distribution, natural history and behaviour of *H. rubra*, but what do we understand about the muscle physiology and biochemistry of this animal? Do *H. rubra*, or any Australian species for that matter, resemble other temperate species by producing the unusual anaerobic end product tauropine? What metabolic effects, if any, does sustained work or deprivation of O_2 have on the muscles and haemolymph of *H. rubra*? How different is abalone radula muscle from the more familiar and economically important pedal muscle?

The general objective of this study is to investigate and describe the metabolic potential of different muscle tissues, a study not yet undertaken for a commercial Australian abalone species, and may be considered as a subset of more specific aims:

i. to examine and describe the metabolic potential of *H. rubra* pedal musculature, and to examine how these results compare to those of other abalone species;

ii. to examine and describe the metabolic potential of *H. rubra* radula muscles, and to resolve their biochemistry *in vivo* and *in vitro*. No consistent literature exists on what one might predict about abalone radula muscles, as some information on these muscles for other gastropods suggests a predominantly aerobic poise, and other literature suggests an enhanced capacity to function anaerobically;

iii. to quantify and examine characteristics of the blood delivery system in *H. rubra*, particularly its role in delivering O_2 to different muscles; and

iv. to consider the application of this knowledge to an increased understanding of the ecology and maintenance of an important commercial species.

CHAPTER TWO

BIOLOGY OF THE BLACKLIP ABALONE, *HALIOTIS RUBRA* LEACH: MORPHOLOGY, BEHAVIOUR AND ECOLOGY

2.1 INTRODUCTION: BIOLOGY OF *HALIOTIS RUBRA* LEACH

Ideally, no biochemical or physiological study should proceed without a thorough understanding of the biology of the animal. The purpose of this chapter is to introduce some general features of the experimental animal, *Haliotis rubra*, and to determine a number of important ecological, behavioural and anatomical parameters that are required to describe, conduct and assess the experiments undertaken in later chapters. Specifically, these include observations on relative muscle and haemolymph weights and volumes, characteristics of and haemolymph supply to different muscle types, food and feeding preferences in *H. rubra*, and examinations of natural behaviour. Both direct observations and measurements, and existing literature, are utilised for these purposes.

Haliotis rubra Leach, or the Blacklip abalone, derives its name from the black foot wall and dark epipodium (Plate 2.1), not to be confused with the black abalone of California (*H. cracherodii*) or the black-foot paua of New Zealand (*H. iris*). *Haliotis rubra* may achieve an average length between 100 mm to 140 mm, before natural death or harvest, according to geographic distribution (McShane 1999). Abalone from different populations are known to vary in maximum size, with largest individuals reported from Tasmania (218 mm long and 3.6 kg weight, McShane 1999). The shell exterior is a characteristic reddish-brown, maculated with green. It is oval in outline, with a greatly enlarged body whorl, covered with fine scaly spiral ridges which overlay oblique wrinkles, and the area between the row of tubercles supporting respiratory tremata and the outer edge is excavated (Shepherd and Thomas 1989). *Haliotis rubra* display a large and distinctive rugose epipodium, a predominantly sensory and protective structure with many nodules and tentacles, often with an intricate or striped pattern.

Given the increasing importance of abalone as an economic and recreational resource, considerable literature exists on the biology of *H. rubra*. In 1973, Shepherd published what must be considered the most often sourced reference on Australian abalone ecology (Shepherd 1973a). Following that initial study the author and associates have maintained an ongoing series of publications on abalone ecology.

Much of the current material concerns aquaculture, and wild stock and population management practices, although a surprising quantity is available on aspects of the physiology of *H. rubra*. Recent literature on *H. rubra* includes the identification of genetic markers both within populations and the individual (*e.g.* Huang *et al.* 1997, 1998, 2000; Conod *et al.* 2002), and fishing management (Gorfine 2001; Gorfine *et al.* 2001; Officer *et al.* 2001a, 2001b). Reviews are available on the aquaculture biology and commercial intake of Australian species (Fleming and Hone 1996; Brown *et al.* 1997; Anon 1998; Hone and Fleming 1998; Freeman 2001). Studies have also investigated the feeding preferences of *H. rubra* (Foale and Day 1992; Shepherd and Steinberg 1992; McShane *et al.* 1994; Fleming 1995a, 1995b), and physical effects of aquaculture (Edwards 1995; Gilroy and Edwards 1998; Harris *et al.* 1999a; Burke *et al.* 2001).

The growth, reproduction and distribution of *H. rubra* has long been of interest (*e.g.* Shepherd 1973a, 1975; Shepherd and Laws 1974; Shepherd *et al.* 1982; Shepherd and Hearn 1983; McShane *et al.* 1986, 1988; McShane 1999), as has the assessment and maintenance of wild populations (McShane and Smith 1988; Prince *et al.* 1988; Hart and Gorfine 1997; Hart *et al.* 1997). The relationship between abalone and their diet is well understood, as are predator interactions (Shepherd 1973a, 1975; Parsons and Macmillan 1979; Thomas and Day 1995; Day *et al.* 1995).

Some literature exists that deals directly with the physiology of *H. rubra*. This literature manages to address a wide variety of subjects, including respiratory or cardiovascular aspects (Ainslie 1977, 1980a, 1980b; Russel & Evans 1989; Edwards 1994, 1996). Less is known about muscle biochemistry, besides the amino acid and heavy metal composition of *H. rubra* tissues (Hyne *et al.* 1992; King *et al.* 1996). James and Olley (1970, 1974), and Olley and Thrower (1977) comment on biochemical changes in meat quality of caught and processed *H. rubra*. On the other hand the genetic variation both within *H. rubra* populations, and between populations of sympatric species, has received considerable attention (Brown 1991a, 1991b, 1993, 1995; Brown and Murray 1992a, 1992b).

Despite the volume of information available on *H. rubra*, a number of important physiological and biochemical questions still exist. This chapter investigates some anatomical, behavioural and ecological features of *H. rubra* that allow these questions to be addressed. Muscle characteristics are examined to determine any physiological or anatomical differences in muscle proportions, size or position within the animal's body plan. Of particular interest are the foot and adductor muscles, and the radula and buccal muscles. The

haemolymph volume of *H. rubra* is estimated to provide information on the contribution of the haemolymph to total weight, and to provide an opportunity to extrapolate later tissue specific metabolite and haemocyanin (Hc) data to the whole animal. Responses to predators, disturbance, exercise and air emersion are observed, to determine appropriate methods of challenging abalone for metabolic experiments and to understand natural situations encountered by *H. rubra* in the wild. Additionally, familiarity with handling, dissection, and general anatomy allows the correlation of physical characteristics of *H. rubra* with physiological and biochemical parameters, which is of importance in this study. The information gained on *H. rubra* can then be compared directly to other abalone species, which aids in interpreting any physiological data. The Discussion in this chapter considers how these data fit in with existing literature on abalone biology in general, and the ecology, behaviour and morphology of *H. rubra* in particular.

2.2 METHODS

2.2.1 Field study sites and behaviour of *Haliotis rubra* in the wild

The Blacklip abalone *Haliotis rubra* was observed and collected subtidally over the period 1999 to 2001 by snorkelling and by SCUBA, under permit (Department of Natural Resources and Environment permit No. RP499A). The primary study site consisted of a series of sheltered reefs at Mornington, Port Phillip Bay, Victoria (38°12.5'S 145°2.9'E; Figure 2.1.). Rare supplementary animal collections were also made from Ricketts Point (37°59.8'S 145°1.7'E), a low relief reef north of Mornington that has been a collection site for other studies on *H. rubra*, and a semi-exposed oceanic site at Tortoise Head, Western Port Bay, Victoria (38°24.3'S, 145°16.4'E; Figure 2.1).

Considerable underwater time was spent observing the natural habitat and behaviour of *H. rubra*, in a variety of tidal and water current situations. Observations were made on the response of *H. rubra* to predation (by presenting them with predatory seastars and gastropods), to inversion, to disturbance and to algae. Information from these preliminary studies is considered in the Discussion, as is the response of *H. rubra* to air exposure.

The principal study and collecting site at Mornington is typified by the bases of granite cliffs forming reefs of moderate relief, accompanied by boulders (Plate 2.2, photo of field site). Algae species composition and cover fluctuated with season, and the macroflora was generally dominated by Phaeophyta (*Hormosira banksii*, *Cystophora moniliformis* and *Ecklonia radiata*) and Chlorophyta (*Ulva australis*.). *Sargassum* sp. cover varied over the course of the study. Any rhodophytes present were typically small.

Haliotis rubra were collected at Mornington from a depth of 0.1-1.5 m (estimated from mean-low-water mark). The shallow sheltered site often experienced periods of low water movement, resulting in the occasional air exposure of some individuals. However, most abalone were distributed subtidally. *Haliotis rubra* were commonly distributed cryptically; on vertical rock faces, in caves, crevices and underhangs. Small individuals (up to 65-70 mm) were more commonly encountered under rocks and deep within crevices. On occasion, *H. rubra* could also be found in the open, on outside edges of reef walls and along bases of walls and large boulders, especially in summer. Abalone exposed in this manner on outer reef walls and boulders were often sheltered by dense algal cover. Individuals were more cryptic on very still and very rough days.

Larger individuals of *H. rubra* were almost always encrusted with epibiota: very regularly bonnet limpets (*Sabia conica*), occasional barnacles, and very rarely juvenile limpets such as *Siphonaria* spp or *Cellana* spp. More typical was the extensive encrustation of shells with coralline and turf algae, sponge, bryozoan, and even macroalgae (*Ulva australis* and *Ecklonia radiata*). *Haliotis rubra* shells, particularly those of large adults, showed signs of damage, probably by boring gastropods and sponge (*e.g. Cliona* sp.).

At the primary field site, *H. rubra* were usually found in their cryptic habitat in association with the crab *Plagusia chabrus*, the small palaemonid shrimp *Palaemon serenus*, and occasionally the related fissurellid limpet *Scutus antipodes*. Abundant in the vicinity, but rarer in caves, was the predatory starfish *Coscinasterias muricata*. Naturally occurring colour variants of *H. rubra*, known as "tiger Blacklip", were distributed evenly with the normal Blacklip at Mornington.

2.2.2 Anatomy, morphology and haemolymph volume of *Haliotis rubra*

Animals were collected subtidally by gently prising off rocks with an abalone tool (blunt flat blade). All animals were checked for any damage, transported live in seawater tanks back to Monash University and held in a large open aquarium (approx. 4.6 m × 1 m × 0.2 m) as part of a 2000 L seawater system maintained at 33-34 ‰ salinity and pH 7-9-8.1, until used for experiments. Animals were allowed to acclimate to tank conditions before experimental use (at least 2-3 days). During captivity, abalone were able to graze freely on the diatom and micro-algal community of the tank walls and floor, and were regularly fed algae collected from the study site (*Ulva australis*.). Animals were collected, transported and maintained in this manner for the duration of the study. The light cycle experienced by captive *H. rubra* was altered according to season, ranging from 14:10 to 10:14 l:d. The water temperature ranged between 13 to 17°C, concordant with changes in external atmospheric temperature and season.

The salinity and health of the aquarium system was monitored regularly, and animals were inspected daily during captivity. Although abalone maintained for long periods (up to 5 months) were observed to lose body weight and condition over time, most animals were used within days or weeks of collection, depending on treatment. No abalone died of natural causes in captivity.

Abalone were dissected to determine gross body composition, haemolymph volume and to gain familiarity with the body plan and muscle types. Trials were performed to determine an appropriate method for bleeding and dissecting animals, the details of which are given in the Discussion.

Prior to dissection, epibiota were removed from the shells, as much as was possible. Initially it was decided to consider any encrusting organisms in the overall weight of the animal and shell, under the presumption that the added weight or drag constituted a natural phenomenon, and one that animals were accustomed to. The degree of infestation of *H. rubra* shells was extremely variable however, and shells were 'standardised' to a degree by the removal of most macro fauna and flora.

Animals were removed from the aquaria, inverted and blotted to remove excess seawater, and the whole animal weight determined. The shell was then rapidly removed by applying thumb pressure to the anterior margin of the shell adductor muscle, where it abuts the shell. After dislodging the adductor muscle from the shell, a blunt probe was carefully run around the margin of the shell to dislocate the mantle and left adductor muscle attachments, and the connective tissue attaching the loop of gonad and digestive gland to whorl of the shell. Abalone were removed from the shell in this manner throughout the course of the study.

The shell was cleaned of any extraneous tissue, and the weight of both the shell and remaining wet tissue were recorded. Shell dimensions (length and width, to the nearest mm) were taken with vernier callipers. Abalone were then slashed across the foot with a scalpel, severing the major pedal blood vessels, and suspended on a wire frame above a collecting tray. Animals were bled for up to 30 minutes, the duration of which was continually assessed by the slowing of bleeding and the production of mucus, of which the risk of contamination increased with longer standing times. To assist bleeding, the heart and major vessels (aorta, ctenidial vessels) were punctured once haemolymph collection from the foot wounds slowed.

Following bleeding, the viscera were removed by careful dissection from the posterior and lateral adductor muscle surfaces and anteriorly from the posterior buccal cavity, and weighed. For the purpose of these dissections, 'the viscera' consist of the digestive gland and gonad, renal, ctenidial and cardiac structures, hypo-branchial glands, mantle and gut posterior to the buccal cavity (Plate 2.3). The muscular mass was further dissected to separate the epipodium and cephalic structures from the foot musculature. The epipodium was taken as the lateral projection from the pedal wall, originating from a small depression running around the animal, rather than the most lateral projections of nodules and tentacles. A similar classification was made by Day *et al.* (1995). The remaining pedal muscle (adductor and foot) could not be separated (Section 2.4.3), and were considered as one muscle mass. The weights of all components were recorded, along with the volume and weight of the collected haemolymph. The head was dissected to reveal the buccal cavity and radula muscles.

After dissection, the ctenidia were carefully removed whole from the mantle cavity. Ctenidia were blotted and the following measurements of both the left and right ctenidium taken: wet weight, and length and height (distance from axial rib to end of filament) using callipers. Width (distance between lamellae tips) was not able to be measured, given the collapsed state of the ctenidia in air.

The ctenidia volume was estimated as the volume of displacement by submerging the organs in distilled water in a 10 mL measuring cylinder, and noting the overall increase in volume. Care was taken to remove bubbles on the ctenidia that may have influenced volume. The dry weight of the ctenidia was determined following drying at 70°C to constant weight.

Anatomical measurements were performed on 21 individuals. Data represents mean ± S.E.M values, given in weight (g) and percentage weight (%), length (mm) and volume (mL). Ctenidial measurements were performed on 6 individuals.

2.3 RESULTS

2.3.1 Tissue weights and volumes

The average weights of *H. rubra* tissues, and proportions of these tissues to wet tissue weight are summarised in Table 2.1. Since extraction of the total haemolymph volume is considered unrealisable by bleeding, haemolymph % was also estimated by considering haemolymph volume as a residual or remainder fraction of total animal weight. Simply, the total weight of the animal minus shell, viscera and all muscle components represents this residual fraction (essentially extracellular fluid). Here we assume an essential haemolymph density of 1 g mL^{-1}. Measurements of collected haemolymph weight versus volume suggest a density of approx. 1.1 g mL^{-1} (pers. obs). Collected haemolymph volumes and calculated residual haemolymph volumes are represented as mL and as % volumes (mL 100 g^{-1} wet tissue weight).

The average abalone weight was 187 g total, with 56 g (30%) attributed to the shell (Table 2.1). The average haemolymph volume collected was 31 mL, or only 24 % mL 100 g^{-1} wet tissue weight. The calculated residual haemolymph volume of approx. 37 mL (28 % mL 100 g^{-1} wet tissue weight) was not significantly different from the collected haemolymph volume (ANOVA; $P>0.05$). Viscera contributed, on average, 25 % of the wet tissue weight, with the remainder being muscle (46.65%), of which the combined adductor and pedal mass comprised 73 %. As a result, at least 24 % total weight, or 34% wet tissue weight, of live *H. rubra* is consumable muscle (Table 2.1).

The ctenidia, on average, make up only 0.8% of wet tissue weight in *H. rubra*. After drying to constant weight, the dry weight of the combined ctenidia was 0.37 g, down from 1.89 g wet weight (water content 80%). The left ctenidium was consistently larger than the right (left ctenidium length 63 ± 3.6 mm, height 5.9 ± 0.8 mm; right ctenidium length 51.5 ± 3.8 mm, height 4 ± 0.4 mm), and around 75% heavier (Table 2.1). The average total volume of the combined ctenidia, estimated from water displacement, was 1.8 ± 0.1 mL.

The total wet weight and shell characteristics for all abalone collected during this study were recorded. The percentage shell weight for most abalone was similar (30 %).

2.3.2 *Haliotis rubra* radula muscles

The radula muscles almost completely surround the odontophores of *H. rubra* (Plates 2.4 to 2.7). These muscles are attached directly to the cartilaginous odontophores, to the sheaths encapsulating the structure, and to the floor of the buccal cavity (Plates 2.4, 2.5, 2.6). Separate muscles are connected to the radula sheath anteriorly and posteriorly, and dorsal and

posterior to the chitinous jaws. A sphincter of muscle, located just behind the lips, was identified in *H. rubra*.

Two pairs of odontophore cartilages were observed in *H. rubra*: a large dorsal pair and a smaller, posterior pair (Plate 2.7). The odontophore halves remain flexible, although they are joined along the anterior ventral margin.

Whilst many of the individual muscles were not easily distinguishable in freshly dissected specimens, the muscle bellies were easily separated and teased apart.

Although weights of radula muscles were not taken during the course of these dissections, Table 2.1 presents the average weight and %wet tissue weight of radula muscles in *H. rubra*. These data were collected over the course of study during dissections and analyses for later chapters, but are calculated and presented here alongside data for other muscles. The radula and buccal muscles, devoid of any connective tissue or sheaths, weighed on average 0.28 g, representing only 0.2 % of wet tissue weight. This figure is probably a slight underestimate of actual weight, as it is very difficult to completely remove all muscle from the odontophores and membranes.

2.4 DISCUSSION

In the following discussion the ecology and behaviour of *H. rubra* is considered first, followed by the physical characteristics, cardiovascular anatomy and haemolymph volume, and muscle anatomy and physiology of *H. rubra* and abalone in general. The relationship between the muscles and cardiovascular system of abalone is then briefly considered.

2.4.1 Ecology and behaviour of *Haliotis rubra*

Haliotis rubra occurs most abundantly in shallow water (<10m in depth) (Shepherd 1973a, 1975), although it is found deeper in the cooler waters of Victoria and Tasmania. Collecting pressure by humans has most probably rendered intertidal populations of *H. rubra* extinct. The distribution of *H. rubra* is concentrated along the southern coastline of Australia (eastern South Australia, Victoria, Tasmania, southern New South Wales), although it ranges from Perth in the west to as far north-east as Coffs Harbour in New South Wales.

Wild *H. rubra* are known to have rather conservative thermal requirements. In the laboratory, Tasmanian *H. rubra* display a preferred temperature and critical thermal maximum of 16.9°C and 26.9°C, respectively (Gilroy and Edwards 1998). The average optimum temperature for growth calculated from these indices was 17.0°C. Victorian abalone experience slightly higher environmental temperatures and *H. rubra* populations in Port Phillip Bay may be subject to wide variations in water temperature. Generally, the water temperature at the entrance of the bay ranges from an average winter minimum of 9-10°C to an average summer maximum of 19°C (McShane *et al.* 1986; Harris *et al.* 1996), although a larger temperature range is observed in shallow embayments (Drew *et al.* 2001; P. Hanna Deakin University, pers. comm).

Although abalone primarily are distributed subtidally, *H. rubra* at Mornington has been observed exposed for up to 3-4 h during especially low tides while inhabiting walls, shallow pools and crevice microenvironments, in full and partial shade. Under these conditions, the majority of tentacles were retracted whereas the epipodium was extended, and at no stage were abalone observed to move or feed whilst emersed. When prodded (finger), abalone displayed mild clamping. Abalone exposed to air in captivity showed similar behaviour.

Haliotis rubra display a preference for a cryptic habitat (Shepherd 1973a, 1975), typically inhabiting caves and crevices. Larger individuals may move into the open, especially in the south-east range of their distribution. Juvenile *H. rubra* emerge from under rocks at night to graze on the upper surface, and are more active than young *H. laevigata* or

H. scalaris (Shepherd 1973a). A size hierarchy exists in which larger adults occupy places nearer the cave or crevice mouth and young abalone live further back (Shepherd 1973a), a pattern observed during the course of this study.

Blacklip abalone usually emerge after sundown to feed, but occasionally can be found out in the open during the day. In some South Australian populations, this emergence is related to the emergence of predators. Predatory fish, crabs and *H. rubra* successfully stagger their exposure and feeding around and after sunset (Shepherd 1973a). As predatory wrasse retire at the onset of dusk, the red bait-crab *Plagusia chabrus*, a food item of fish, emerges to feed and forage. *Plagusia chabrus*, an apparent predator of *H. rubra*, juveniles particularly, returns to its cryptic habitat after sunset, at which time *H. rubra* become active and begin foraging.

Predators of *H. rubra* include cartilaginous and teleost fish, decapod crustaceans and cephalopod and gastropod molluscs. The predatory seastar *Coscinasterias muricata*, previously known as *C. calamaria* (Edgar 2000), is often associated with *H. rubra*, but some conjecture exists over whether this seastar constitutes a major predator of *H. rubra*. For example, Shepherd and Breen (1992) conclude that *C. muricata* poses little threat to *H. rubra*. Certain reefs in Port Phillip Bay support large numbers of both species however, and *H. rubra* are consumed in large numbers, especially when usual prey items of *C. muricata* are rare (Litchen 1986; McShane and Smith 1986; Day *et al.* 1995).

Haliotis rubra are also susceptible to shell damage from boring sponges (*Cliona* sp.) and annelid worms (*Polydora* sp.), and gastropods (*e.g. Dicathais orbita* and *Haustrum baileyanum*, Shepherd 1973a; Thomas and Day 1995).

Preliminary trials conducted in the field to determine responses of *H. rubra* to physical or physiological challenge included presenting the animal with predators, and inversion. Parsons and MacMillan (1979) described complex avoidance responses for *H. laevigata* and *H. rubra* presented with the predatory whelk *Lepsiella vinosa*. These stereotyped behaviours are elicited by centrally programmed neural responses (Parsons 1978). *Haliotis rubra* displays multiple responses to the predatory asteroid *C. muricata*, as opposed to the highly choreographed response of *H. laevigata* (Parsons and Macmillan 1979; Day *et al.* 1995). It is thought that the cramped cave and crevice habitats of *H. rubra* prevent a highly active escape response. During trials in the field, *H. rubra* would detect the seastar by sweeping their epipodial and cephalic tentacles and exposing the epipodium, then twisting the shell to break contact, or rapidly crawling away. Alternatively, animals would clamp down, withdrawing all sensory tentacles. It was more difficult to elicit a response from *H. rubra* presented with the predatory gastropods *Dicathais orbita*, *Cominella lineolata* or *Lepsiella vinosa*. This

variability in behaviour of *H. rubra* in the presence of predators has been reported previously (Parsons and Macmillan 1979; Day *et al.* 1995).

In addition to clamping, shell twisting or escape, *H. rubra* is known to produce a chemical deterrent from the epipodial epithelium when under attack from *Coscinasterias muricata*, and may expand the epipodium upwards and outwards towards this predator (Day *et al.* 1995; pers. obs). Additionally, *H. rubra* may release a milky mucus, presumably from the mantle tissues, as a result of contact with predators (Day *et al.* 1995). The release of a milky substance from the respiratory tremata was observed during rough handling of *H. rubra* in the field.

Whole animal inversion however produced repeatable righting behaviour in *H. rubra*, both in the field and in the laboratory. Successful righting manoeuvres depended on gaining a proper purchase on the substrate. After initial trials where captive abalone often slipped and lost footing on the aquarium floor, inversions were performed on a large flat rock collected from the field site. In the field, abalone are usually situated on vertical crevices or in caves and would rarely encounter totally flat surfaces, or a vertical surface not within reach of the tentacles and foot. Briefly, righting behaviour is initiated by the extension of epipodial and cephalic tentacles and the twisting of the adductor muscle, orienting the lengthened foot muscle until contact is made with the substrate. Once sufficient purchase is gained, the abalone contracts both the foot and adductor muscle, twisting the latter to shift the body mass and shell over the foot. A more detailed description of abalone righting behaviour is given by Minchin (1975). Repeated inversion caused *H. rubra* to slow its twisting movements, and it appears that *H. rubra* may be exhausted by constant righting.

Haliotis rubra responded very quickly to tactile and photo stimuli by clamping down. Some individuals escape though, especially the juveniles which show a strong negative phototaxic response. If removed from the substrate then replaced, animals tended to move away briefly and either continue moving (*e.g.* towards back of cave) or clamp. Clamping consisted of the spreading of the epipodium, and withdrawal of all cephalic, epipodial and respiratory tentacles. In this state, abalone were virtually impossible to remove by hand from the substrate without considerable injury to the animal.

The diet and feeding habits of *H. rubra* have been described by a number of authors, and are addressed in more detail in Chapter 5. The intake of algae by *H. rubra* is affected by numerous properties of the algae itself, including nutritional quality and toughness (Foale and Day 1992; McShane *et al.* 1994; Fleming 1995a, 1995b). It appears that *H. rubra* from Port

Phillip Bay demonstrates a preference for the red alga *Jeannerettia lobata*, although Blacklip abalone have been known to ingest brown algae (*e.g. Phyllospora*, McShane 1999).

While occasionally feeding during daylight hours, *H. rubra* usually emerge at night to forage for algae, the majority of which is in the form of drift algae. Abalone display distinct drift feeding behaviour, by orientating themselves vertically on rock faces in areas of water movement that guarantees the presence of drift and grasping passing algae. *Haliotis rubra* is most successful during moderate surge, and feeds poorly with strong surge or rough water (Shepherd 1973a, 1975). This may explain the more cryptic behaviour of *H. rubra* at Mornington during rough weather. Adult *H. rubra* are also known to graze upon epilithic species opportunistically (Shepherd 1973a, 1975; pers. obs).

The response of *H. rubra* to being hand-fed algae in the field was variable, given the usually day light hours during which animals were studied. On occasion, abalone would respond positively to a blade of 'drift' algae, usually *Ulva australis*, by protracting the cephalic tentacles and head and anterior lobes of the foot, and grasping the algae. In captivity, abalone were induced to feed after sunset on blades of algae, and were observed to also graze upon microalgal growth on the aquaria walls. *Haliotis rubra* were not often active in aquaria during daylight hours.

In Victoria and southern New South Wales, grazing pressure by urchins not only structures algal communities, but also directly influences abalone distribution. Competition from Black sea urchins (*Centrostephanus rodgersii*) and Purple urchins (*Heliocidaris erythrogramma*) often results in a decrease in *H. rubra* numbers, or a migration of abalone to deeper water (Shepherd 1973b; McShane 1999). This phenomenon was witnessed during collections of abalone made at Tortoise Head, Western Port Bay, Victoria. During a repeat dive 18 months later, a dramatic increase in the density of urchins had displaced abalone, with no *H. rubra* or *H. laevigata* observed down to 3-4 m depth. Similar relationships between *H. rufescens* and urchins are observed in northern California (Karpov *et al.* 2001).

2.4.2 Physical characteristics of *Haliotis rubra*

Haliotis rubra is known to display a number of different morphs. Occasionally, abalone whose appearance is intermediate between the two commonly fished species, *H. rubra* and *H. laevigata*, are observed in southeastern Australia. These rare individuals are regarded as genetic hybrids (Brown 1991a, 1995) and display a shell morphology, epipodial development and colour intermediate between *H. rubra* and *H. laevigata*. Additionally, the haemocyanins (Hcs) of these hybrids display antigenic properties identical to a mixture of *H. rubra* and *H. laevigata* Hc (Ainslie 1977).

These hybrids may be confused with other naturally occurring colour variants of *H. rubra*. Although originally considered as separate species, the relationship between *H. rubra* and the Brownlip abalone, *H. conicopora* (*H. rubra* var. *conicopora*) is now generally regarded as conspecific (Brown and Murray 1992a; Brown 1993; McShane 1999). The Brownlip probably represents an allopatric (Western Australian) population of *H. rubra* (Shepherd 1975). Brownlip abalone are found across southern Australia, from Perth to Melbourne, and support a small fishery in Western Australia (Freeman 2001).

"Tiger" Blacklips, common in Port Phillip Bay (pers. obs), are indistinguishable from the more usual Blacklip other than by the distinctive striated lateral foot wall and epipodium. These surfaces are usually light brown with darker streaks. In the course of this study, considerable numbers of 'tigers' were observed and collected from the main study site. While also found in northern Tasmania, far-south New South Wales and the gulfs of South Australia, little else is known of the causes or distribution of this colour variant (McShane 1999). Studies suggest that some genetic variation exists among Victorian *H. rubra* populations (Brown 1991c, b, 1993, 1995; Brown and Murray 1992a, b), and bay abalone may be distinct from oceanic populations (Huang *et al.* 2000; Drew *et al.* 2001).

The majority of the anatomical characteristics of *H. rubra* appear identical to the general descriptions given for the genus in the Chapter 1. According to available scientific and documentary literature, and anecdotal verbal sources, the biology and anatomy of *H. rubra* appears to closely match that of many other temperate abalone species.

Almost half of the wet tissue weight of the animal is pedal muscle (adductor, epipodium and foot muscle), whereas the radula and buccal muscles powering feeding in *H. rubra* contribute very little to the weight of the animal. This buccal and radula muscles mass is a complex arrangement of muscle fibres and sheaths, intimate with the odontophore cartilages and buccal structures. In contrast to the dense foot and adductor muscle, radula muscles are dark red and can be easily separated.

The haemolymph comprises approx. 30% of the wet tissue weight of *H. rubra*. Abalone were initially prepared for haemolymph volume and anatomy dissections by anaesthetising on ice, or at 4°C. However, accessing haemolymph via syringe proved difficult following cooling and excessive handling, as *H. rubra* show a tendency to clamp or contract the foot following any disturbance (including cooling), resulting in muscular occlusion or vasoconstriction of vessels and sinuses. Alternatively, haemolymph was collected by making a number of deep transverse slashes with a scalpel blade across the anterior and middle of the pedal surface, severing the pedal vessels. Although cutting the foot

resulted in a higher yield of haemolymph than manual withdrawal, the yield was low until, and only if, the animal relaxed muscle contractions.

Alternatively, relaxation by Magnesium sulphate was investigated. Despite having been used as a muscle relaxant in previous studies (*e.g.* White *et al.* 1996), and being relatively easy to access and administer, saturated saline solutions were avoided due to any possible osmotic effect on extracellular volume. As a result, the simple method of minimal animal handling and dissection at room temperature was employed.

The contribution of the shell to the overall weight of the animals (29.8%) was affected by the differing degree of infestation and encrustation. Encrusting coralline algae, bryozoan growths and sponges often dominated the dorsal surface of the shell and were difficult to remove. *Haliotis rubra* shells often displayed signs of repair (as nacreous nodules on the inner surface) after heavy attacks by boring sponge (*e.g. Cliona* sp.) or by drilling gastropods (*e.g. H. baileyanum*, Thomas and Day 1995), which also affected shell weight.

The body components expressed as percentages determined in this study are relatively crude estimates, and probably require a larger sample size of abalone before drawing strong conclusions. *Haliotis rubra* from Port Phillip Bay are known to display a different shell shape and topology from oceanic conspecifics. Bay abalone are also reported to bleed longer than oceanic abalone when wounded, and to tolerate higher seawater temperatures (Huang *et al.* 2000). Also, after exposure to a hyposaline seawater (80%), bay abalone are apparently able to regulate their osmolarity quicker than oceanic abalone (Drew *et al.* 2001), and can survive higher temperatures and a longer emersion period than oceanic abalone.

McShane *et al.* (1988) collected data from 5461 individual *H. rubra* sampled from around the Victorian coastline, and the foot muscle was found to constitute 42.5% of total animal wet weight. It is assumed the adductor, head and epipodium were included as part of the foot muscle, as no mention of the removal of these structures is made. If the current data were to be calculated as such, 'foot muscle' makes up around 32.8% of total animal wet weight, considerably lower than for oceanic samples (McShane *et al.* 1988). James and Olley (1974) arrived at a similar figure. After shucking and evisceration of caught *H. rubra*, the recovery of meat is only of the order of 35% (James and Olley 1974).

Although this large difference may be related to the discrepancy in sample size, other possibilities may explain the difference. The maximum abalone weight and shell length examined in this short study were 300 g and 111 mm, respectively, far smaller than maximum data (576 g and 164 mm, respectively) obtained by McShane *et al.* (1988). It is also possible that the shells of exposed oceanic abalone are not as encrusted with epibiota, given the

possibly higher water velocities, and hence contribute a lower percentage to total weight. Furthermore, oceanic abalone are more likely to encounter conditions of high surge and water current, and may require a larger, more muscular foot to maintain adhesion. This hypothesis was tested between *H. iris* populations in exposed and sheltered locations. However, no environmentally-induced differences in shell and body proportion were observed between these sheltered and exposed populations (Wells *et al.* 1998b). Lastly the foot muscle, as measured by McShane *et al.* (1988), may have retained haemolymph, contributing to the weight.

Foot muscle weight in *H. rubra* varies according to season, with weight yields lowest in summer. This correlates with the known reproductive cycle in Victorian *H. rubra* (McShane *et al.* 1986, 1988).

Estimates by Just (2002) on *H. iris* suggest around 39% of the wet tissue weight is foot, and about 15% adductor muscle, with a total muscle proportion of about 54% wet body weight. This value approaches the 66% calculated for *H. cracherodii* by Jorgensen *et al.* (1984). The epipodium and head of *H. iris* account for a further 17% wet tissue weight (Just 2002). This is higher than the 9% observed for *H. rubra* (total muscle weight minus adductor and foot weight). The data on *H. iris* were calculated not by direct weights, but rather from the proportionate distribution of haemolymph injected tracers (Just 2002), where labelled microsphere entrapment within tissues represents the haemolymph perfusion of these tissues.

The gills, although large in *H. rubra*, contribute only around 1% of wet tissue weight. The left ctenidium is consistently larger and heavier than the right ctenidium, probably as a result of the displacement of the mantle cavity and hypertrophy of the shell adductor muscle.

2.4.3 *Haliotis rubra* muscle anatomy

The pedal musculature of *H. rubra* is, again, typical of the genus, consisting of the stout adductor or columella, dorsal to the broad flat foot, or tarsos. The lateral foot wall is heavily pigmented, as is the sensory epipodium. The plantar sole is creamy yellow coloured in live active individuals, but may take on a dirty grey appearance in moribund or dead abalone. The adductor muscle is a soft creamy white, whereas the foot muscle is a dirty white colour. The high collagen levels in the foot muscle give it its characteristic texture.

As outlined in the Chapter 1, the morphology and function of abalone pedal musculature is documented and understood. Sato *et al.* (1991) separate the muscle tissue of *H. discus hannai* into 3 parts: upper columella, lower columella (which includes the plantar of the foot) and the foot-rim (tarsic muscle). Crofts (1929) regards the columellar/adductor and foot of *H. tuberculata* as separate, whereas Gäde (1988) regards the adductor muscle of *H.*

lamellosa as embedded into the foot (similar to Sato *et al.* 1991). Trueman and Brown (1985) identify retractor fibres running from the top of the adductor muscle to sole of the foot in *H. midae*. Olley and Thrower (1977) suggest differences in abalone foot muscle protein compositions are best understood by considering the foot muscle as three distinct anatomical features: adductor, sole and epipodium.

No reference is given to the relative contributions or proportions of these distinct regions to the pedal musculature. However, from sketches of the major pedal muscle bundles in sagittal and transverse sections of *H. kamtschatkana* (Voltzow 1986), it appears that the adductor and foot contribute roughly equally to the mass of the pedal musculature. Based on this generalisation, the pedal musculature (minus epipodium) of *H. rubra* is assumed to be 50% each adductor muscle and foot.

The general toughness of abalone foot muscle, especially surfaces such as the sole and epipodium which are in constant contact with the environment, is well known. This texture is related to the high collagen content of this muscle (Kimura and Kubota 1968; Olley and Thrower 1977). James and Olley (1971a) were able to show that less than 1% of the solids in the adductor muscle of *H. rubra* were collagen, whereas collagen contributed 9% and 28% of the solids in the foot and epipodium, respectively.

Whereas the pedal musculature of abalone is familiar to science and commerce, the radula muscles are not. A brief description of the feeding apparatus of *H. rubra* follows, and a more detail on gastropod radula and buccal anatomy is provided by Hyman (1967) and Fretter and Graham (1962), and Crofts (1929) for *Haliotis* specifically. The actions of feeding in *H. rubra* are elaborated upon in Chapter 5, which specifically deals with feeding.

The short cylindrical snout is oriented vertically and bears the mouth cleft. The buccal cavity contains what is referred to as the 'odontophore or radula complex' consisting of the cartilages, muscles and membranes involved in feeding, and the radula itself. A pair of amber coloured chitinous jaws guard the opening to the mouth and provide a firm surface against which the radula can work (Crofts 1929). The buccal cavity merges with the oesophagus and receives paired salivary glands and buccal pockets (Plate 2.8). In *H. rubra*, these structures are yellow-white and flowery, resembling little cauliflowers.

The radula in *H. rubra* is easily identified, and the radular sheath, which secretes the ribbon and teeth, is partly enclosed in the anterior aorta. The radula is of the primitive rhipidoglossan variety, characteristic of lower Archaeogastropoda (*e.g.* Halitotidae, Fissurellidae, Trochidae, and Turbinidae, Fretter and Graham 1962; Plate 2.9). The radula rests on the basal membrane, which covers the anterior edge of the odontophore cartilages like

a hood, and provides attachment for muscles (Plates 2.4 to 2.7). The pearl-white odontophore cartilages themselves provide attachment for the associated muscles and the supporting structure over which the radula moves. According to Crofts (1929), the odontophore of *H. tuberculata* consists of a large dorsal pair of cartilages, a small lateral posterior pair and a vestigial anterior ventral pair. This third vestigial pair was not found in *H. rubra* (Plate 2.7). Gastropod odontophore cartilage resembles vertebrate hyaline cartilage histologically, but differs chemically (Lash 1959; Hyman 1967).

The muscles of this apparatus, hereto referred as radula and buccal muscles, are many and complex, and have been described in detail for *Haliotis* by Fleure 1905 (as cited in Crofts 1929) and Crofts (1929). Crofts (1929) identifies up to 21 single, paired or groups of multiple retractor, protractor, tensor, constrictor and intrinsic odontophore muscles in *H. tuberculata*, which are "very distinctly striped and are flesh coloured" (Crofts 1929). The radula muscles of abalone perform various functions during feeding: the attachment of odontophore and radula membranes to the buccal cavity and each other, the movement of these structures (protrusion and retraction), and the opening and closing of jaws (Crofts 1929).

For simplification, no individual muscles are identified or described in this study. The gross dissections did not allow for this, and the detail is unnecessary for the investigations planned. As such, all muscles are considered as one entity, the 'radula muscle mass'.

2.4.4 *Haliotis rubra* cardiovascular anatomy and haemolymph volume

The description of abalone cardiovascular anatomy given here is based on the excellent descriptions of Crofts (1929) for *H. tuberculata*, Bevelander (1988) for *H. rufescens*, and Russell and Evans (1989) for *H. rubra* specifically. Similar descriptions are available for *H. cracherodii* (Jorgensen *et al.* 1984), *H. corrugata* (Bourne and Redmond 1977a, 1977b), *H. kamtschatkana* (Bourne *et al.* 1990), and *H. iris* (Taylor 1993).

The general cardiovascular anatomy of *H. rubra*, typical of the genus, is outlined in Figure 2.2. The heart is located in a posterolateral position on the left of the dorsal surface, at the posterior of the mantle cavity (Russell and Evans 1989), and lies within the pericardium. The bilaterally symmetrical heart, consisting of two atria and one ventricle, is typical of dibranchiate archaeogastropods (Jones 1983), with the rectum passing through the ventricle (Russell and Evans 1989). The ventricle pumps haemolymph via the common aortic trunk, which immediately bifurcates to supply the mantle and mantle cavity organs, the viscera medially, and continues forward as the anterior aorta, running ventral to the mantle cavity. Along its length, it parallels the oesophagus and encompasses the posterior radula sac. As the aorta approaches the anterior of the shell adductor muscle, it gives off fine branches to the

oesophagus, intestine and the adductor muscle. Anteriorly, it suddenly widens, forming the cephalic arterial sinus (CAS). This sinus provides the haemolymph for the head and pedal structures, and almost completely surrounds and bathes the feeding apparatus (odontophore cartilages, muscles and the radula), except for the most anterior of the radula in the buccal cavity. The CAS may also be involved in the protrusion of tentacles, and even the feeding apparatus, under the action of increased haemolymph pressure (Russell and Evans 1989; Bourne *et al.* 1990).

The CAS gives off branches that supply the cephalic sensory and neural structures (Fig 2.2), and forms a depression in the floor (dorsal foot surface), from which the paired pedal and epipodial arteries arise (Russell and Evans 1989). The paired pedal arteries, which accommodate the pedal nerves in their lumen, and epipodial arteries run parallel and posteriorly through the foot muscle. Branches extending laterally from these arteries ramify through the tissue, forming a network of lacunar tissue spaces (LTS). varying between 10 and 50 μm, which are not lined with endothelium.

Venous haemolymph from the pedal LTS eventually collects in the posterior pedal sinus, a large sinus located medially between the pair of pedal arteries, running the length of the foot. This sinus drains the pedal muscles. As the sinus reaches the anterior of the adductor muscle, it expands to form the cephalic (or cephalopedal) venous sinus (CVS), which lies adjacent to the CAS. The CVS also receives haemolymph from the cephalic and buccal circulation and, it is hypothesised for *H. rubra*, the CAS (Russell and Evans 1989).

The afferent renal vein then passes posteriorly to the right kidney, which also receives haemolymph from the viscera (Fig 2.2). The right kidney serves as a renal portal system, from where the haemolymph progresses to the basibranchial sinus, before travelling along the length of each of the large bipectinate ctenidia in the afferent ctenidial veins. After traversing the gill lamellae, the oxygenated haemolymph travels back to the atria via the efferent ctenidial veins, collecting the mantle-oxygenated haemolymph from the pallial circulation on the way.

In many archaeogastropods, including *Haliotis*, it has been proposed that the mantle is a more important respiratory organ than the ctenidia (Hyman 1967). A circumpallial arterial and venous circulation is present in the mantle of abalone. Oxygenated haemolymph from the mantle eventually flows into the right atrium. Measurements of tissue-specific haemolymph flow reveal the mantle receives as much haemolymph as the digestive gland in *H. cracherodii* (Jorgensen *et al.* 1984). The high haemolymph requirement of the mantle probably reflects the active role of this tissue in mucus production and the construction of the shell. Just (2002) hypothesised a possible respiratory role of the mantle in *H. iris* during impaired O_2-uptake via

the gills, a notion rejected after observing no increase in perfusion of this structure during air exposure.

The position of the radula muscles in the CAS is important, as they have access to large arterial and, potentially, venous flows (Fig 2.2). Russell and Evans (1989) were able to identify the CAS and CVS in *H. rubra*, and a possible vascular connection between the two. Interestingly, muscular valves have been reported between the CAS and pedal arteries in *H. corrugata* (Bourne and Redmond 1977a), and between the CVS and pedal venous sinus for *H. tuberculata* (Crofts 1929). These valves could therefore function to regulate flow of haemolymph. The fact that these valves are positioned to guard both inflow and outflow from the foot suggests that pedal muscle region of the cardiovascular system could be by-passed or isolated from the rest of the circulation (Russell and Evans 1989), with haemolymph passing from the CAS to the CVS. This may be achieved when either the peripheral pressure or resistance is increased (as in foot and adductor movement), or the conditions in these muscles are deleterious to the rest of the circulation and O_2-delivery (*e.g.* tissue damage and haemolymph loss). To date, this valve system has not been identified in its entirety in any one species of abalone.

The gross dissections revealed the presence of pedal arteries, a pedal sinus, and the entrance of these vessels into cephalic region of *H. rubra* (pers. obs). However the presence of either a vascular connection between the cephalic sinuses, previously identified for *H. rubra* (Russell and Evans 1989), or valves guarding the sinuses from the pedal circulation, were not highlighted. Nevertheless, a flap of muscle continuous with the anterior wall of the adductor was identified. This flap appeared to separate the pedal sinuses: a single vessel, the pedal venous sinus, was located above the projection, whereas paired vessels with structures occupying the lumen (the pedal arteries with nerves inside) were identified ventrally. Compared to the solid foot ventrally and adductor muscle posteriorly, this flap was considerably loose and pliable, and only about 5 mm across (pers. obs). Immediately anterior to these vessels the floor of the cavity (dorsal surface of foot) formed a depression, into which a large vessel opened from the left. This vessel was followed posteriorly beneath the mantle cavity, and was positively identified as the aorta. As such, the cavity was identified as the CAS.

The intimate association of buccal mass with the buccal cavity, and the corruption of any space during dissection, make the visualisation of the CAS and CVS *in situ* rather difficult. The space posterior to the odontophore in *H. rubra* and the depression in the floor of the buccal cavity suggest that in an intact abalone, room is available to accommodate the

working structure, and to serve as a haemolymph sinus. Corrosion casting of the cephalic circulation of *H. iris* reveals an extensive haemolymph supply surrounding the odontophores and radula muscles (pers. obs).

The relative haemolymph volume of molluscs varies greatly within the phylum, and also between individuals of a species. As such, it is estimated that the haemolymph of a marine mollusc constitutes from 30 to up to 80% of the soft parts (Burton 1983). The method of bleeding as a direct estimate of haemolymph volume is generally regarded as unreliable in most circumstances (Martin *et al.* 1958; Jones 1983). Detailed and protracted methods for estimating haemolymph volume, by the use of injected and monitored tracer substances, were beyond the scope of this brief study.

As mentioned above, contraction of *H. rubra* is likely to reduce haemolymph flow, although the relatively low volume of haemolymph collected probably reflects the trapping of haemolymph within the tissues. Drainage of haemolymph from the viscera proceeds very slowly, and is compromised by the increasing contamination of mucus (pers. obs). As a result, weights for the viscera are probably over-estimated at the expense of haemolymph volume.

During preparation of *H. rubra* for commercial processing, haemolymph loss is initially rapid, and drainage slows until up to 40% of the original muscle weight may be lost (James and Olley 1974; Olley and Thrower 1977). A residual volume of haemolymph, which may account for up to 15% of weight, may remain in the foot after draining. The volume of haemolymph obtained by bleeding to circulatory standstill usually represents approx. half the total volume (Taylor 1993). If this is the case for *H. rubra*, then haemolymph volume is more likely to be 40-50% of wet tissue weight.

The haemolymph volumes of a number of marine gastropods are shown in Table 2.2. The average haemolymph volume of *H. fulgens*, estimated as inulin space, is around 41% (Pilson 1963, as cited in Pilson 1965). Indirect *in vivo* haemolymph volume studies on *H. iris* using ^{51}Cr-EDTA (Taylor 1993) or [^{14}C] inulin (Ragg *et al.* 2000) as volume markers suggest that more than half of the volume of *H. iris* is haemolymph (52-57 mL 100g^{-1} wet wt tissue). Simple bleeding resulted in haemolymph volume estimates for the tropical species *H. asinina* of around 55 mL 100g^{-1} (Baldwin *et al.* in prep), higher than observed for *H. rubra*.

Although considerably higher than the simple estimates here for *H. rubra*, these figures of around 50 % concur with numerous other data on marine gastropod haemolymph volumes (Table 2.2). It is evident that different methods of provide different estimates. Estimates of haemolymph volume range from 60 to 79 % for the opisthobranch *Aplysia*

californica (Martin *et al.* 1958), whilst the same method (Amaranth dye) gave haemolymph volumes of 57 to 66% for the gastropod *Bullia* sp. (Brown 1964).

During the course of this study, *H. rubra* have regularly sustained haemolymph removal of over 20 mL, and a 200g individual remained apparently healthy even after the removal of 35 mL (pers. obs). Taylor (1993) and Taylor *et al.* (1994) report that *H. iris* may lose up to 65% of total haemolymph volume and still recover, although generally any loss over 60% is fatal. *Haliotis rubra* and *H. iris* display similar morphologies and physiology, and it is quite possible that the standing haemolymph volume of *H. rubra* is close to 50 %.

This study estimates the density of *H. rubra* haemolymph at around 1.1 g mL^{-1}, lower than the figure of 1.3 g mL^{-1} calculated by Hyne *et al.* (1992).

2.4.5 The haemolymph supply to *Haliotis rubra* muscles

The difference in haemolymph perfusion of various tissues in *H. rubra* is important when considering major physiological and biochemical differences may exist between these tissues. Some understanding of the potential differences in haemolymph supply and therefore fuel and O_2-supply to different muscles is required for interpreting results presented. These differences in haemolymph delivery are revisited later in Chapter 6.

The haemolymph supply to the pedal musculature of abalone primarily is via a single adductor artery and the paired pedal arteries (Crofts 1929; Voltzow 1990), a condition noted for *H. rubra* (Figure 2.2; Russell and Evans 1989). This is in contrast to the favourable position of the radula muscles in the CAS. This association has led to suggestions that an increase in CAS haemolymph pressure may aid in the protraction of cephalic structures of abalone, like the tentacles (Bourne *et al.* 1990), or the odontophore and radula during feeding (Russell and Evans 1989).

Furthermore, attempts to access haemolymph from the adductor muscles of abalone has proven difficult (Chen 1996), and the perfusion of the pedal musculature is reduced further during periods of muscular contraction and air exposure (Taylor *et al.* 2001; Just 2002).

Haemolymph in abalone does not appear to be directed to the tissues in direct proportion to the percent total body weight the tissues represent (as might be expected in an open vascular system), but apparently in proportion to tissue metabolic rate (Jorgensen *et al.* 1984). Coulson *et al.* (1977) considered that the metabolic rate of tissues, as estimated from enzyme reaction rates, should be proportional to the delivery of substrate and O_2, and hence blood or haemolymph flow. Churchwell (1972, as cited in Jorgensen *et al.* 1984) found the radula muscles of *H. cracherodii*, at only 0.8% of the wet body weight, had a high O_2-uptake,

second only to the kidneys. The weight specific haemolymph flow to these muscles should then be proportionate. However, the radula muscle receives considerably less haemolymph than the metabolic data suggests (only 1% of the cardiac output), which is lower than most other organs. Russell and Evans (1989) believe this discrepancy is due to a failure of the method used by Jorgensen *et al.* (1984). The technique of counting the distribution of labelled microspheres (15 µm) in the tissues and vessels, and hence estimating haemolymph supply, requires the entrapment of these microspheres in very small vascular channels. However the CAS, bathing the radula muscles, may not facilitate microsphere entrapment; hence, the value given may be an underestimate of relative haemolymph flow, and therefore O_2-supply, to this tissue (Russell and Evans 1989). In contrast, the foot muscle of *H. cracherodii* may account for about two-thirds of whole wet body weight, but at rest receives only 27% of the cardiac output (Jorgensen *et al.* 1984).

The pedal musculature of *H. rubra* differ in size, texture, colour and form to the radula muscles. While the pedal musculature plays important roles in animal locomotion, adhesion, and posture, the radula muscles power the repetitive movements of the radula associated with feeding. The position of these muscles in respect to the general circulation of the animal may reflect their physiology. Considering these differences, it is now of interest to examine the biochemistry and physiology of these muscles. Specifically, what biochemical parameters reflect the ability of muscle to perform aerobic and anaerobic metabolism, and do they illustrate differences among muscles in *H. rubra*? This question forms the basis of the next chapter.

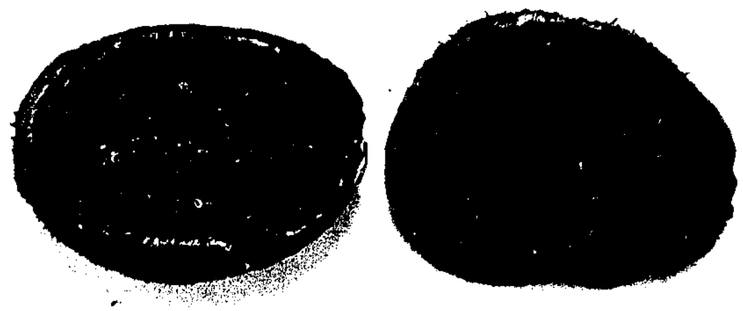

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Plate 2.1. The Blacklip abalone, *Haliotis rubra*. Ventral view of pedal sole (left); dorsal view of shell (right). Anterior of animals is to the right. Note black foot wall and epipodium, and row of shell respiratory tremata.

1985). Both monomeric and dimeric Mbs coexist in polyplacophoran radula muscles (Terwilliger and Read 1970a).

However, abalone appear to possess a structurally and genetically distinct Mb that differs from the globin-based forms present in most other gastropods. The Mb is present as a dimer of 40-41 kDa Mw, and more closely resembles, and is thought to have evolved from, the tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO). The globin-based and IDO-type Mbs are functionally similar and, as reviewed by Suzuki *et al.* (1998a), most oxyMbs isolated in a purified state share the common characteristic that the absorbance ratio of α- to β-peaks (579 to 544 nm, α/β ratio) ranges from 1.02 to 1.08. However, the α/β ratio of IDO-type Mbs are higher. The Mb from the radula muscles of the abalone *Sulculus diversicolor* shows a α/β ratio of 1.14 (Suzuki *et al.* 1998a). The spectrophotometric properties of the IDO-type Mb from the archaeogastropod *Turbo cornutus* are very similar to those of *S. diversicolor* Mb (Suzuki *et al.* 1998b). The mean α/β ratio of a crude *H. rubra* Mb preparation was 1.11. Although not as high as *S. diversicolor*, it is slightly higher than for other purified gastropod and vertebrate globin-based Mbs. Furthermore, the absorption spectra of *H. rubra* oxy-, deoxy- and metMb are similar to that for *S. diversicolor*, which in turn bears strong similarity to vertebrate oxyHb and oxyMb (Suzuki and Takagi 1992; Suzuki *et al.* 1998a).

Despite these similarities in absorption spectra and α/β ratios to other abalone and archaeogastropod Mbs, does *H. rubra* possess an IDO-type Mb? Whereas IDO-type Mbs have been identified for the abalones *Sulculus diversicolor* and *Nordotis madaka*, other reports suggest abalone Mb is a more traditional globin-based Mb. Earlier work on archaeogastropod radula muscle Mb indicated that *H. kamtschatkana* possess a homodimeric Mb of about 34 kDa (Terwilliger and Read 1970b). Suzuki and co-workers suggest that in at least *S. diversicolor*, *N. madaka*, *Turbo cornutus* (Turbinidae) and *Omphalius pfeifferi* (Trochidae), the gene for a "normal" Mb was lost, and modified 2,3-IDO evolved as a substitute during evolution. They maintain that the diversion of these lineages of archaeogastropods occurred about 220 million years ago, an event that isolated the IDO-type Mb and suggests the IDO-type Mb must have been present in a common ancestor (Kawamichi and Suzuki 1998; Suzuki *et al.* 1998a, 1998b). It stands to reason then that all abalone would possess the IDO-type Mb. Having expressed this belief, it is curious for the authors to then accept that a separate abalone species (*H. kamtschatkana*, Terwilliger and Read 1970b) possesses a normal globin-type Mb (Suzuki *et al.* 1996). This could be feasibly possible if, contrary to the above, the Mb arose separately and subsequent to the diversification of an ancestral abalone species (*i.e.* it only developed in a few species). To date, IDO-type Mbs

have not been identified outside of the Archaeogastropoda, and only a number of families within the Archaeogastropoda are known to possess the unusual Mb. The Patellidae, Acmaeidae and Neritidae of the Archaeogastropoda all retain the conventional 16 kDa globin-based Mb (Suzuki *et al.* 1998a).

Properties and function of Mb

In conjunction with the current data, a more integrated concept of how *H. rubra* radula muscles and Mb work may be obtained by examining literature on gastropod Mbs. The primary role of Mb in gastropod muscles is to facilitate the diffusion of O_2 from the haemolymph to the muscle mitochondria, as for vertebrate muscle. Just as O_2-equilibrium curves have been determined for molluscan Hc (see Chapter 6), so too have the O_2-binding properties of a number of molluscan Mbs. *Sulculus diversicolor* Mb shows a high O_2-affinity (P_{50} = 3.8 mmHg at 20°C and pH 7.4), is insensitive to pH (no Bohr effect pH 7.0-7.9), and does not display cooperative binding (n=1.02, Suzuki *et al.* 1996; Suzuki and Imai 1997). Oxygen-binding properties of another archaeogastropod IDO-type Mb, that of *Turbo cornutus*, are similar to those for *S. diversicolor* (P_{50}=3.5 mmHg, n=1.04; Suzuki *et al.* 1998b). These O_2-affinities are much higher than those of *H. rubra* Hc, at around 25-28 mmHg at 20°C and normoxic PCO_2 (Ainslie 1977, see Chapter 6).

Gastropod Mbs are known to bind O_2 reversibly, although little allosteric modulation by protons, CO_2 or organic phosphates has been demonstrated (Read 1966; Terwilliger and Read 1971; Geraci *et al.* 1977). The O_2-affinity and sigmoid nature of the O_2-dissociation curve of *Buccinum undatum* Mb are not affected by the concentration of the Mb, or by the presence of 2,3-DPG, α-glycerophosphate, ATP, phosphocreatine or lactate (Terwilliger and Read 1971). It is this last metabolite that is of potential importance in the current study on *H. rubra* metabolism. It is assumed that the form of lactate added in the above study was L-lactate, in keeping with the use of predominantly vertebrate Hb modulators. It is not certain whether these Mbs are equally insensitive to D-lactate, formed in whelk radula muscles during contractions under anoxia (Wiseman and Ellington 1987; Wiseman *et al.* 1989). The high O_2-affinity of abalone Mb, and the relative insensitivity of gastropod Mb to effectors, suggests that any muscle or haemolymph changes associated with anaerobic metabolism in *H. rubra* may not greatly influence the O_2-uptake of, or delivery of O_2 to, the radula muscles (see below).

What does the level of Mb say about the function of the radula muscle, considering the correlation between muscle work and Mb? Fänge and Mattisson (1958) conclude the richness

in respiratory enzymes and Mb in the radula muscles of *Buccinum undatum* is of great importance to this whelk in regards to the efficacy of feeding. The rapidity of food acquisition is imperative to a carnivore and scavenger taking its food in competition with other organisms. Manwell (1960a) further illustrates the point of a functional requirement in comparing the Mb levels in tectibranch gastropods. The sea hares *Aplysia* sp. feed almost continually on algae, with radula muscle Mb levels of 5-6 % dry wt (Rossi-Fanelli and Antonini 1957). In contrast, the related carnivore *Navanax inermis* has no Mb in its pharyngeal muscles, which it uses only occasionally to engulf prey whole (Manwell 1960a).

The relationship between Mb and feeding mode however is often irregular and not always so obvious. Even with the few examples given in the text and Table 3.14, it is evident that both herbivorous and carnivorous gastropods can possess not only elevated radula muscle enzyme activities, but high levels of Mb also. Moreover, the herbivorous Fissurellidae do not appear to possess a radula muscle Mb. A more appropriate correlation may be found with actual action of the radula itself, rather than feeding mode (carnivory, herbivory): the action of the radula and radula muscles would presumably be heavily influenced by the anatomy of the buccal cavity. The relatively short and broad snout of the Haliotidae presents a shorter mechanical distance than the extended proboscis of carnivorous or scavenging neogastropods.

Prosobranch gastropods and chitons possess both Hc and Mb, and the transfer of O_2 between the two proteins presumably is analogous to haemoglobin and Mb in vertebrates. For the diffusive transfer of O_2 to be efficient, the O_2-equilibrium curve of Mb must lie to the left of that of Hc. As a consequence, Mb becomes saturated at a low PO_2, maintaining a low O_2 tension and high O_2 gradient (Manwell 1958). Oxygen-binding data suggests that radula Mb has a higher affinity for O_2 than Hc for at least the chiton *Cryptochiton stelleri* (Manwell 1958), and the whelks *Busycon canaliculatum* and *Buccinum undatum* (Terwilliger and Read 1971). If this were the case for *H. rubra*, then the arrangement of the radula muscles in the cephalic sinuses would provide a constant supply of O_2 to the muscles. Oxygen-binding curves of the respiratory proteins would overlap, benefiting the delivery of O_2 to the muscles. At normoxic pH and CO_2, the affinity of *H. rubra* Hc is moderate, with P_{50} between 22 and 28 mmHg (Ainslie 1977, 1980b). The oxygen affinity of *Sulculus diversicolor* radula Mb is considerably higher, with a P_{50} of around 3.8 mmHg. If a favourable O_2 supply and gradient is maintained in *H. rubra*, radula muscle work, theoretically, should not be compromised and the muscles should not suffer hypoxia. This may hold true during normoxia (rest or sub-maximal activity), but what about during air exposure or exercise or clamping? Is Mb O_2-binding affected by changes in O_2-affinity of abalone Hc during hypoxia? These questions

stimulate further investigation into abalone respiratory proteins, and the effects of hypoxia on them.

3.4.8 Overview of indices of aerobic and anaerobic metabolism in *Haliotis rubra* muscles

It has been demonstrated that the biochemical profiles of *H. rubra* muscles can be correlated with their observed functions. The foot and, especially, the adductor muscle are known to power righting after inversion, shell clamping and locomotion in abalone. These muscles are well equipped to deal with high ATP demands and alterations in proton flux associated with anaerobic burst muscle work, by possessing high activities of TDH, AK, large glycogen and arginine phosphate stores, as well as high pH buffering capacities. In this respect, *H. rubra* pedal musculature is biochemically similar to the pedal musculature of other abalone species, and an obviously low scope for sustained aerobic work in white muscle is apparent for the genus. TDH and D-LDH are probably the only important pyruvate reductases in the genus, and whilst structural and maintenance costs in the resting foot and adductor may be met aerobically, any increased energy demands of these muscles are probably sustained anaerobically.

In marked contrast to the pedal musculature, highest activities of some glycolytic and mitochondrial enzymes indicate enhanced abilities of radula muscles to function aerobically. These properties are similar to those for other gastropod radula muscles. Unlike whelk muscles however, abalone radula muscles do not show high activities of pyruvate reductase enzymes, a feature also reflected in their low pH buffering capacity. The relatively higher activities of PFK in the radula muscles may indicate a higher flux of aerobic glycolysis in this muscle, an assumption corroborated by higher PK and CS activities. Higher activities of HK and HOAD in the radula muscles suggest an enhanced capacity for both glucose and fatty acid use, and radula muscles may rely more heavily on haemolymph-borne fuels than the pedal musculature.

The kinetic and electrophoretic properties of *H. rubra* foot and radula muscle D-LDH do not differ, and presumably indicate the same molecular form of the enzyme is present in both muscles. Pyruvate inhibition of D-LDH in *H. rubra* suggests the enzyme may be more like a vertebrate H-type than M-type isozyme, a feature noted for other abalone and gastropod muscle D-LDHs. However, the production of D-lactate by the pedal muscles of abalone concomitantly suggests a similar potential for the radula muscles to accumulate this metabolite. Despite these assays being run with crude D-LDH preparations, with possible interfering compounds present, the results suggest that any further investigation into the possibility of different isozymes probably is not warranted.

Levels of arginine phosphate suggest hydrolysis of the phosphagen may be less important in radula muscles compared to adductor and foot muscles, although the activity of AK in gastropod radula muscles does not entirely support this conclusion. The concentrations of carbohydrates in the muscles point to a largely anaerobic and possible storage role of the pedal musculature.

These conclusions drawn from the enzymatic, pH buffering and substrate profiles are further supported by the presence and amount of Mb in the radula muscles of *H. rubra*. The possession of a respiratory protein greatly increases access to circulating O_2, important in the aerobic function of these muscles. Absence of Mb in the pedal musculature correlates with their higher anaerobic poise.

The constant muscle work required to protrude and retract the radula during extended feeding bouts of abalone probably are supported by a greater flux of carbohydrate, especially glucose, through glycolysis and the TCA cycle, and the provision of O_2 via Mb. The ability to utilise lipid intermediates and the importance of the mitochondria are elevated in the radula muscle. The biochemical profiles of *H. rubra* muscles suggest that the radula muscle is a predominantly aerobic tissue, a conclusion entirely in keeping with its function in the intact animal.

The indices of metabolic potential suggest that the adductor and foot muscles have a greater scope and capacity for anaerobic metabolism, and probably rely more on facultative anaerobic metabolism than do the radula muscles, which appear predominantly aerobically poised. The next step in this study is to test these predictions made about muscle biochemistry and function. This is achieved by examining changes in the levels of anaerobic metabolites in these muscles during situations where anaerobic metabolism might be expected, such as during elevated muscle work in the form of exercise and feeding, and environmental hypoxia. This forms the basis of Chapters 4 and 5.

Table 3.1. Enzyme activities (μmol substrate min^{-1} g^{-1} wet wt tissue) in *H. rubra* tissues at 20°C

Enzyme	Adductor	Foot	Ctenidia	Radula
Alanopine dehydrogenase	1.01 ± 0.29^{a}	0.90 ± 0.36^{a}	0.11 ± 0.07^{b}	1.02 ± 0.17^{a}
β-Alanopine dehydrogenase	0.61 ± 0.37^{a}	1.00 ± 0.11^{a}	0.11 ± 0.05^{a}	0.64 ± 0.10^{a}
D-Lactate dehydrogenase	1.92 ± 0.13^{a}	7.19 ± 0.41^{b}	5.87 ± 0.32^{c}	6.98 ± 0.37^{bc}
Lysopine dehydrogenase	0.23 ± 0.08^{a}	0.89 ± 0.41^{b}	1.46 ± 0.13^{b}	1.69 ± 0.85^{b}
Octopine dehydrogenase	0.17 ± 0.09^{a}	0 ± 0^{b}	0.07 ± 0.06^{a}	0.77 ± 0.15^{a}
Strombine dehydrogenase	0.23 ± 0.04^{a}	0.34 ± 0.18^{b}	0.34 ± 0.15^{b}	0.50 ± 0.50^{b}
Tauropine dehydrogenase	36.97 ± 5.52^{a}	19.38 ± 2.35^{b}	0.16 ± 0.09^{c}	0.22 ± 0.22^{c}
Arginine kinase	153.49 ± 17.82^{a}	113.20 ± 14.81^{a}	19.84 ± 0.98^{b}	165.85 ± 8.43^{a}
Phosphorylase	2.40 ± 0.01^{a}	2.05 ± 0.17^{a}	0.66 ± 0.01^{b}	2.17 ± 0.23^{a}
Pyruvate kinase	20.24 ± 2.41^{a}	20.59 ± 2.76^{a}	7.64 ± 1.50^{b}	35.75 ± 3.84^{c}
Phosphofructokinase	0.42 ± 0.11^{a}	0.20 ± 0.07^{ab}	0.16 ± 0.04^{b}	0.54 ± 0.16^{a}
Hexokinase	0.17 ± 0.03^{a}	0.25 ± 0.03^{a}	0.88 ± 0.03^{b}	1.67 ± 0.16^{c}
HOAD*	0.06 ± 0.01^{a}	0.19 ± 0.08^{b}	0.53 ± 0.06^{c}	0.75 ± 0.16^{d}
Citrate synthase*	0.18 ± 0.05^{a}	1.04 ± 0.13^{b}	4.15 ± 0.29^{c}	17.07 ± 1.00^{d}

*=sonicated

Values given as Mean ± S.E.M for N=6

Common superscripts indicate no significant difference within muscle groups, and different superscripts are significant at $P<0.05$ (except where double superscripts indicate no significant difference with groups sharing superscripts)

Table 3.2. Activities (μmol substrate min^{-1} g^{-1} wet wt tissue) of CS and HOAD in sonicated and non-sonicated *H. rubra* tissues at 20°C.

Enzyme	Foot	Adductor	Ctenidia	Radula
CS non-sonicated	0.52 ± 0.07	0.15 ± 0.03	0.89 ± 0.10	3.26 ± 0.35
CS sonicated	1.04 ± 0.13*	0.18 ± 0.05	4.15 ± 0.29*	17.07 ± 1.00*
HOAD non-sonicated	0.10 ± 0.05	0 ± 0	0.21 ± 0.03	0.1± 0
HOAD sonicated	0.19 ± 0.08	0.06 ± 0.01*	0.53 ± 0.06	0.75 ± 0.16*

* = significantly different from non-sonicated sample at $P<0.05$

Values given as Mean ± S.E.M for N=6

Table 3.3. Ammonium sulphate purification of *H. rubra* foot and radula muscle D-LDH

Muscle	Optimal AS%	Preparation	Specific activity (i.u. mg^{-1})	Purification (n-fold)
Foot	70-80%	Initial homogenate	0.17	
		Final preparation	0.73	4.29
Radula	60-75%	Initial homogenate	0.18	
		Final preparation	0.45	2.50

Values given as Mean for N=3

Table 3.4. K_m values of *H. rubra* foot and radula muscle D-LDH in the direction of pyruvate reduction at 15°C.

Muscle	pH	Substrate	Co-substrate	K_m (mmol L^{-1})
Foot	6.8	Pyruvate	0.15 mmol L^{-1} NADH	0.16 ± 0.01
		NADH	0.5 mmol L^{-1} Pyruvate	0.01 ± 0.01
	7.3	Pyruvate	0.15 mmol L^{-1} NADH	0.22 ± 0.05
		NADH	0.75 mmol L^{-1} Pyruvate	0.01 ± 0.01
Radula	6.8	Pyruvate	0.15 mmol L^{-1} NADH	0.33 ± 0.08
		NADH	0.75 mmol L^{-1} Pyruvate	0.01 ± 0.01
	7.3	Pyruvate	0.15 mmol L^{-1} NADH	0.20 ± 0.02
		NADH	0.75 mmol L^{-1} Pyruvate	0.01 ± 0.01

Values given as Mean ± S.E.M. for N=4

Table 3.5. K_m values of *H. rubra* foot and radula muscle D-LDH in the direction of lactate oxidation at pH 9.0 and 35°C.

Muscle	Substrate	Co-substrate	K_m (mmol L^{-1})
Foot	D-lactate	1.4 mmol L^{-1} NAD^+	2.43 ± 0.18
	NAD^+	4 mmol L^{-1} D-lactate	0.14 ± 0.01
Radula	D-lactate	1.4 mmol L^{-1} NAD^+	5.11 ± 0.26
	NAD^+	4 mmol L^{-1} D-lactate	0.16 ± 0.01

Values given as Mean ± S.E.M. for N=4

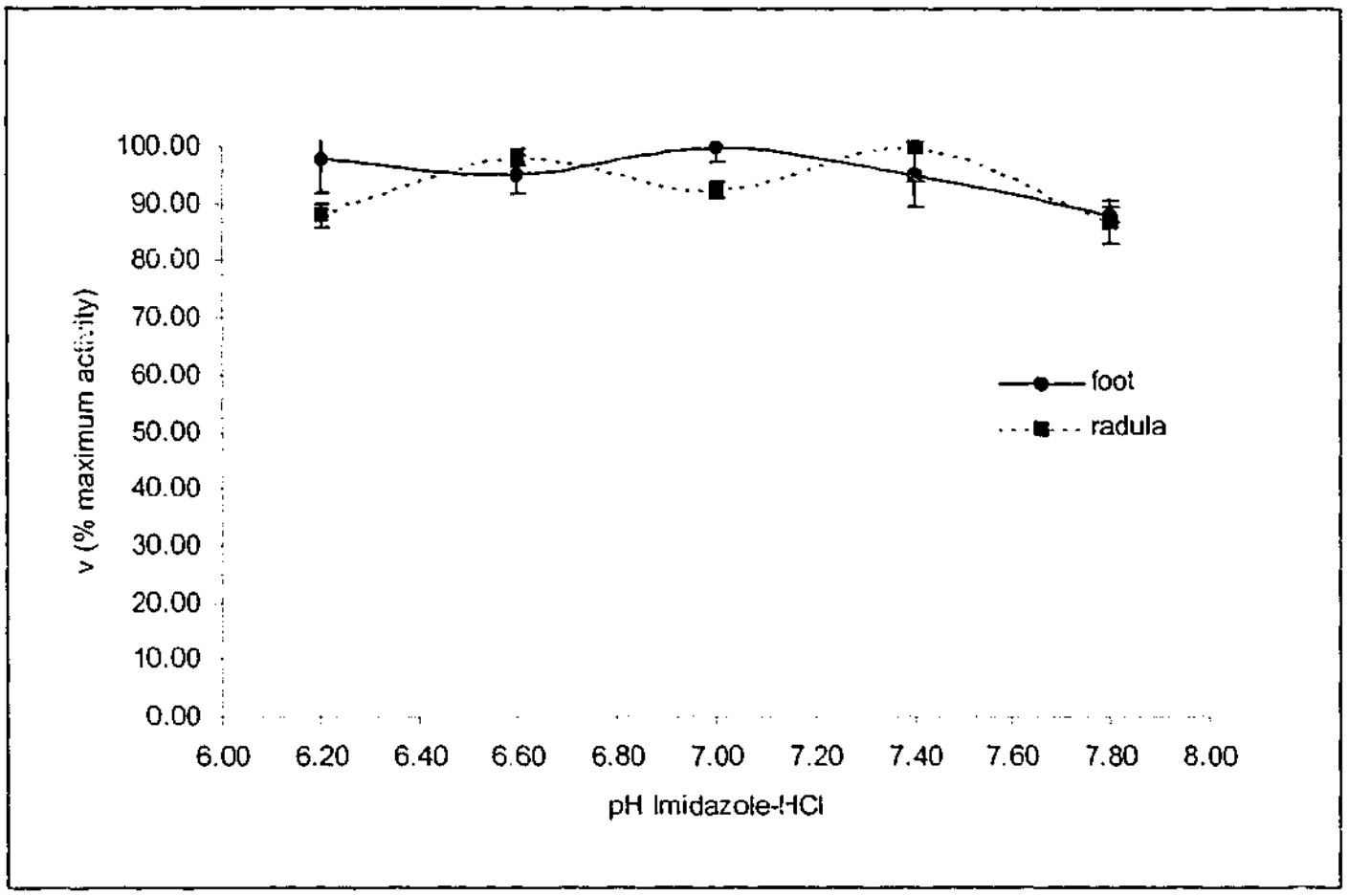
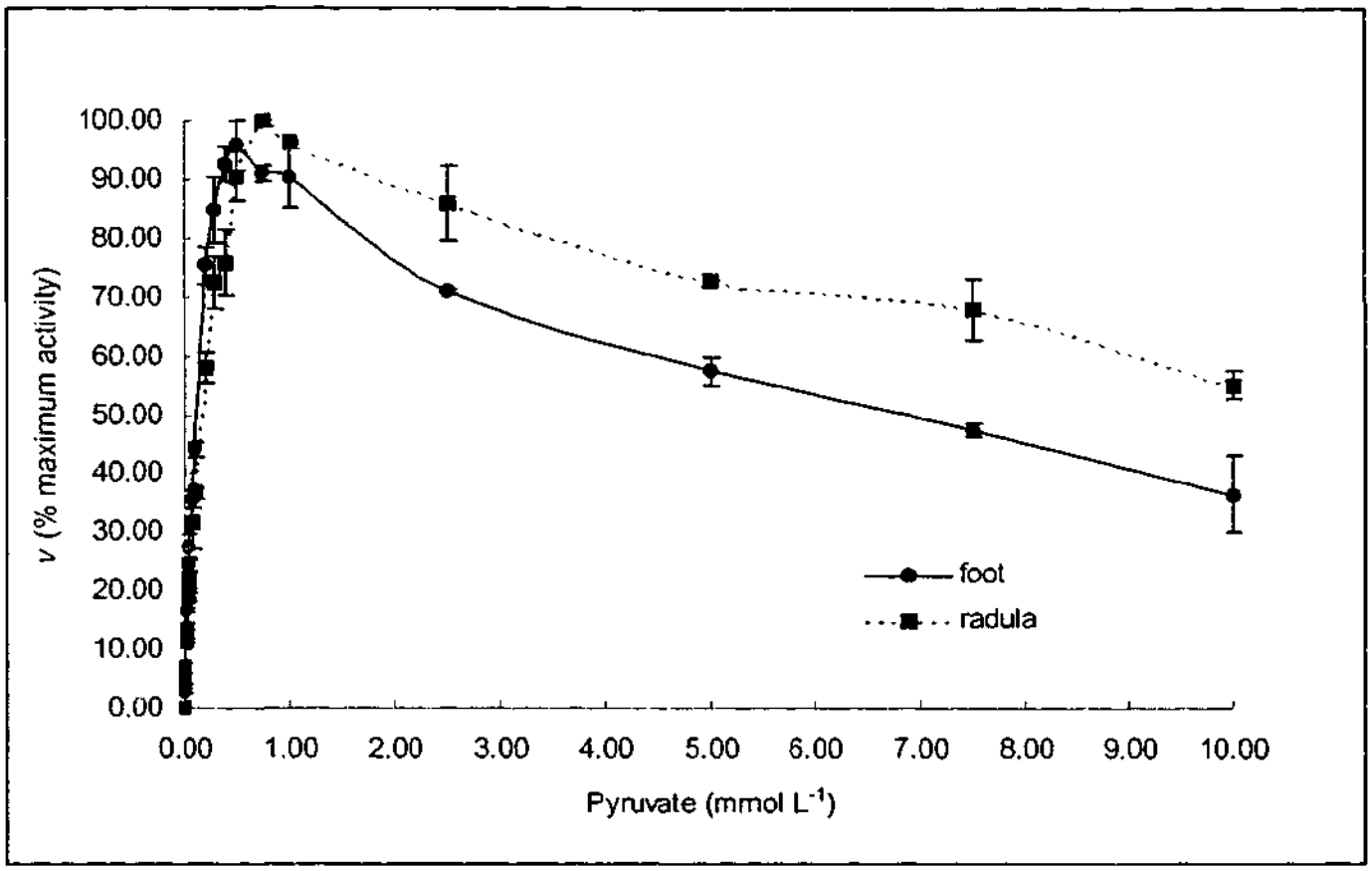


Figure 3.1. Substrate saturation curve of *H. rubra* D-LDH: effect of pH on D-LDH activity. Assay conditions: 0.75 mmol L^{-1} pyruvate, 0.15 mM NADH, 50 mmol L^{-1} Imidazole-HCl buffer, pH 6.2-7.8, 20°C.

(A)



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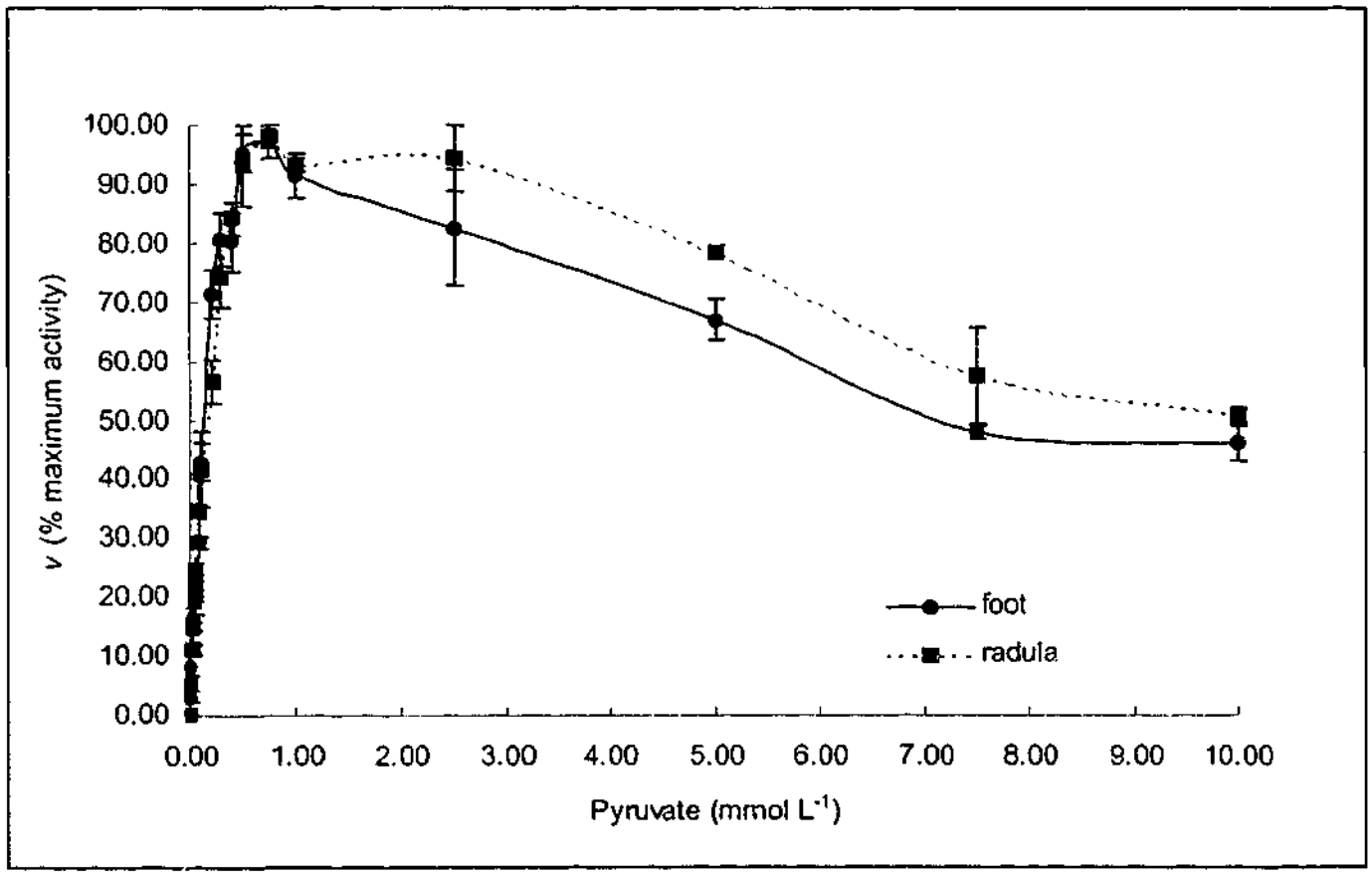
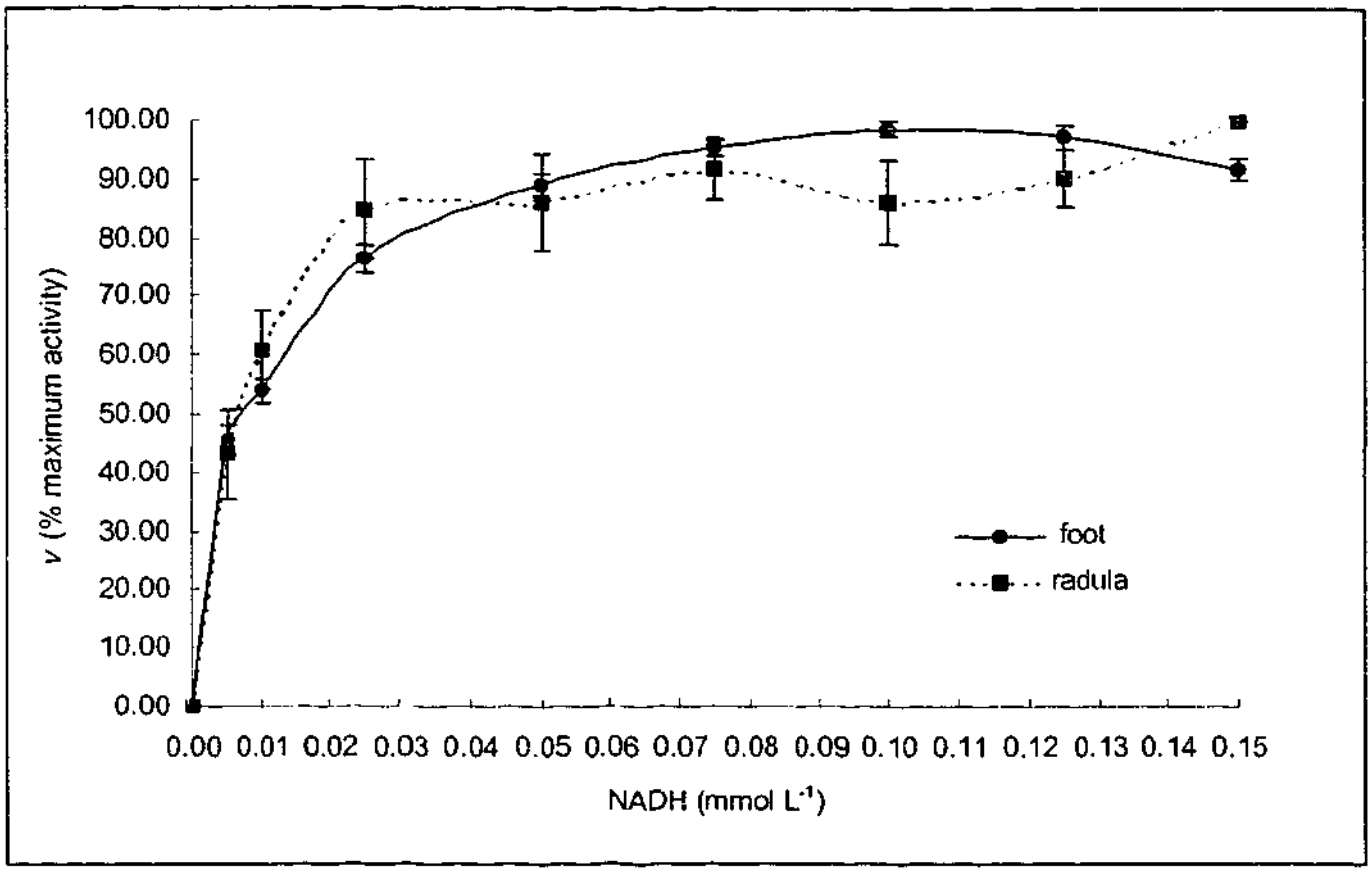


Figure 3.2. Substrate saturation curve of *H. rubra* D-LDH: pyruvate saturation curve at (A) pH 6.8 and (B) pH 7.3. Assay conditions: 0.005-10 mmol L^{-1} pyruvate, 0.15 mM NADH, 50 mmol L^{-1} Imidazole-HCl buffer, pH 6.8 and 7.3, 20°C.

(A)



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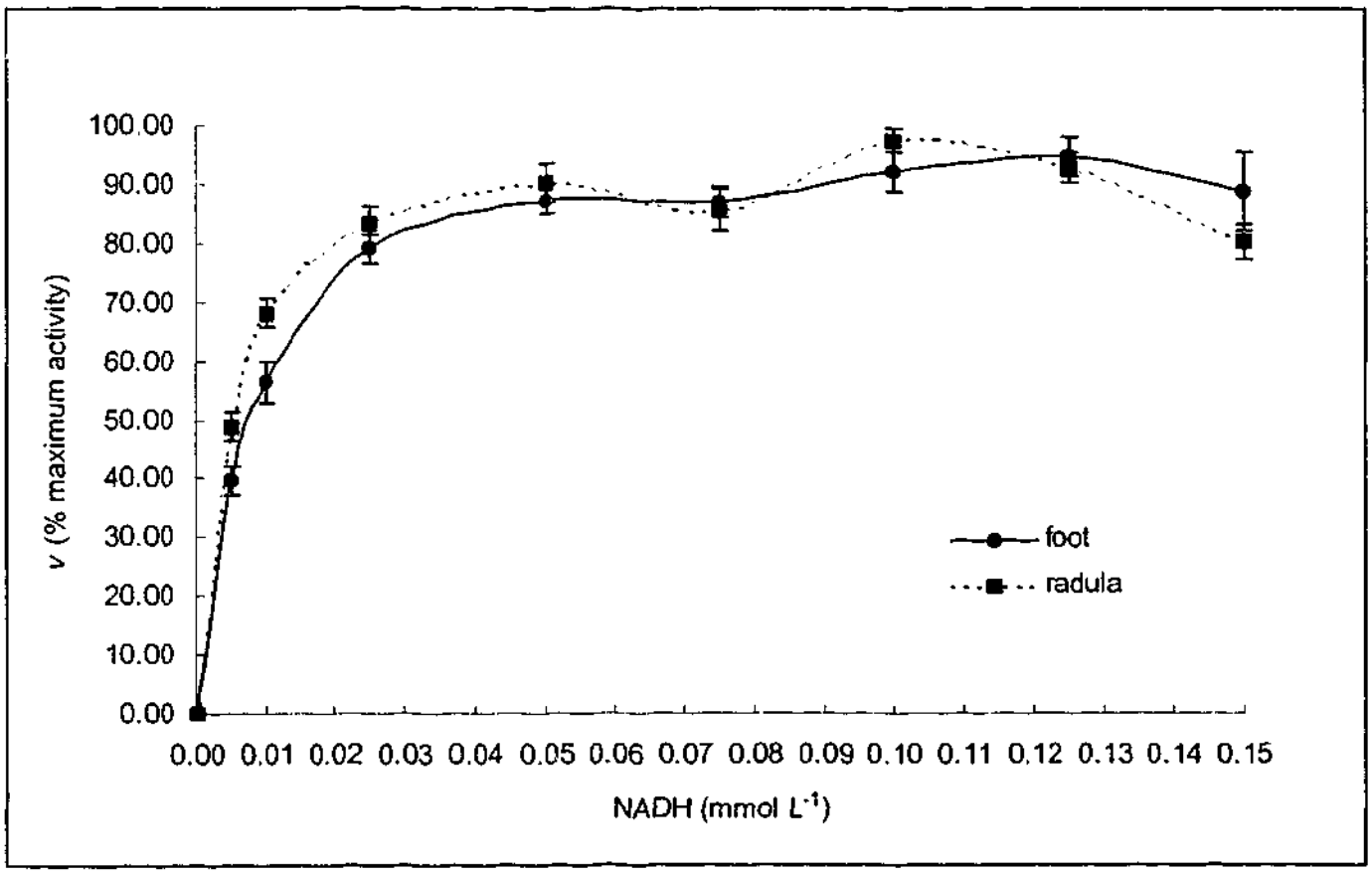
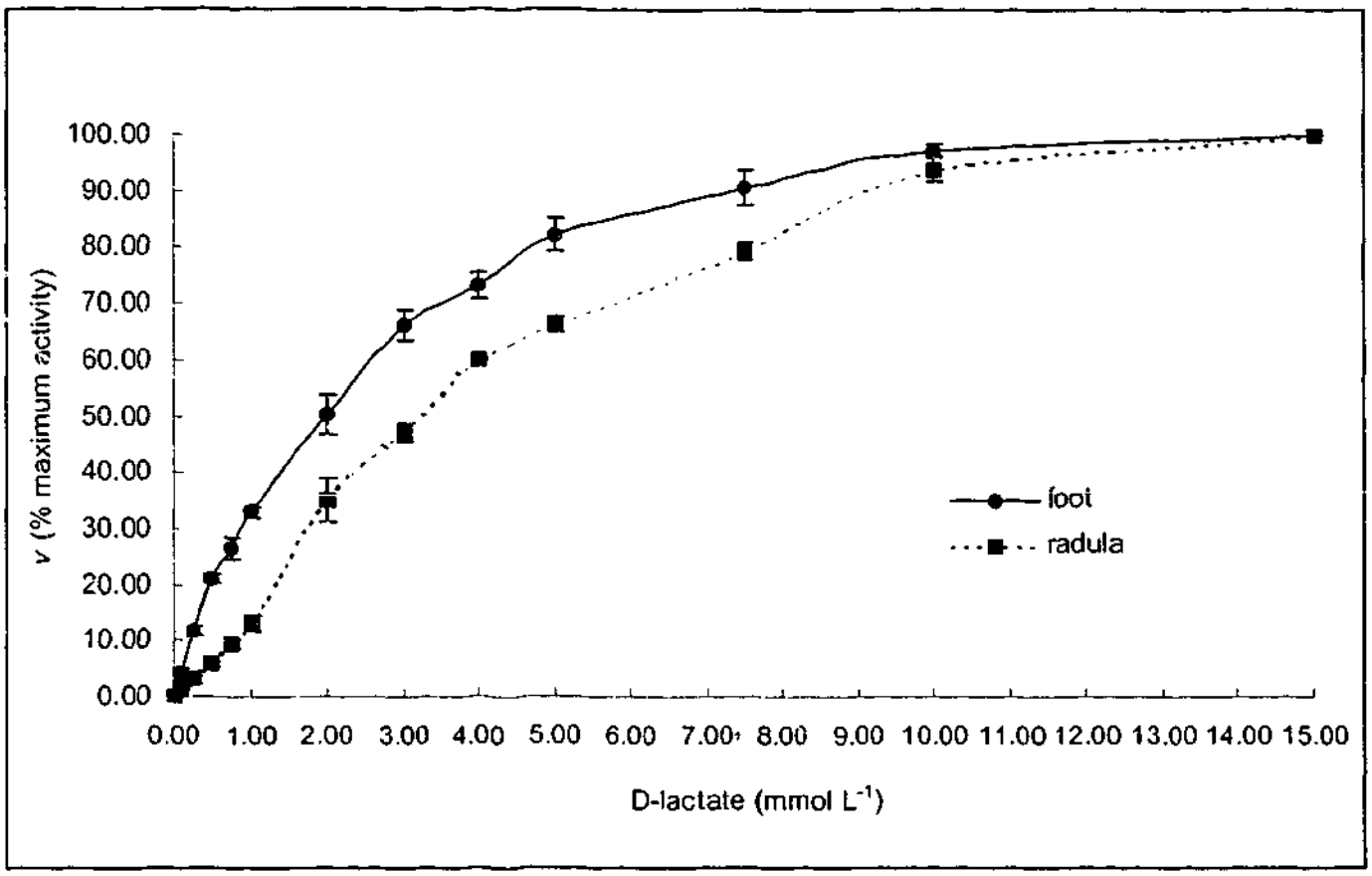


Figure 3.3. Substrate saturation curve of *H. rubra* D-LDH: NADH saturation curve at (A) pH 6.8 and (B) pH 7.3. Assay conditions: 0.75 mmol L^{-1} pyruvate, 0.005-0.15 mM NADH, 50 mmol L^{-1} Imidazole-HCl buffer, pH 6.8 and 7.3, 20°C.

(A)



(B)

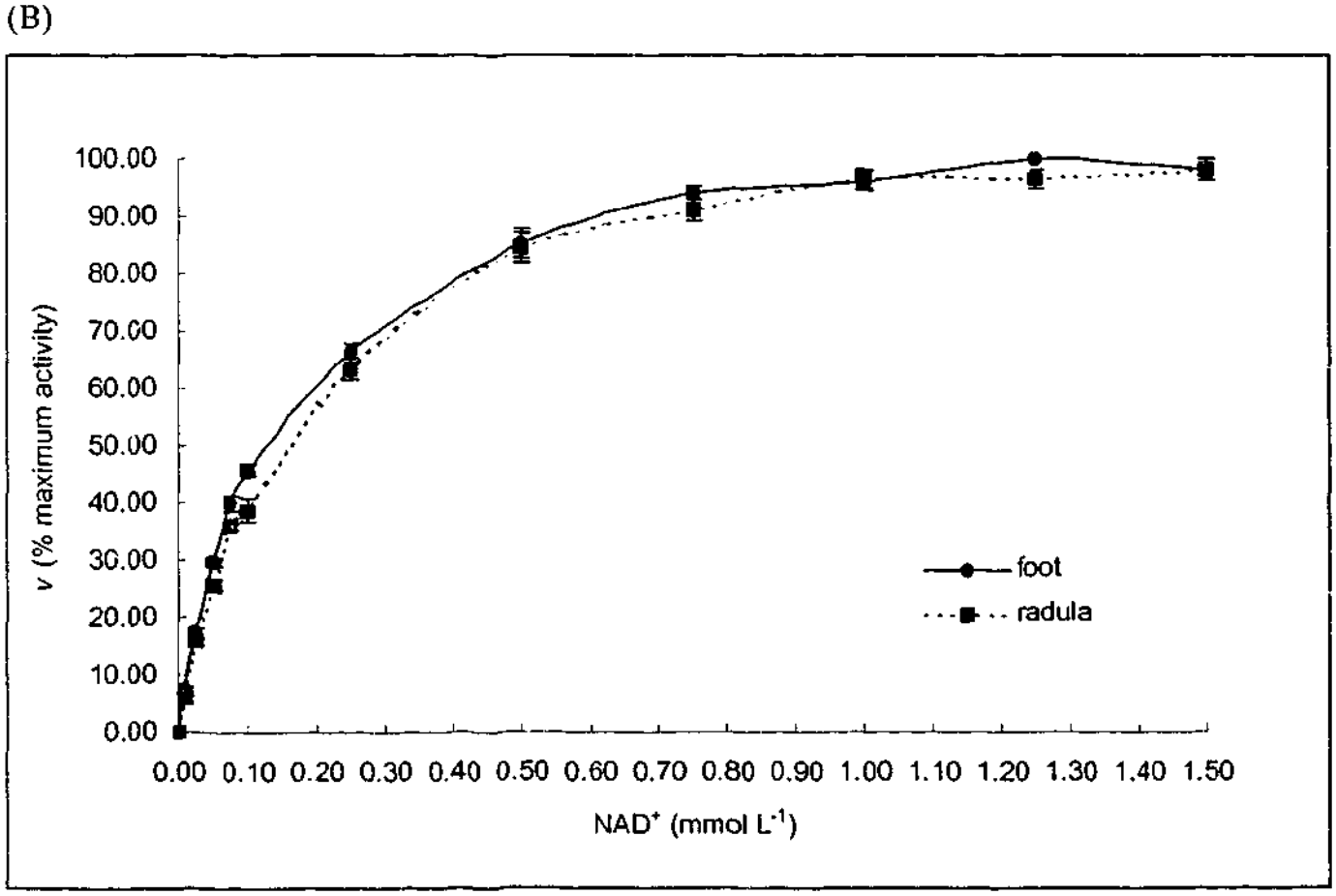
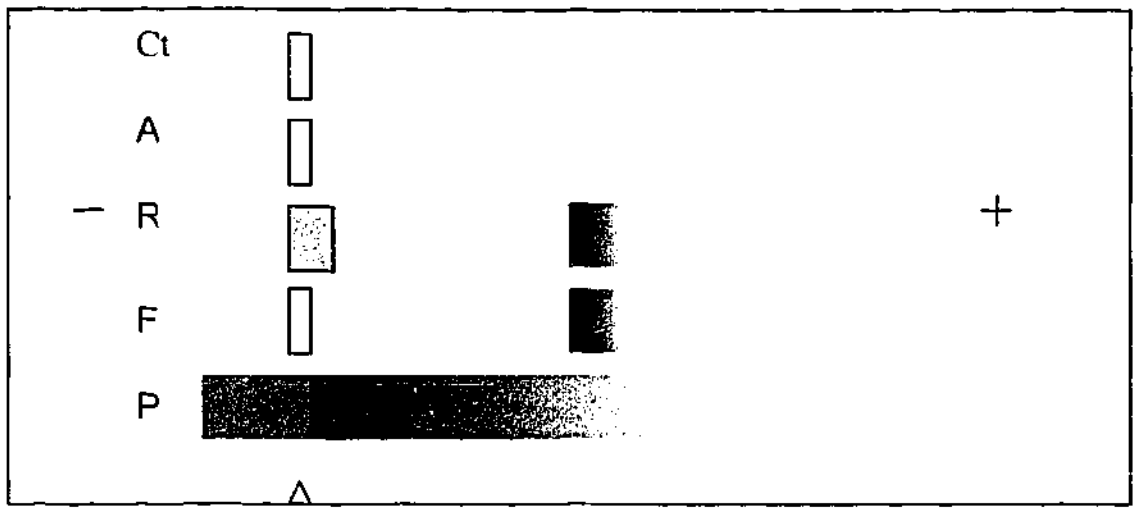
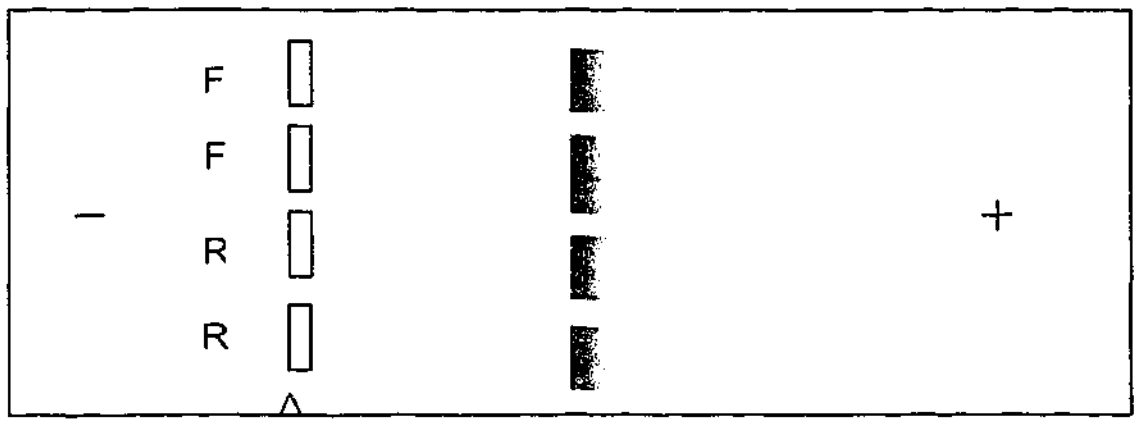


Figure 3.4. Substrate saturation curve of *H. rubra* D-LDH: (A) D-lactate saturation curve and (B) NAD^+ saturation curve at pH 9.0. Assay conditions: (A) 0.1-15 mmol L^{-1} D-lactate, 0.14 mmol L^{-1} NAD+; (B) 4 mmol L^{-1} D-lactate, 0.03-1.5 mmol L^{-1} NAD+, 100 mmol L^{-1} Tris-HCl buffer, pH 9.0, 20°C.

(A)



(B)



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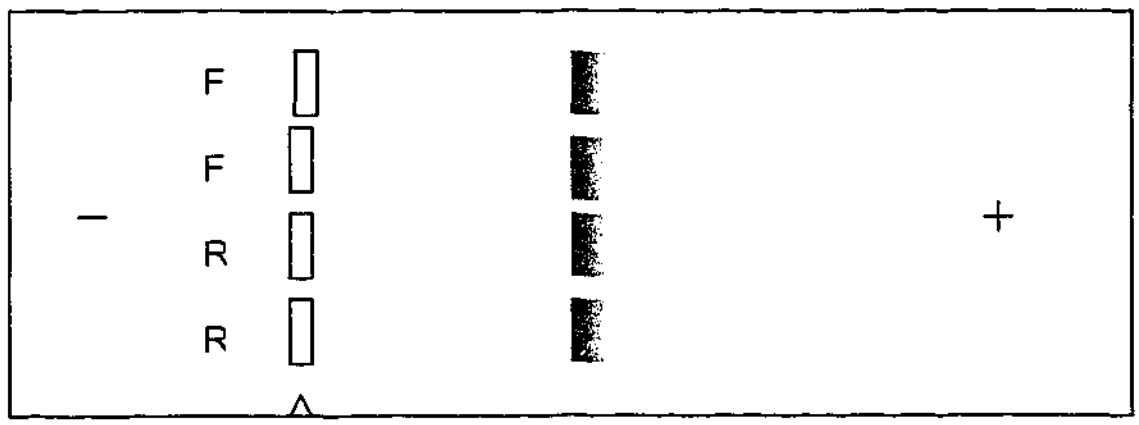
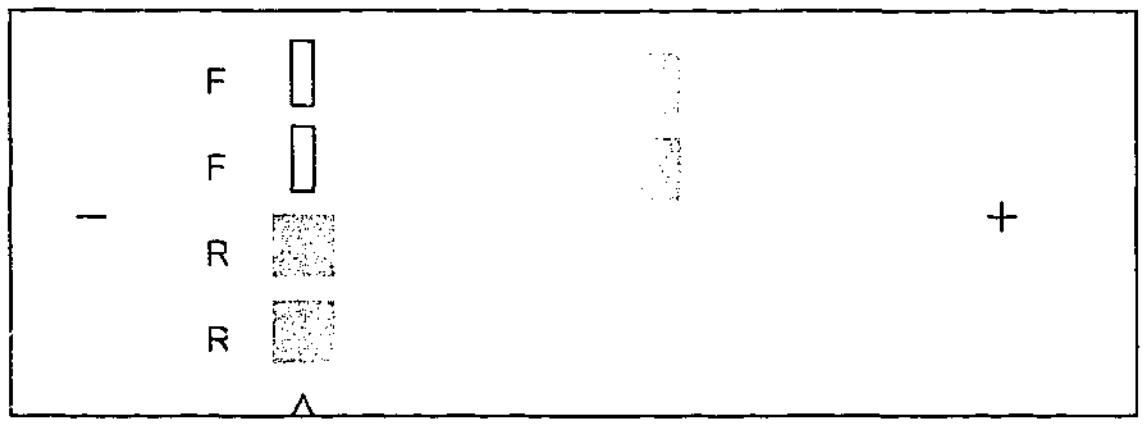


Figure 3.5. Zymograms of *H. rubra* D-LDH cellulose acetate gel electrophoresis. Abbreviations are as follows: Ct = ctenidia; A = adductor muscle; R = radula muscle; F = foot muscle; and P = chicken pectoralis muscle. Intensity of bands reflects intensity of staining on cellulose acetate gel. Shading gradient indicates smearing of bands on cellulose acetate gel. Origin as indicated by Δ. (-) = cathode, (+) = anode. (A) Initial gel on crude adductor, foot radula muscle and ctenidia homogenates in 100 mmol L^{-1} Tris-EDTA-borate buffer pH 8.7. (B) Second gel on foot and radula muscle supernatants. Note presence of faint activity bands for radula muscle only. (C) Third gel on foot and radula muscle supernatants. Note lack of secondary bands.

(D)



(E)

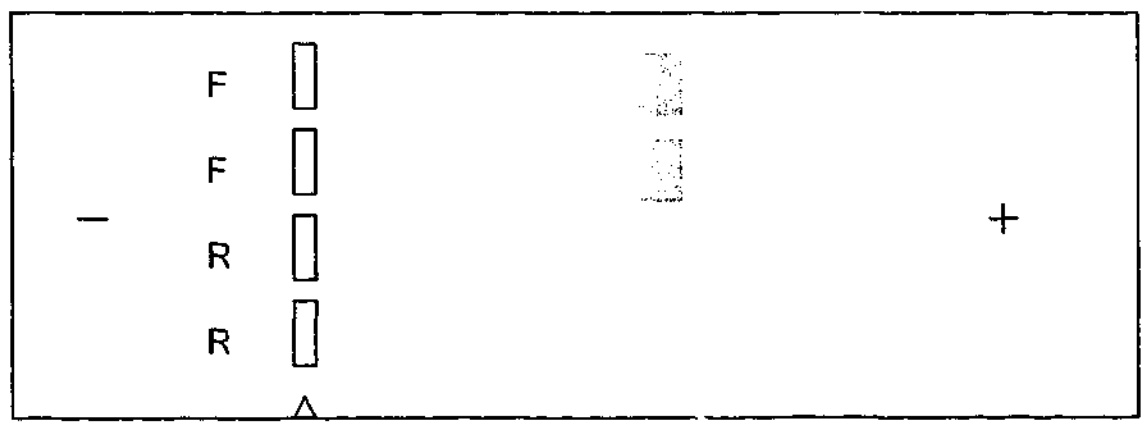


Figure 3.5 (continued). Zymograms of *H. rubra* D-LDH cellulose acetate gel electrophoresis. Abbreviations are as follows: Ct = ctenidia; A = adductor muscle; R = radula muscle; F = foot muscle; and P = chicken pectoralis muscle. Intensity of bands reflects intensity of staining on cellulose acetate gel. Shading gradient indicates smearing of bands on cellulose acetate gel. Origin as indicated by Δ. (-) = cathode, (+) = anode. (D) Typical gel using dialysed ammonium sulphate samples in 50 mmol L^{-1} Tris-maleate-EDTA-$MgCl_2$ buffer pH 7.8. Note staining at origin for radula muscle only. (E) Dialysed ammonium sulphate samples in 100 mmol L^{-1} Tris-EDTA-borate buffer pH 8.7. Note lack of staining at origin for radula muscles.

Table 3.6. Pyruvate inhibition ratios of *H. rubra* foot and radula muscle D-LDH

Muscle	Assay pH	Inhibition ratio (mmol L^{-1}:mmol L^{-1})	
		0.3:10	1:10
Foot	6.8	2.292	2.462
	7.3	1.799	2.044
Radula	6.8	1.320	1.756
	7.3	1.357	1.756

Table 3.7. pH buffering capacity (β, slykes) of *H. rubra* muscle.

Muscle	Adductor	Foot	Radula
Buffering capacity (β)	37.23 ± 1.76^{a}	28.66 ± 1.46^{b}	20.81 ± 1.06^{c}

Values given as Mean ± S.E.M for N=6

Common superscripts indicate no significant difference within muscle groups, and different superscripts are significant at $P<0.05$ (except where double superscripts indicate no significant difference with groups sharing superscripts)

Table 3.8. Free arginine and arginine phosphate content (μmol g^{-1} wet wt) of *H. rubra* muscles

Muscle	Adductor	Foot	Radula
Free arginine	16.75 ± 5.45^{a}	14.17 ± 6.32^{a}	10.12 ± 0.40^{a}
Arginine phosphate	24.42 ± 3.87^{a}	14.93 ± 3.96^{ab}	10.55 ± 1.10^{b}

Values given as Mean ± S.E.M for N=6

Common superscripts indicate no significant difference within muscle groups, and different superscripts are significant at P<0.05 (except where double superscripts indicate no significant difference with groups sharing superscripts)

Table 3.9. Concentrations of glucose and glycogen (as µmol glucosyl units g^{-1} wet wt) in *H. rubra* muscles at rest.

Muscle	Treatment	Substrate	
		Glucose	Glycogen
Adductor	non-centrifuged	-	243.94 ± 34.08^{a}
Foot		-	92.67 ± 24.11^{b}
Radula		-	53.93 ± 5.73^{b}
Adductor	centrifuged	2.65 ± 0.34^{a}	138.45 ± 51.71*
Foot		1.46 ± 0.27^{b}	29.48 ± 6.85*
Radula		2.25 ± 0.27^{ab}	12.37 ± 2.81*

* = significantly different from non-centrifuged sample

Values given as Mean ± S.E.M for N=6

Common superscripts indicate no significant difference between muscles, and different superscripts are significant at $P<0.05$ (except where double superscripts indicate no significant difference with groups sharing superscripts)

Table 3.10. Myoglobin concentration of *H. rubra* muscles (% = g $100g^{-1}$ wet wt muscle)

Muscle	Adductor	Foot	Radula
% myoglobin	0.04 ± 0.01	0.05 ± 0.01	0.99 ± 0.04

Values given as Mean ± S.E.M for N=8 for radula muscle and N=4 for adductor and foot muscle

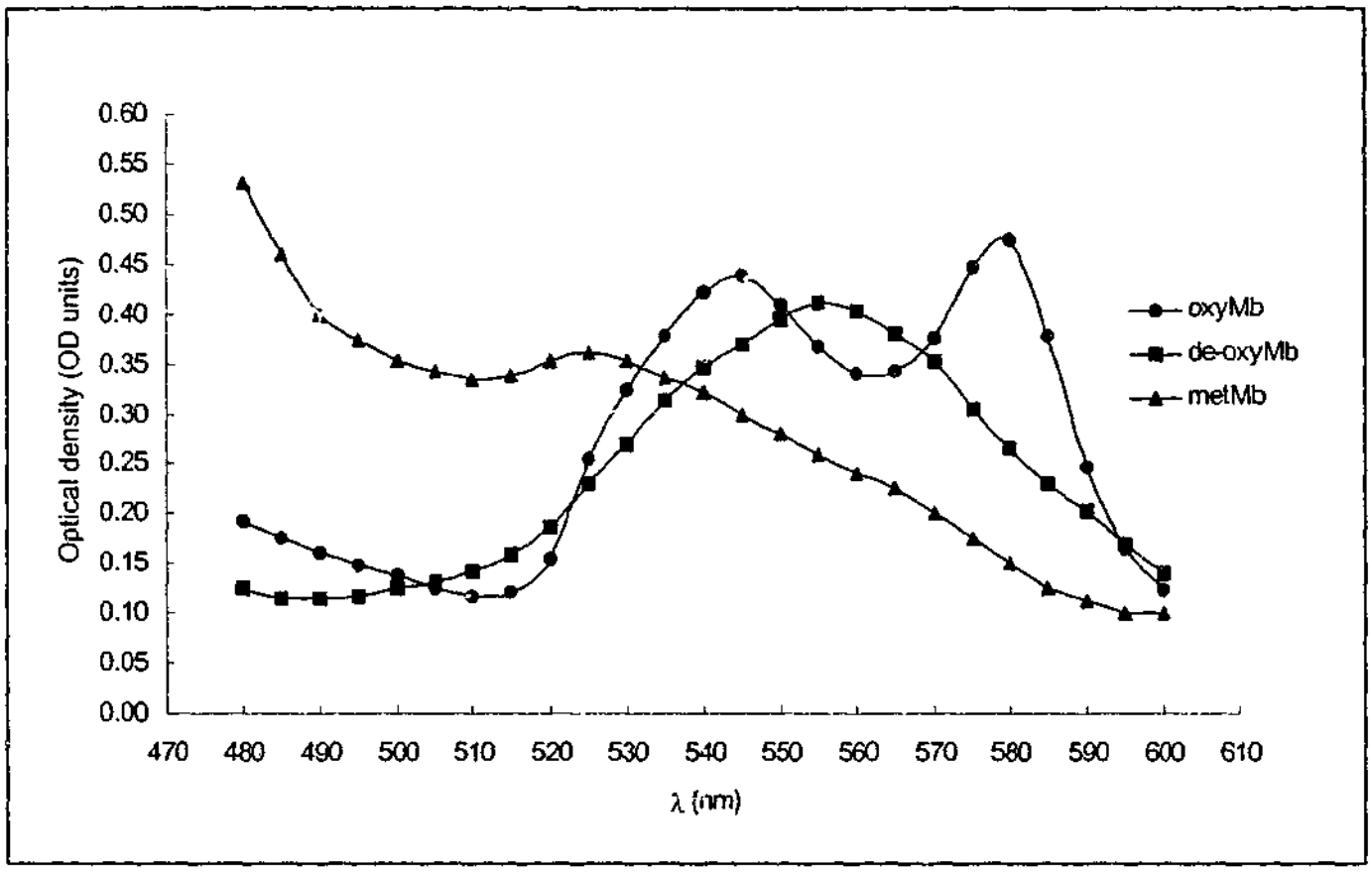


Figure 3.6. Absorption spectra of *H. rubra* radula muscle oxy, de-oxy and metmyoglobin in 20 mmol L^{-1} Tris-HCl buffer pH 8.0 at 20°C.

Table 3.11. Mean activities of D-LDH and TDH (μmol min^{-1} g^{-1} wet wt tissue) for *Haliotis*.

Species	Tissue	Enzyme		Reference
		D-LDH	TDH	
H. asinina	Adductor	4.5	48.3	Baldwin *et al.* (in prep.)
	Foot	9.1	19.4	
H. australis	Adductor	2.6	65.8	Wells and Baldwin (1995)
	Foot	4.3	28.1	
H. cracherodii	Foot	8.1		Bowen (1987)
H. corrugata	Foot	5.6		
H. fulgens	Foot	5.8		
H. rufescens	Foot	3.1		
H. rufescens	Adductor	1.0	1.7	Hammen and Bullock (1991)
	Foot	2.2	0	
H. discus hannai	Adductor	5.3	45.1	Sato *et al.* (1991)
	Foot	3.0	3.3	
H. iris	Adductor	4.3	93	Baldwin *et al.* (1992)
	Foot	5.6	78	
H. kamtschatkana	Adductor	1.2	29.0	Donovan *et al.* (1999)
	Foot	9.1	20.1	
H. lamellosa	Adductor	2.0	44.4	Gäde (1986)
	Foot	10.8	6.9	
	Mantle	1.7	0.9	
	Radula retractor	4.3	<0.1	
	Ctenidia	2.6	<0.1	
	Residual tissue	4.0	<0.1	
H. rubra	Adductor	1.9	37	This study
	Foot	7.2	19.4	
	Ctenidia	5.9	0.2	
	Radula	7.0	0.2	

Table 3.12. K_m values for *Haliotis* D-LDH

Species	Tissue	Substrate	K_m (mmol L^{-1})	Reference
H. rubra	Foot	Pyruvate	0.22	This study
		NADH	0.01	
		D-lactate	2.43	
		NAD^+	0.14	
	Radula	Pyruvate	0.20	
		NADH	0.01	
		D-lactate	5.11	
		NAD^+	0.16	
H. cracherodii	Foot	Pyruvate	0.16	Long *et al.* (1979)
		NADH	0.02	
		D-lactate	1.90	
		NAD^+	0.1	
H. discus hannai	Foot	Pyruvate	0.50	Sato *et al.* (1991)
		NADH	0.01	

Table 3.13. pH Buffering capacity (β, Slykes) of *Haliotis* muscle, and radula muscle of prosobranch and polyplacophoran molluscs. (All determinations made by the titration method).

Species	Muscle	Buffering capacity	Reference
H. asinina	Adductor	30.2	Baldwin *et al.* (in prep)
	Foot	26.4	
H. australis	Adductor	35.9	Wells and Baldwin (1995)
	Foot	31.5	
H. iris	Adductor	34.2	
	Foot	32.4	
H. rubra	Adductor	37.2	This study
	Foot	28.7	
	Radula	20.8	
B. canaliculatum	Radula retractor	36.7	Eberlee and Storey (1984)
		47	Wiseman and Ellington (1989)
D. textilosa (*orbita*)	Radula	24	Morris and Baldwin (1984)
I. australis	Radula	14	

Table 3.14. Myoglobin concentration in molluscan muscles (% = g $100g^{-1}$ wet wt muscle).

Species	Muscle	Amount (%)	Reference
Gastropoda			
H. rubra	Adductor	0.04	This study
	Foot	0.05	
	Radula	0.99	
B. canaliculatum	radula	3-4	Manwell (1963)
	heart	0[a]	
B. contrarium	radula	<1	
	heart	2-3	
Aplysia depilans[a]	buccal	4.9-6.2[b]	Rossi-Fanelli and Antonini (1957)
Polyplacophora			
Cryptochiton stelleri	radula	1-2	Manwell (1958)
Bivalvia			
Teredo sp.	adductor	≤2	Manwell (1963)
Bankia sp.	adductor	≤2	

[a]=*B. canaliculatum* found to have little Mb in heart (Ball and Meyerhoff 1940)

[b]=% dry weight basis

CHAPTER FOUR

ENERGY METABOLISM IN *HALIOTIS RUBRA*: EFFECTS OF HYPOXIA ASSOCIATED WITH WHOLE ANIMAL EXERCISE AND EMERSION ON PEDAL MUSCLE AND HAEMOLYMPH METABOLITES

4.1 INTRODUCTION

It was concluded from the data presented in Chapter 3 that the poise of the adductor and foot muscles of *H. rubra*, unlike that of the radula muscles, is toward a low aerobic scope and considerable dependence on facultative anaerobic metabolism. The pedal musculature of other abalone species has been shown to utilise facultative anaerobiosis during both elevated sustained activity, and exposure to air (emersion). Therefore one would predict that whole animal muscle work and air exposure should lead to the accumulation of the anaerobic metabolites D-lactate and tauropine in the pedal musculature of *H. rubra*. However, little is known about changes in the haemolymph during these hypoxic events. This chapter aims to examine the effect of hypoxia on the pedal musculature, and haemolymph, of *H. rubra*. The effects of whole animal exercise and air emersion, and the specific effects of muscle work in the form of feeding, on radula muscles are considered in Chapter 5.

4.1.1. Hypoxia in *Haliotis*

Abalone are generally subtidal, although their distribution may extend into the littoral zone (Bowen 1984, 1987; pers. obs). For abalone, environmental hypoxia would manifest as a result of low tide or migration into the littoral zone, during periods of slack water, or as a result of an increasing temperature and decreasing dissolved O_2 in rock pools (Newell 1973; Mangum and van Winkle 1973; Truchot and Duhamel-Jouve 1980). Environmental hypoxia would also ensue during emersion and handling procedures associated with commercial transport and aquaculture. The mode of gas exchange in abalone precludes making effective use of aerial O_2, as the ctenidia collapse in air, rendering gas uptake and transfer difficult (Houlihan *et al.* 1981; McMahon 1988a, 1988b).

Functional hypoxia can arise during muscular activity associated with locomotion or exercise during righting after inversion. As noted in Chapter 1, the sessile appearance of abalone belies their activity, since abalone display fast gliding rates during locomotion

(Donovan and Carefoot 1997; Donovan *et al.* 1999), and rapid and impressive shell twisting behaviour utilised during predator avoidance and righting after dislodgment. The constant contraction of the pedal muscle mass during clamping and exercise not only involves sustained and elevated muscle work, the demand of energy during which may outstrip aerobic energy provision, but potentially also occludes vessels and sinuses. This could effectively decrease the delivery and hence availability of O_2 to working muscles. During both clamping and emersion, there is also an inability to irrigate the ctenidia efficiently due to decreased mantle cavity volume or lack of ambient O_2.

The response of abalone to hypoxia is quite conservative. Both exposure to air and burst muscle work result in the hydrolysis of high-energy phosphagen and production of the pyruvate reductase end products D-lactate and tauropine in the pedal musculature of haliotids (*H. lamellosa* Gäde 1988; *H. discus* Sato *et al.* 1991; *H. iris* Baldwin *et al.* 1992; *H. australis* Wells and Baldwin 1995; *H. kamtschatkana* Donovan *et al.* 1999). In conjunction with the production of D-lactate and tauropine, muscle and haemolymph pH of abalone decreases as a result of anaerobiosis (Tjeerdema *et al.* 1991a, 1991b; Baldwin *et al.* 1992; Wells *et al.* 1998a). The metabolic poise of abalone tissue (Chapter 3) and the response to hypoxia does not appear to be as variable as other physiological parameters of the genus, such as haemolymph haemocyanin (Hc) content (Chapter 6).

Currently, the response of *H. rubra* to hypoxia is not documented, although it is probable that, based on the metabolic indices (Chapter 3), *H. rubra* expresses a metabolic response similar to that observed in other temperate abalone species. To this effect, we would expect *H. rubra* to demonstrate the reduction of pyruvate to both D-lactate and tauropine during anaerobic glycolysis.

Tissue specific responses to hypoxia

As the energy metabolism of gastropods predominantly is carbohydrate based (Livingstone and De Zwaan 1983), monitoring changes in the levels of fuels may augment the use of anaerobic end product assays to determine effects of hypoxia. Abalone are known to store large amounts of glycogen, particularly in the foot (Chapter 3), which may be mobilised during times of stress. Although symptomatic of various stresses in fish and crustaceans, change in haemolymph-glucose titre is not a common measurement in marine molluscs (Carefoot *et al.* 1993; Carefoot 1994), with the exception of studies on starvation stress. It appears that to date, no data are available on the effects of hypoxia on levels of haemolymph glucose in abalone, although alterations in salinity and food availability are known to affect haemolymph glucose (Carefoot *et al.* 1993; Boarder *et al.* 2001).

The accumulation of D-lactate as an end product, and the release of D-lactate into the haemolymph during periods of exercise and air emersion, is a feature of many invertebrates. Pulmonate gastropods are known to release metabolites, notably D-lactate, into the haemolymph both during and following hypoxia and anoxia (von Brand *et al.* 1950, 1955; Wieser 1978; Kluytmans and Zandee 1983). In contrast, the presence of D-lactate in the haemolymph of prosobranch gastropods does not appear to have been reported to date, and it is generally held that following hypoxia in abalone, metabolites are retained and re-metabolised within the muscles (Gäde 1988; Ryder *et al.* 1994). Evidence suggests however that marine gastropod muscle is capable of releasing anaerobic end products. For example, *in vitro* work on isolated whelk muscle has revealed that D-lactate is released during electrically stimulated contractions (Wiseman and Ellington 1987; Wiseman *et al.* 1989).

Whilst D-lactate may accumulate in incubation media of isolated preparations, it is not clear whether this occurs during or following *in vivo* hypoxia or exercise for prosobranch muscle. Furthermore, reports of opine end products in invertebrate haemolymph are rare. The presence of an opine in molluscan haemolymph has been conclusively demonstrated only for cephalopods, which are known to accumulate octopine in the haemolymph during and following exhaustive exercise (Storey and Storey 1979b). Given the levels to which abalone muscle may accumulate both D-lactate and tauropine it was considered of interest to examine the role of the haemolymph during hypoxia in *H. rubra*. D-lactate in the haemolymph of some pulmonate gastropods is known to serve as a fuel for tissues other than the foot muscle, and it is possible that anaerobic metabolites in the haemolymph of *H. rubra* may be used as a fuel for more aerobic tissues.

Recovery from hypoxia

A number of specific metabolic events are associated with recovery from hypoxia in molluscs. These include the recharging of high-energy phosphates, the oxidation of end products and the resynthesis of anaerobic substrates (Ellington 1983). Numerous studies have investigated changes in marine bivalve and gastropod metabolite levels during recovery from exercise or air emersion (*e.g.* Koormann and Grieshaber 1980; Ellington 1983; Meinhardus-Hager and Gäde 1987; Eberlee and Storey 1988; Isani *et al.* 1989).

Studies by Gäde (1988), Tjeerdema *et al.* (1991b) and Ryder *et al.* (1994) have examined the levels of anaerobic metabolites and pH in abalone pedal muscles during recovery from environmental hypoxia or anoxia. D-lactate concentrations decrease relatively quickly, while the return of pH and tauropine to resting values follows a more protracted time course. The actual time taken for metabolites to reach control levels depends on the severity of hypoxia, and these authors assume that re-metabolism of these metabolites occurs *in situ*. However, the role of the haemolymph in possibly clearing these metabolites was not investigated. To date, no anaerobic metabolites have been identified in the haemolymph of abalone during or after exercise or emersion. Aquatic and terrestrial pulmonate gastropods however show a reduction in muscle and haemolymph D-lactate levels during recovery from hypoxia (von Brand *et al.* 1955; Wieser 1981).

It was of interest therefore to examine the recovery of *H. rubra* from a short hypoxic episode (exercise), in order to determine, as with the hypoxia treatments themselves, if metabolites enter the haemolymph and may therefore act as potential fuels for other tissues, such as the presumably aerobic radula muscles. Another aim of the recovery experiment was to monitor a short time course of D-lactate and tauropine change in both the muscles and haemolymph, which may indicate a 'wash-out' of metabolites from the muscles.

Considering that the metabolic response to hypoxia has not been wholly established for an Australian temperate abalone, the major aim of this chapter is to examine, and confirm, the effects of environmental and functional hypoxia on *H. rubra* pedal muscle and haemolymph. Primarily this is achieved by examining the changes in metabolite levels and pH in the pedal muscles and haemolymph. Besides a known change in pH, the effects of hypoxia on *Haliotis* haemolymph are not clear. It is possible that marine prosobranchs, like terrestrial gastropods, accumulate anaerobic metabolites in the haemolymph. If anaerobic metabolites are observed in abalone haemolymph, the possible significances of this result must be considered. Changes in haemolymph glucose as a result of feeding state or adverse environmental conditions have been observed for other marine gastropods, although it is unclear whether hypoxia affects haemolymph glucose in abalone.

In addition, a brief examination of the fate of anaerobic metabolites over a short recovery period following functional hypoxia in *H. rubra* is described, in order to determine not only the effects of hypoxia on tissue metabolites, but the possible time-course of appearance and disappearance of these metabolites.

Finally, the relevance of these findings to both abalone and molluscan metabolism is considered.

4.2 MATERIALS AND METHODS

4.2.1 Simulating environmental and functional hypoxia in *Haliotis rubra*

This chapter is concerned with the effects of environmental and functional hypoxia on energy metabolism in *H. rubra*. Environmental hypoxia in the form of air emersion and functional hypoxia in the form of exercise (repeated righting after inversion under water) were chosen following observations on *H. rubra* made in the wild (Chapter 2), and literature on effects of these types of hypoxia on other species of abalone, which are detailed in the Discussion.

Abalone were collected from Mornington and maintained in the aquaria as previously described (Chapter 3).

Control treatment animals consisted of quiescent non-feeding abalone. Control treatment animals were selected during the middle of the day, when activity was usually lowest.

Emersion treatment animals were removed from the aquarium and inverted to drain the mantle cavity of residual seawater. They were placed in high edged plastic trays (approx 800 × 500 × 150 mm) containing a thin film of seawater, and wet algae or wet blotting paper. These trays were then placed back in the aquarium to maintain constant temperature. Animals were exposed to air for 12 h.

Functional hypoxia treatment animals were forced to repeatedly right themselves after inversion under water, until the animals appeared to tire and declined to right themselves after 5 min. This time was arbitrarily set as the experimental end point, after initial trials suggested abalone may be immobile for up to 5 min before commencing activity again. Successful righting manoeuvres depended on gaining a proper purchase on the substrate, and after initial trials where abalone often slipped and lost footing on the aquarium floor, inversions were performed on a large flat rock collected from the field site. The time taken until the experimental end point, and the number of successful and unsuccessful manoeuvres, were recorded for each animal. In addition, behaviour and physical appearance was noted. Most of the righting manoeuvres were preformed using the adductor muscle and posterior of the foot, although a number were attempted using an anterior foot flap.

4.2.2 Recovery of *Haliotis rubra* from exercise

A short experiment was conducted to monitor the levels of anaerobic metabolites in pedal muscle and haemolymph during an aquatic recovery phase following exercise. It was of interest to examine the relationship between muscle and haemolymph metabolite levels, and the time course of removal of these metabolites.

Abalone were collected and held as described previously. A group of 8 animals was evenly divided into two treatment groups: those that were allowed to recover for 1 h after exercise, and those that were allowed to recover for 2 h. All animals had a portion of shell removed 2-3 days prior to the experiment to allow access to the aorta for sampling (Section 4.2.3). Animals were exercised until the experimental end point, and then left undisturbed for the allotted recovery period in the aerated aquarium system.

4.2.3 Muscle and haemolymph sampling

Just as preliminary trials were conducted to determine the most appropriate methods for subjecting abalone to hypoxia, trials in the acquisition and removal of haemolymph samples were performed. Whilst familiarity with the anatomy of *H. rubra* had been gained during experiments outlined in earlier chapters on morphology and biochemical profiles, the efficient accessing and treatment of haemolymph had to be developed. These trials, and the development of appropriate sampling techniques, are addressed in the Discussion.

Abalone were separated into three groups of 12 animals each for control, exercise and emersion treatments (total of 36), from which adductor and foot muscle, and two haemolymph sites (pedal sinus and aorta) were sampled. In addition, adductor, foot muscle, pedal sinus and aorta haemolymph samples were taken from eight exercise recovery treatment animals.

Aorta haemolymph samples were taken in addition to pedal sinus samples not only to determine if tissue metabolites entered the haemolymph, but also to see if there was any difference in haemolymph metabolite levels retrograde to the renal and ctenidial circulation. The right kidney and atria of abalone are sites of haemoconcentration and excretion (Behrens 1999), and it was possible that any metabolites may have been filtered or excreted at these sites.

Obtaining haemolymph from the aorta of *H. rubra* required prior treatment of the shell before commencing the hypoxia experiments. Excessive handling of abalone, especially during haemolymph sampling and shell removal, results in increased body movements and

muscle contractions, thus potentially influencing levels of muscle and haemolymph metabolites. Removing a section of the shell overlying the aorta and sampling via this window was considered less intrusive than removing the shell and sampling directly from the exposed vessel. This procedure also had the benefit of allowing muscle samples to be taken immediately upon shell removal.

The aorta of *H. rubra* was exposed for sampling by making a hole in the left postero-lateral edge of the shell overlying the heart and major vessels. Extreme care was exercised. An engraver's tool (Dremel®, USA) equipped with a diamond-tipped bit was used to carefully remove a section of shell of approximately 25 mm by 15 mm, containing the closed posterior respiratory tremata. This exposed the heart, anterior extremity of the digestive gland, the left kidney and ctenidial vessels. Underlying these structures are the left efferent ctenidial vein and aorta. The window was cut as far back as possible, in an attempt to avoid disturbing water flow dynamics through the mantle cavity. The surrounding shell was carefully burnished to remove sharp edges, and the exposed viscera gently irrigated with seawater to remove any fine shell dust.

All animals survived the surgery and were placed back in the aquaria to acclimatise and to ensure the removal of any stress response or metabolites accumulated during the 5 minute handling regime. Animals with shell windows appeared to suffer no ill effects and were maintained in the aquaria as long as untreated abalone. It was noted however that some 'shifting' of exposed viscera occurred, a likely response initiated to protect vital organs. Animals with shell windows were allowed to re-acclimatise in the aquaria for 2 to 3 days before being exposed to control or hypoxic treatments.

Following treatment (control resting, exercise, recovery or emersion), animals were quickly blotted and weighed. Abalone were inverted and for those with a shell window a blunt probe was carefully inserted through the shell window and used to lift the overlying structures away from the aorta. A 1 mL syringe with a 27-gauge needle was introduced orthograde into the anterior aorta, approx 20-25 mm from the base of the ventricle, and haemolymph slowly withdrawn. Gentle pressure was applied to avoid occlusion of the aorta. Haemolymph samples taken were often as low as 0.3-0.5 mL.

Haemolymph (3-4 mL) was then immediately taken from the medial pedal sinus using a separate 5 mL syringe and 23-gauge needle introduced into the midline of the foot, posterior to the junction of the head. Aorta samples and 0.5-1 mL of the pedal haemolymph samples were immediately dispensed into separate eppendorf tubes on ice and treated with 1:10 vols

(acid:haemolymph) ice-cold 6 mol L^{-1} perchloric acid (PCA). The remaining 2.5-3.5 mL pedal haemolymph sample was reserved for blood pH measurements (see below).

All 36 animals (12 animals per treatment) had pedal sinus samples taken, whereas successful aorta samples were collected from only 18 of the 36 (6 per treatment). Successful aorta haemolymph samples were taken from all eight of the exercise recovery animals.

The animals were then removed from the shell, and approximately 0.5-1 g samples from the dorsal surface of the adductor muscle, and the posterior portion of the foot muscle (excluding sole epithelium and majority of the mucus glands) were rapidly excised on ice, blotted, and snap-frozen in liquid N_2.

4.2.4 Haemolymph pH measurements

After the appropriate experimental treatment, haemolymph samples were taken by syringe as described previously. These were transferred to a glass test tube, and placed in a circulating water bath maintained at aquarium temperature (14.9 ± 0.1°C). Haemolymph pH was measured with an Activon 205 meter coupled to a Hanna HI 1230 electrode, which had been previously calibrated (Selby Biolab pH buffers, Melbourne) at experimental temperature. For reference, the pH of the seawater in which the animals were contained was also determined. It was noted that initial (immediate) pH reading was stable for a few minutes, after which the pH would slowly drift and rise for 5 to 6 minutes before finally stabilising. The pH reading was taken as the initial recording.

4.2.5 Tissue extracts and metabolite assays

The preparation of tissue extracts for metabolite studies, and assay of these extracts, was identical for all samples taken from *H. rubra* throughout the course of this study, unless otherwise stated. The only difference involved the non-neutralisation of the haemolymph PCA samples, given the neutralisation process (see below) had the potential to excessively dilute the metabolite levels in such small samples (as low as 0.3 mL). Neutralisation was deemed unnecessary, as the addition of haemolymph PCA samples did not alter metabolite assay pH conditions. Trials using 0.6 mol L^{-1} PCA, neutralised and non-neutralised haemolymph samples gave the same cuvette assay pH, even up to the addition of 100 µl (four times volume used in standard assay) for D-lactate and tauropine assays, and up to 20 µL for glucose assays, in a total volume of 1 mL.

Frozen adductor and foot muscle (approximately 0.4 g) were powdered in a stainless steel tissue crusher pre-chilled in liquid nitrogen, and homogenised in 10 volumes of ice-cold 0.6 mol L^{-1} PCA. Tissue extracts were centrifuged at 13000 × *g* and 4°C for 5 min, and the supernatants neutralised with 5 mol L^{-1} K_2CO_3. After standing on ice for several hours, the supernatant was drawn off and centrifuged at 13000 × *g* and 4°C for 5 min to remove the excess precipitated $KClO_4$. The supernatant was stored at –80°C until assayed.

Haemolymph samples were centrifuged at 13000 × *g* for 5 mins to remove denatured protein, and the supernatant stored at –80°C until assayed.

D-lactate and tauropine assays

D-lactate and tauropine were assayed in tissue extracts following modification of the methods outlined by Gutmann and Wahlfield (1974) and Engel and Jones (1978). The selection of methods is considered in the Discussion (Section 4.4.1). The reactions are as given:

$$\text{D-lactate} + NAD^+ \xleftrightarrow{\text{D-LDH}} \text{pyruvate} + NADH$$

$$\text{pyruvate} + \text{hydrazine} \longleftrightarrow \text{pyruvate hydrazone}$$

$$\text{Tauropine} + NAD^+ \xleftrightarrow{\text{TDH}} \text{taurine} + \text{pyruvate} + NADH$$

$$\text{pyruvate} + \text{hydrazine} \longleftrightarrow \text{pyruvate hydrazone}$$

(the removal of pyruvate by hydrazine ensures completion of reaction in direction of D-lactate or tauropine oxidation)

The assay concentrations were as follows: glycine/hydrazine/EDTA buffer (333 mmol L^{-1} glycine, 133 mmol L^{-1} hydrazine sulphate, 10 mmol L^{-1} EDTA, pH 9.0), 50 mmol L^{-1} NAD^+, 25 µL muscle or haemolymph extract and 3 i.u. (2-10 µL) of either D-LDH (Boehringer Mannheim) or TDH. The latter was obtained from *H. rubra* adductor muscle by ion-exchange chromatography as described by Gäde (1987) and Baldwin *et al.* (1992) (Section 4.2.5). Reactions were initiated by addition of enzyme, and suitable controls consisting of water or appropriately diluted PCA were run to allow for non-specific activity. Duplicate measurements were run on all samples. EDTA was included in the assay buffer to reduce interference from metal cations (Section 4.4.1).

Haemolymph glucose assays

It was considered of interest to follow changes in haemolymph glucose levels along with other metabolites in *H. rubra* following hypoxia. Haemolymph glucose titres were determined in *H. rubra* at rest, and following exercise and 12 h emersion. Haemolymph PCA samples examined for D-lactate and tauropine were assayed for glucose, as described previously (Chapter 3, Section 3.2.4).

Controls for non-specific background activity

In addition to controls for non-specific activity in assays (water or PCA substituting for tissue sample), assay conditions were checked by the addition of the appropriate metabolite; either a commercial racemic mixture of D/L-lactate, or tauropine that had been synthesised in the laboratory (Section 4.2.7), was used.

In addition, the possibility of L-lactate accumulating as a metabolite in *H. rubra* tissue was examined by assaying tissue extracts with L-LDH (Boehringer Mannheim) in place of D-LDH.

4.2.6 Preparation of tauropine dehydrogenase from *Haliotis rubra* for use in tauropine assays

All preparative steps were carried out at 0-4°C. Buffer pH values were recorded as the pH of solutions when prepared at 20°C. Briefly, fresh *H. rubra* adductor muscle (10g) was finely diced on ice with a scalpel, suspended in 50 mmol L^{-1} imidazole-HCl buffer, pH 7.2 (1:5 w/v), containing 1 mmol L^{-1} EDTA and 0.1 mmol L^{-1} DTE, and homogenised on ice with a Sorvall Omnimixer for three 30-second bursts. The homogenate was centrifuged for 30 min at 10,000 × *g* and 4°C, the supernatant collected, and the pellet re-homogenised in 50 mL of buffer and centrifuged as before. The pooled, filtered supernatants were applied to a column (2.5 × 37 cm) of DE22 Cellulose (Whatman), equilibrated with the same buffer used for homogenisation. The column was washed with 250 mL of equilibration buffer, and the enzyme was eluted using a linear gradient of 0-250 mmol L^{-1} NaCl in 800 mL of equilibration buffer. Eluted fractions (14 mL) were assayed for LDH, TDH and malate dehydrogenase (MDH). Fractions containing TDH only were pooled (70-90 mL), concentrated by membrane filtration (PM-10, Amicon Corp. Lexington, MA), and dialysed against 85% saturated ammonium sulfate made up in 50 mmol L^{-1} Imidazole-HCl pH 7.2, 1 mmol L^{-1} EDTA, 0.5 mmol L^{-1} DTE buffer. The partially purified enzyme was stored at 4°C.

The TDH yield was typically around 350-450 i.u., and free of LDH or MDH activity. An average preparation resulted in 80% recovery of TDH activity in the original muscle supernatant. Fresh enzyme was isolated prior to most series of tauropine assays.

4.2.7 Chemical synthesis of tauropine

In order to check tauropine assay conditions, tauropine (D-rhodoic acid) was produced in small quantities based on the methods of Abderhalden and Haase (1931). Translation of this text was generously provided by Mr. Steve Langford (Department of Chemistry, Monash University).

Approximate molar ratios of D-Alanine and 2-Bromoethanesulfonic acid were mixed (1 g and 2.8 g respectively, slight acid excess), dissolved in a minimum volume (10 mL) of 2 mol L^{-1} NaOH and left to stir at room temp for 3 weeks. After several days, a precipitate had formed, which was subsequently dissolved upon the addition of a further 10 mL aliquot of 2 mol L^{-1} NaOH.

This volume (about 20-25 mL) was added to a large excess of ether (50 mL) to dissolve excess acid, gently agitated in a separating funnel, and left to separate. The lower aqueous phase was collected in a volumetric flask, neutralised with conc. HCl, and freeze-dried.

4.2.8 Data analyses

Statistical analyses were undertaken using MS Excel and Systat statistics packages. The levels of metabolites between tissues within treatments, and between treatments were compared (muscle vs muscle, haemolymph vs haemolymph). Also, any difference in metabolites in the haemolymph sampled at different sites was examined. The changes in haemolymph pH following hypoxia were compared, as were the levels of metabolites during and after recovery (to control levels and exercise levels). Significant differences were tested by ANOVA on data following checks for normality, and accepted at $P< 0.05$.

A possible relationship between animal size and exercise capacity was investigated by regression analysis. Correlations were also run between metabolite levels in haemolymph and muscle. All results are reported as Mean ± S.E.M., unless otherwise stated.

4.3 RESULTS

4.3.1 Animal behaviour during exercise and recovery

All animals remained alive during exercise, recovery and air emersion. During exercise, inverted animals were observed to 'shudder' or 'cough', which appeared to be a contraction of the adductor muscle and mantle cavity. Debris on the aquaria floor in front of the animal was often swept away by this cough.

Generally, abalone performed up to two or three times as many twists or failed attempts as successful rightings, particularly as the duration of exercise increased. For 26 animals exercised in total (12 for exercise, 8 for recovery from exercise and 6 for exercise haemolymph pH), the mean exercise period was 40.2 ± 1.8 min, during which an average of 9.1 ± 0.9 successful righting manoeuvres were performed. For the entire data set, there was no relationship between time taken and number of successful manoeuvres (r^2=0.31, P>0.05), whole animal weight and time taken (r^2=0.01, P>0.05) or whole animal weight and number of successful manoeuvres (r^2=0.001, P>0.05).

During recovery, abalone tended not to move far after exercise, and were mostly found on the nearby vertical walls of the aquarium. Abalone were alert and responded to any disturbance during recovery.

4.3.2 Animal behaviour during air emersion

Abalone rarely moved during emersion. After 12 h the animals appeared flaccid, with the epipodium and foot maximally extended from the body, exposing the dorsal surface of the foot and epipodium (usually hidden beneath shell). The viscera were not observed. On a number of animals the epipodium took on a buckled or wrinkled appearance. All emersion treatment animals responded to tactile stimulation during and at the completion of 12 h emersion. Although many of these animals displayed pedal tonus, little resistance was offered upon removal from the plastic trays. Pedal sinus haemolymph samples were unusually difficult to acquire, and haemolymph from all sample sites displayed variable degrees of visually assessed oxygenation (blue oxygenated to almost clear deoxygenated haemolymph).

4.3.3 Tissue metabolite levels and haemolymph pH in resting animals

The levels of D-lactate, tauropine and glucose in the muscles and haemolymph of resting animals, and those exposed to hypoxic conditions, are given in Table 4.1. In control animals, no D-lactate or tauropine were detected in the haemolymph, and D-lactate was not

observed in the adductor muscle or foot muscle of control animals. In contrast, tauropine up to 1.67 μmol g^{-1} was found in the adductor muscle. Mean control haemolymph glucose levels were 0.09 ± 0.02 μmol mL^{-1}. Control animals resting in water had a haemolymph pH of 7.29.

4.3.4 Effects of exercise on tissue metabolite levels and haemolymph pH

Following exercise, concentrations of both D-lactate and tauropine were significantly elevated in adductor muscle, foot muscle and haemolymph from the pedal sinus and aorta (ANOVA, $P>0.05$; Table 4.1). A significantly higher concentration of D-lactate accumulated in the foot of exercising animals than in the adductor or haemolymph.

The highest metabolite level encountered during the study was that of tauropine in the adductor of exercising abalone (up to 7.38 μmol g^{-1}). As with control animals, significantly more tauropine is observed in the adductor muscle than the foot. Taking resting concentrations into account, concentrations of tauropine are relatively higher in the adductor than foot muscle following exercise.

Mean concentrations of tauropine are higher in both the adductor and foot muscles following exercise than they are after air emersion (Table 4.1), although only exercise adductor muscle tauropine concentrations are significantly different (ANOVA. $P>0.05$).

Exercise significantly elevated the concentration of glucose in the haemolymph of *H. rubra*. Levels rose over three-fold from 0.09 μmol mL^{-1} to 0.32 ± 0.03 μmol mL^{-1}.

Following exercise, haemolymph pH had fallen significantly from 7.29 to 7.21 (ANOVA, $P<0.05$).

4.3.5 Effects of recovery from exercise on tissue metabolite levels

The results of the short recovery experiment are given in Table 4.2 and Figure 4.1. As previously determined, muscle levels of D-lactate and tauropine are elevated following exercise. Over the 2 h recovery time course, the muscle levels of both metabolites fall. Little change in adductor muscle metabolite concentrations are noted in the first hour, whereafter they fall rapidly (Figure 4.1). In contrast, foot muscle D-lactate and tauropine concentrations fall almost linearly over time. Haemolymph metabolite concentrations remain relatively unchanged: haemolymph D-lactate falls after 1 h recovery, and then begins to increase, and haemolymph tauropine remains very low. D-lactate in both the foot and adductor after 2 h is

significantly lower than concentrations in muscles following exercise (ANOVA, $P<0.05$), whereas only foot concentrations reach control levels (no D-lactate detected).

After 2 h recovery, the concentration of tauropine in the adductor significantly decreased. As seen with D-lactate, both adductor and foot tauropine concentrations fell after 2 h to values not significantly different from controls (0.29 and 1.0 μmol g^{-1} respectively; Table 4.2, Figure 4.1).

4.3.6 Effects of air emersion on tissue metabolite levels and haemolymph pH

Levels of D-lactate, tauropine and glucose in the haemolymph and muscles of emersion treatment animals display a different distribution to that found in exercise animals. Haemolymph values of these metabolites are higher following emersion, with D-lactate approaching foot muscle concentrations after 12 h emersion (Table 4.1). Haemolymph levels of D-lactate are not significantly different between sampling sites (Table 4.1). Just as tauropine was the predominant metabolite accumulated in the muscles following exercise, D-lactate was detected in higher amounts in the foot muscle and haemolymph following emersion.

After 12 h air emersion, the concentration of D-lactate in the haemolymph of *H. rubra* (0.78 to 1.09 μmol mL^{-1}) is higher than in the adductor muscle, and approaches foot values.

The highest D-lactate concentrations observed were within the foot muscle. The concentration of D-lactate in the haemolymph represents 70% to 78% of the concentration in the foot. Haemolymph D-lactate levels may reach 1.5 μmol mL^{-1}.

Whereas D-lactate appears in the haemolymph of *H. rubra* during emersion, most of the tauropine remains in the muscles (Table 4.1). A ratio of the concentration of each metabolite in the haemolymph versus muscle, for each treatment, should give some indication of the different distribution of these metabolites. Such ratios do not take into account total amounts, which requires incorporation of total muscle mass and haemolymph volume. These mean haemolymph (mean aorta and pedal sinus values) to mean muscle (mean adductor and foot values) ratios for D-lactate and tauropine are, for exercise animals, 0.18 ± 0.06 and 0.02 ± 0.01 respectively, and for emersion animals 0.91 ± 0.06 and 0.13 ± 0.02 respectively. Both D-lactate and tauropine occur at significantly higher concentrations in the haemolymph following air emersion, compared to exercise, and D-lactate concentrations in the haemolymph were significantly higher than for tauropine (ANOVA, $P<0.05$). Ratios were

calculated for animals from which values for both haemolymph sites and both muscles were available.

Air emersion resulted in a significantly higher accumulation of D-lactate in the adductor muscle and haemolymph compared to exercise, but not so for the foot muscle (ANOVA, P=0.07).

A significantly higher concentration of tauropine was found in the adductor muscle of exposed abalone than in the foot and haemolymph, and higher in the foot than haemolymph (Table 4.1). Similarly, haemolymph and adductor tauropine levels were elevated as compared to control levels.

In contrast to D-lactate and tauropine, L-lactate was not detected in muscle or haemolymph of control, exercise or emersed *H. rubra*.

Emersion stimulated a significant large increase in the haemolymph glucose titre of *H. rubra*. After 12 h of air emersion, concentrations had risen to 0.93 ± 0.26 μmol mL^{-1}, a 10-fold increase from resting control levels of 0.09 μmol mL^{-1}. Air emersion raised glucose levels significantly higher than did exercise (ANOVA, P<0.05).

Emersion resulted in a large decrease in haemolymph pH. Following 12 h air emersion, *H. rubra* haemolymph pH had decreased from pH 7.29 to pH 6.82 (Table 4.1), significantly lower than both control and exercise values (ANOVA, P<0.05).

4.3.7 Correlations between haemolymph and muscle metabolites in *Haliotis rubra*

The strongest correlation of metabolite concentrations in the muscle and haemolymph of *H. rubra* is that between the foot muscle and haemolymph from the pedal. Correlation between foot muscle and haemolymph D-lactate after air emersion gave a correlation r-value of 0.66. A value of 0.64 was obtained for foot muscle and aorta haemolymph. A much lower correlation was noted for adductor muscle and pedal sinus haemolymph D-lactate (r=0.37). These correlations however are similar to the muscle:haemolymph metabolite ratios in not taking into account tissue volumes.

4.4 DISCUSSION

The main aims of this chapter were to investigate the effects of environmental hypoxia and muscle work on the energy metabolism of *H. rubra* muscle. More specifically, this chapter was concerned with: 1. determining whether, as indicated from the metabolic indices determined in Chapter 3, the pedal musculature of *H. rubra* responds in a way similar to that of other abalone to exercise and emersion by displaying a capacity for facultative anaerobiosis; and 2. to see if anaerobic metabolites enter the haemolymph and, if so, to examine any relationship between levels of the metabolites in haemolymph and muscle. The movement of anaerobic metabolites from the pedal muscles to the haemolymph of *H. rubra* is important, as they may act as potential fuels for other more aerobic tissues.

The hypoxic treatments and their effects on tissue metabolism are addressed in this discussion, beginning with a consideration of some of the methodological issues encountered. The responses of *H. rubra* muscles and haemolymph to exercise and emersion, and their differences, are considered separately, followed by the effects of recovery on muscle and haemolymph metabolite levels. The effects of hypoxia on tissue pH and haemolymph glucose are then considered. The data are approached in this manner to include discussions of other gastropods and their physiological responses. The role of the haemolymph during hypoxia is then examined, as are associated effects of hypoxia on respiratory and cardiovascular systems of abalone.

4.4.1 Methodology

Animal treatment

The methods used to impose hypoxia on *H. rubra* have a direct bearing on the interpretation of the results, and are discussed in relation to other studies on abalone.

Repeated righting after inversion has been used in previous studies on abalone, as it is a convenient way to induce muscle work and exhaust animals (Gäde 1988; Baldwin *et al.* 1992; Pirker and Schiel 1993), and is consistent and inducible both in the field and the laboratory. During exercise, *H. rubra* performed, on average, 2-3 unsuccessful righting attempts for every successful flip. These unsuccessful twists are assumed to elicit a similar biochemical response from the muscles, as they involved violent muscular contraction, and as such the data may be considered to include effects of unsuccessful righting attempts. Exercised animals occasionally contracted muscle and mantle cavity, resulting in a shudder.

This shudder or 'coughing' has also been observed for *H. iris*, and is thought to aid in the expulsion of matter from the mantle cavity (N. Ragg University of Canterbury, pers. comm.).

Previous studies have found abalone apparently exhaust after an exercise period of 10-15 minutes (Gäde 1988), and have assigned exhaustion as a failure of the animal to right itself within 2-5 minutes (Baldwin *et al.* 1992; Donovan *et al.* 1999). Average work time for *H. rubra* in this study was 40 min. The primary reason for a long time until apparent exhaustion in *H. rubra* was the setting of an arbitrary time of 5 min between righting movements. As noted, animals often lay still (inverted) for up to 5 min before re-commencing activity. Although it is possible that animals may have been exhausted, they may alternatively have been either recovering slowly or respiring aerobically during these 'rest' periods.

Although most previous studies have attributed an exhausted state to an abalone unable to right itself after 2-5 minutes, Pirker and Schiel (1993) set a time limit for righting at 10 min. They describe stressed abalone as displaying sluggish behaviour, which is usually evident as a slow righting response. As a result, they argue that abalone taking longer than 10 min are usually incapable of righting themselves.

Given the variability in both time to apparent exhaustion, and method in assessing exhaustion in abalone, 5 minutes was chosen as an arbitrary time in assessing exhaustion in *H. rubra*. Leaving animals for periods longer than 5 minutes may have allowed them to recover.

A number of studies on abalone metabolism have subjected abalone to 24 h of air emersion (Baldwin *et al.* 1992; Ryder *et al.* 1994; Wells and Baldwin 1995), or exposure to anoxic seawater (Gäde 1988). Other studies examining the extent to which abalone can withstand emersion suggest *H. rubra* can survive up to 40 h air emersion, as long as a low temperature is maintained (James and Olley 1974). Given that abalone may be observed in rock crevices or under-hangs when exposed during tidal movements, and commercial individuals can be transported or stored emersed for up to 20 h plus (Fleming 2000; Gorfine 2001), a mean duration of 12 h was chosen to subject *H. rubra* to emersion in this study. The abalone appeared able to sustain this duration of air emersion, based on preliminary trials where animals were exposed and allowed to recover in water. This time frame was preferred to a shorter emersion regime (*e.g.* 6 h), as it was thought that any metabolic changes may be more prominent and easily detected after 12 h.

Haliotis rubra exposed to air, both naturally in the wild and during experimental emersion treatments, exhibited distinctive behaviour and body posture. Abalone often

displayed a rippled epipodium, with the shell displaced laterally, exposing muscle usually beneath the shell (Section 4.3.2). The appearance of exposed animals resembled previous descriptions of stressed *H. rubra* and *H. iris* (see below). In addition to this buckled appearance, *H. rubra* tends to raise the shell from the foot, perform shell twisting movements, and eventually lose footing on experimental surfaces as water PO_2 decreases, and temperature or air emersion increases (Ainslie 1977; Gilroy and Edwards 1998; Gorfine 2001; Drew *et al.* 2001). Abalone are also known to escape hypoxic water conditions by creeping up to the surface of experimental vessels (Nakanishi 1978).

During emersion treatment *H. rubra* tended to expose the dorsal surface of the foot muscle, epipodium and the mantle, which appeared 'puffy'. Whilst this is probably due to a relaxed state of the muscle, it is also possible that haemolymph has accumulated in these tissues, perhaps having been redirected to the exposed muscle to increase viable surface area for O_2-uptake. For example, more haemolymph is sent to the mantle and body surface during air emersion in the limpet *Acmea* spp. (Kingston 1968, as cited in Just 2002), and the thin-walled foot is considered an important respiratory organ in the whelk *Bullia digitalis* (Brown 1964, 1984). Integumentary gas exchange, augmenting ctenidial respiration, normally accounts for 8-14% of total O_2-uptake in the keyhole limpet *Diodora aspersa* (Bourne 1987). When exposed to hypoxic water, cutaneous gas exchange increases to 30%, which the animal facilitates by expanding the mantle dorsally over the shell.

It is not known whether *H. rubra* augments ctenidial respiration through the body surface, or indeed takes up O_2 from air. This same mantle and epipodium exposure behaviour has been observed in *H. iris* (pers. obs). In *H. iris* however, the haemolymph perfusion of the mantle and epipodium does not increase during air emersion, suggesting these structures do not play an important role as accessory respiratory structures (Just 2002).

There was some initial concern that any locomotion or movement of the abalone during the recovery period following exercise might jeopardise the effects of hypoxia by introducing a further anaerobic component. Any metabolites formed as a result of locomotion may give a false reading for hypoxia. However, the confinement of the animals may also serve as an added stress, and locomotion (or at least access to aerated and flowing water) may be part of any natural recovery process in the wild. As a result, animals were not restrained during recovery following exercise treatment.

Tissue sampling

Abalone haemolymph can best be sampled from the medial pedal sinus or cephalic sinuses, and abalone can and do recover from these bleeding methods (Ainslie 1980a, 1980b; Taylor *et al.* 1994; Chen 1996; pers obs.). Abalone haemolymph does not possess clotting factors, so caution must be exercised when accessing sinuses for haemolymph samples in non-terminal experiments. In these exercise and emersion experiments, tissue samples were also taken in terminal experiments so excessive bleeding was not an issue. As mentioned earlier, haemolymph samples are occasionally difficult to take due to the contraction and writhing of the pedal muscle, and if the pedal vessels or sinus are not accessed at the first attempt, a latent period may be required before another attempt is made.

While the removal of a section of shell to afford access to the aorta was regarded as sufficient for these experiments, the cannulation of the aorta with small diameter polyethylene or PVC tubing, resulting in reduced handling of the animal when sampling for haemolymph, was also considered. However this manipulation was discarded due to the highly probable dislocation of the cannulae in free-roaming control and exercising individuals.

Cutting a window in the shell of animals from which aorta haemolymph samples were taken may initiate a metabolic response in *H. rubra*. An increase in overall animal metabolism is a likely response to the added disturbance of handling and surgery, or an alteration to water flow (and hence oxygenation) through the mantle cavity. Similarly, metabolism may be elevated during active repair of the shell. Attempts were made to reduce the influence of the surgery by allowing a period of recovery, and cutting the window posteriorly and removing unused respiratory tremata so as not to interfere with flow over ctenidia. The use of a shell window to access abalone cardiovascular structures is common (Bourne and Redmond 1977a, 1977b; Jorgensen *et al.* 1984; Krajniak and Bourne 1988; Behrens *et al.* 2002).

Metabolite assays

NAD^+ dependent dehydrogenases are used widely in specific assays for metabolites such as lactate, malate and opines, due to the ease with which NADH production can be followed by absorbance measurement at 340 nm. As the equilibrium constants for these reactions favour the oxidation of NADH rather than the reduction of NAD^+, one way of ensuring quantitative reduction of NAD^+ is to employ auxiliary trapping reactions to remove one of the reaction products and allow the reaction to run to completion in the desired direction. The most widely used trapping agent in pyruvate producing reactions is hydrazine,

which reacts with the carbonyl group of the oxidised product, pyruvate, to create pyruvate hydrazone. Assays are also typically carried out at alkaline pH, to trap any protons produced in the reaction.

It appears that in alkaline hydrazine-glycine buffers used for many metabolite assays, NAD^+ undergoes side reactions ('blank' reactions) that interfere with the optical absorbance and undermine the accuracy of such assays. The first reaction is rapid and strongly pH dependent (Engel and Jones 1978). Gutmann and Wahlfield (1974), having revised Hohorst's (1963) procedures for assaying lactate and malate, employed a buffer at pH 9 rather than pH 9.5. This decrease in pH reduces the initial 'blank' reaction.

The second 'blank' reaction slowly emerges over time, resulting in a drifting end point of these assays. This reaction is potentiated by metal ions, for example the Cu^{2+} in arthropod and molluscan haemolymph (Graham *et al.* 1983). This drift might be due to a metal ion catalysed reduction of NAD^+ by hydrazine. Consequently, it becomes important for any metal ions present to be sequestered (Engel and Jones 1978).

After reviewing assay procedures, Engel and Jones (1978) suggest not only performing the assay at pH 9, but also the inclusion of a high concentration (at least 10 mmol L^{-1}) of ethylenediamine tetra-acetic acid (EDTA) to chelate any metal ions present. These assay conditions were employed in the current examination of metabolite accumulation in abalone tissues following appropriate treatment.

4.4.2 Effects of exercise and emersion on muscle pyruvate reductase end products

During both functional and environmental hypoxia, *H. rubra* accumulates the pyruvate reductase end products D-lactate and tauropine in the pedal musculature. Although both metabolites are produced during both hypoxic events, tauropine is accumulated predominantly in the adductor muscle and during exercise, and D-lactate is the major end product in the foot and during emersion.

These metabolic responses of *H. rubra* pedal musculature to functional and environmental hypoxia are very similar to those of other abalone species, inferring similar capabilities for facultative anaerobiosis. The production of D-lactate and/or tauropine has been demonstrated for *H. australis* and *H. iris* (Baldwin *et al.* 1992; Wells and Baldwin 1995; Ryder *et al.* 1994; Behrens *et al.* 2002), *H. discus hannai* (Sato *et al.* 1991; Watanabe *et al.* 1993, 1994a, 1994b), *H. kamtschatkana* (Donovan *et al.* 1999), and *H. lamellosa* (Gäde 1988). Tauropine may accumulate to mean levels as high as 5.8 μmol g^{-1} in adductor muscle of *H. iris* following air emersion (Baldwin *et al.* 1992; Behrens *et al.* 2002), or adductor

muscle of *H. lamellosa* following exercise (Gäde 1988). To date, the highest detected concentration of tauropine was noted for the adductor muscle of *H. discus hannai*, but this was following electrical stimulation rather than exercise (9.60 μmol g^{-1}, Sato *et al.* 1991).

The production of tauropine during limited O_2-availability is not restricted to the Haliotidae. The related keyhole limpet *Scutus antipodes* (Fissurellidae) is, as far as the author is aware, the only mollusc other than abalone that has been conclusively shown to accumulate tauropine (up to 1.87 ± 0.67 μmol g^{-1}) during muscle work and emersion (Elias 1996; Elias *et al.*, in prep). To date, the production of tauropine during hypoxia has been demonstrated in only one other non-molluscan organism, the seastar *Asterina pectinifera* (Kan-no *et al.* 1998).

The persistence of tauropine in abalone muscles supposedly at rest is not unique to *H. rubra*. Resting levels of tauropine from *H. australis* and *H. iris* were as high as 2.1 μmol g^{-1} (Wells and Baldwin 1995), and even higher for *H. discus hannai* (4.89 μmol g^{-1}, Sato *et al.* 1991). Whilst this suggests that these muscles may not be actually at rest, but rather continuously contracted or involved in locomotion, it also highlights the limited aerobic scope of these muscles, such that anaerobic metabolites accumulate when the animals are simply maintaining body posture, or during moderate locomotion (Donovan and Carefoot 1997; Donovan *et al.* 1999). Anaerobic metabolism accounted for approximately 54% of COT_{min} (minimum cost of transport) during locomotion in *H. kamtschatkana* (Donovan *et al.* 1999). The pre-experiment conditions that treatment abalone were exposed to in the above studies can not be quantified however, and may be equally responsible for the elevated metabolite levels. In contrast, D-lactate levels are low or absent in abalone tissues at rest (Sato *et al.* 1991; Baldwin *et al.* 1992; Donovan *et al.* 1999; and this study). It should be noted however that not all exercise or air emersion treatments result in anaerobiosis, as evidenced by the lack of anaerobic metabolites or arginine phosphate depletion in the foot of *H. kamtschatkana* during righting (Donovan *et al.* 1999).

Nonetheless a broad tissue-specificity persists, in that tauropine generally typifies adductor muscle work, and D-lactate foot muscle work. Likewise, exercise usually accounts for the major production of tauropine, and emersion for D-lactate, although this pattern is not entirely strict. The extent to which abalone muscles resort to anaerobic glycolysis during periods of low O_2 varies considerably. Direct comparisons should be made cautiously, as the handling and hypoxia treatment protocols often differ across studies. Environmental hypoxia ranges from total anoxia (2-6 h N_2 bubbled seawater, Gäde 1988; Shofer *et al.* 1997b) to moist air emersion (1-36 h emersion, Tjeerdema *et al.* 1991b; Watanabe *et al.* 1994a, 1994b), with exercise usually in the form of repeated righting after inversion.

Exposure of *Haliotis discus* to air resulted in increases in muscle D-lactate (Watanabe *et al.* 1993, 1994a, 1994b). Surprisingly L-lactate, previously unobserved in abalone, is present not only in muscle and soft tissue of *H. discus*, but also appeared to accumulate in the foot muscle to higher concentrations than D-lactate, especially at night (Watanabe *et al.* 1993; 1994a). Whilst the levels of metabolites in *H. discus* muscle may be correlated with muscle activity associated with foraging during feeding at night, the appearance of high levels of L-lactate, especially during normal quiescence during daylight, is questionable. L-lactate was not observed in tissue extracts of *H. rubra* following hypoxia, and was very low in *H. iris* tissue and haemolymph (Behrens *et al.* 2002). Earlier work on the distribution of pyruvate reductase enzymes and end products in *H. discus* failed to identify L-LDH or L-lactate (Sato *et al.* 1991).

As the pattern of metabolite accumulation is different both between *H. rubra* muscles and between treatments, this reflects both the different functions of the muscles and the activities of D-LDH and TDH. The shell adductor is particularly involved in exercise in abalone, as evidenced by observations of righting in abalone. Gäde (1988) calculates the rate of adductor muscle energy consumption and glycolytic flux as 8- to 9-fold higher than for the foot. Sato *et al.* (1991) conclude that the higher TDH activity and tauropine contents of adductor muscle show that abalone "may consume a lot of energy during shell fixation" (Sato *et al.* 1991). The amount of ATP produced for exercising and exposed abalone was calculated for *H. lamellosa* (Gäde 1988), *H. kamtschatkana* (Donovan *et al.* 1999) and the tropical species *H. assinina* (Baldwin *et al.*, in prep), and it was found a higher glycolytic flux persisted during exercise.

Despite higher activities of TDH, D-lactate accumulates in *H. rubra* foot muscle during air emersion. D-lactate has been observed to accumulate preferentially in the foot of the jumping cockle *Cardium tuberculatum* despite a relatively low D-LDH activity (7 i.u), even in competition with higher activities of ODH (120 i.u, Gäde 1980b). In *H. rubra*, similar results suggest an increased ability of D-LDH compared to TDH to reduce pyruvate under conditions of air emersion, and potentially low glycolytic flux, at least in the foot. Conversely, TDH appears to function preferentially during burst muscle work. These differences may reflect kinetic properties favouring D-lactate accumulation during periods of environmental hypoxia, and a lower rate of energy production (Gäde 1988; Kreutzer *et al.* 1989; Sato *et al.* 1991). As both enzymes terminate glycolysis with the reduction of pyruvate, competition between D-LDH and TDH would be direct for pyruvate and NADH. If reaction

kinetics differences do not separate these enzymes temporally or functionally, then anatomy may separate them spatially.

From consideration of mass action ratios (ratios of substrates and products) and equilibria of pyruvate reductase reactions, Gäde and Meinhardus-Hager (1986), Meinhardus-Hager and Gäde (1986), Kreutzer *et al.* (1989) and Meinhardus-Hager *et al.* (1989) propose that all LDHs and OpDHs present in a tissue catalyse near-equilibrium reactions during situations of low glycolytic flux. However, during burst muscle work, only those enzymes present in high activities can approach reaction equilibrium and keep pace with this increase in flux (Kreutzer *et al.* 1989). This may explain the co-occurrence of D-lactate and tauropine in the muscles of *H. rubra* and other abalone species.

4.4.3 Effects of exercise and emersion on haemolymph metabolites

D-lactate and tauropine

Exercise and emersion of *H. rubra* also resulted in a significant increase in haemolymph metabolite concentration, particularly D-lactate and glucose. Emersion elicited a larger increase in the haemolymph metabolite concentrations than did exercise. It is assumed that the major source of haemolymph D-lactate and tauropine are the foot and adductor. However, the potential of other tissues such as the mantle or even digestive gland as sources of D-lactate and tauropine can not be entirely excluded.

The majority of the work investigating the effects of hypoxia on *H. rubra* muscle and haemolymph was completed in 1999 and 2000. The author was also involved with a recent study of similar nature on *H. iris*, which has subsequently been published (Behrens *et al.* 2002) during the completion of this thesis. As had been established for *H. iris* previously, air emersion resulted in the accumulation of D-lactate and tauropine in the foot and adductor muscles. More interestingly however, both metabolites were found to enter the haemolymph of *H. iris* (D-lactate 1.88 μmol mL^{-1}, tauropine 1.58 μmol mL^{-1}), supporting the current findings in *H. rubra*. Air emersion occurred over 24 h, which probably explains the higher tissue and haemolymph levels of D-lactate and tauropine in *H. iris* than reported here for *H. rubra*.

In *H. iris*, haemolymph was sampled from three separate sites: the pedal sinus, the aorta and the adductor muscle (Behrens *et al.* 2002). Adductor muscle haemolymph was accessed by drilling through the shell, creating a well in the muscle from which haemolymph was subsequently aspirated. Interestingly, it is this adductor muscle interstitial haemolymph that displays the highest amount of D-lactae during rest in water (0.47 μmol mL^{-1}), and

displays as much D-lactate as the foot muscle during 24 hr emersion (1.74 μmol mL^{-1}, Behrens *et al.* 2002). In contrast to these adductor haemolymph metabolite levels, D-lactate was not detected in resting *H. rubra* haemolymph, and only low levels of D-lactate was found in *H. iris* haemolymph sampled from the other sites (0.02-0.12 μmol mL^{-1}, Behrens *et al.* 2002). It is the contention of the author that these high adductor D-lactate levels observed for *H. iris* may be a response of tissue damage associated with the creation of a well in the adductor muscle surface.

Given the similarity in haemolymph metabolite concentrations between different sampling sites in *H. rubra*, it is not possible to determine if any filtration of the metabolites is occurring in the kidneys or atria. This could be more appropriately addressed by assaying haemolymph from these potential filtration sites.

Until the current study, D-lactate had not been observed in the haemolymph of an archaeogastropod mollusc (c/f Behrens *et al.* 2002). In comparison to marine gastropods, D-lactate has been found in the haemolymph of terrestrial and freshwater pulmonate and operculate gastropods, particularly those undergoing severe hypoxia or anoxia (von Brand *et al.* 1950, 1955; De Zwaan *et al.* 1976; Wieser 1978; Wieser and Wright 1978, 1979; Kluytmans and Zandee 1983). Haemolymph D-lactate concentrations may reach 60 mmol L^{-1} in the pulmonate *Helix pomatia* during periods of anoxia lasting up to 45 h (Wieser 1981). During 24 h anoxia, Wijsman *et al.* (1985) observed a haemolymph D-lactate concentration 4 times higher than in the muscles and viscera of the gastropod *Lymnaea stagnalis*, a ratio not seen in abalone, and suggest this indicates an active transport of the metabolite into the haemolymph. Likewise, more D-lactate is often observed in the haemolymph or incubation media than the tissues of these gastropods (De Zwaan *et al.* 1976).

What is the significance of D-lactate in the haemolymph of *H. rubra*? It has been proposed that the appearance of D-lactate in the haemolymph of gastropods may relate to pH regulation. In conjunction with intracellular buffering, gastropods may regulate intracellular pH by releasing acidic end products from their tissues. The fates of these anaerobic end products, during sustained hypoxia/anoxia or during recovery, are varied. End products could be excreted to the external environment. Alternatively, end products may be released in to the haemolymph and may be selectively taken up by certain organs enzymatically geared for end product metabolism. These compounds could then be utilised as aerobic fuels or converted back to anaerobic substrates. Finally, tissues producing anaerobic end products may not

release these compounds at all, but instead reoxidise them for energy or for reconversion to substrate *in situ* (Ellington 1983).

The release of D-lactate from *in vitro* marine gastropod muscle preparations into experimental incubation media, as described by Wiseman and Ellington (1987), may have a number of functional advantages were it to occur *in vivo*. Firstly, the removal of the end product would favour the forward reaction (production of D-lactate), ensuring any end product inhibition is avoided, and maintaining the redox flux. Also, the removal of an end product in a symport (H^+) or anitport (OH^-) system would aid in regulation of intracellular pH (Wiseman and Ellington 1987). It is not certain whether this same pattern is observed during muscle contractions *in vivo*.

Only very low concentrations of tauropine were observed in the haemolymph of *H. rubra*. With an extended emersion protocol however, haemolymph tauropine levels may rise. Compared to resting *H. rubra* haemolymph samples in which neither D-lactate nor tauropine were detected, resting *H. iris* displayed haemolymph D-lactate and tauropine concentrations high as 0.47 and 0.38 μmol mL^{-1}, respectively (Behrens *et al.* 2002). After 24 h emersion, haemolymph tauropine levels increased to concentrations almost equal with D-lactate (1.58 mmol L^{-1}, Behrens *et al.* 2002), a level not seen previously for an opine.

To date, the only other conformation of opines leaving the cell and entering molluscan haemolymph are studies on octopine in cephalopods. Octopine produced in mantle muscle of *Loligo vulgaris* and *Sepia officianalis* during elevated or sustained work is found in the haemolymph, where it may be transported to nervous tissue (brain and optic lobe) and ventricle and oxidised to pyruvate and arginine by ODH isozymes (Storey 1977b; Storey and Storey 1979a, 1979b; Gäde 1980a). Having established this pattern *in vivo*, it was suggested that the cycling of ODH may be completed via a metabolic cycle, analogous to the Cori cycle observed for L-lactate in vertebrates. Given the low levels of tauropine in *H. rubra* haemolymph and TDH in tissues such as radula muscle and ctenidia, it is unlikely that tauropine and TDH participate in any cycling between tissues. In terms of relative concentrations however, tauropine is actually found in abalone haemolymph at very similar concentrations to octopine in the haemolymph of *S. officinalis* (0.02 to 0.16 mmol L^{-1}, Storey and Storey 1979b).

Why is it that D-lactate enters the haemolymph of these organisms, whereas opines largely appear to be are retained in the cell? Ellington (1983) and Wiseman and Ellington (1987) suggest that the retention of opines is related to the fact they are nitrogen-containing

compounds. Any loss of carbon as a result of the release and potential excretion of D-lactate on the other hand may be buffered by the stocks of glycogen in molluscan muscle. Wiseman and Ellington (1987) argued that there is no direct evidence for the release of opines from any molluscan tissue, and consider the rise in haemolymph octopine and concomitant decrease in the muscle arginine pool during contractile activity in cephalopods as an indirect suggestion. Similarly, De Zwaan and Dando (1984) concluded that no study to date had convincingly revealed a release of opines from molluscan muscle.

The production of opines results in no net increase in the internal osmolarity, as the respective amino acid condenses with pyruvate derived from the large glycogen polymer (Fields 1983; Gäde and Grieshaber 1986; Bowen 1987; Wiseman and Ellington 1987). The formation of lactate on the other hand increases the number of intracellular osmotically active particles. It would therefore seem disadvantageous to transport out opines (Wiseman and Ellington 1987). However, given the small fraction by which accumulated end products are thought to contribute to cell osmolarity, the removal of these compounds for the sake of cell volume seems unlikely (Grieshaber and Kreutzer 1986). It is more likely that no mechanism coupling the removal of opines and pH regulation (H^+ symport or OH^- antiport) exists (Wiseman and Ellington 1987). The appearance of tauropine in abalone haemolymph does not concur with these suggestions however, and should be investigated more thoroughly.

Glucose

Exercise and emersion in *H. rubra* appear to stimulate a mobilisation of tissue glycogen stores that results in an increase in haemolymph glucose from a resting concentration of 0.09 μmol mL^{-1} to as high as 0.93 μmol mL^{-1}. Resting haemolymph glucose levels of *H. rubra* are generally lower than previous measurements for abalone. The haemolymph glucose level of both adult and juvenile *H. iris*, from sheltered or exposed habitats, ranges from 1.20 to 1.73 mmol L^{-1} (Wells *et al.* 1998b). Boarder *et al.* (2001) found resting haemolymph glucose at levels around 0.78 mmol L^{-1} for the Greenlip abalone, *H. laevigata*, a concentration almost as high as *H. rubra* levels after 12 h air emersion. Resting haemolymph glucose levels of both *H. kamtschatkana* and the sea hare *Aplysia dactylomela* are more similar to *H. rubra*, at around 0.14 mmol L^{-1} (Carefoot 1991, 1994; Carefoot *et al.* 1993).

All abalone used for experiments were well fed and checked regularly, as haemolymph glucose levels in abalone are known to respond to the fed state of the animal. Haemolymph glucose titre drops to about half of the normal resting level of 23-27 μg mL^{-1}

(0.13-0.15 μmol mL^{-1}) after 6 days of starvation in *H. kamtschatkana* (Carefoot *et al.* 1993). Associated with this starvation is the depletion of glycogen reserves in the digestive gland, with foot reserves depleting at a much slower rate (27 days, Carefoot *et al.* 1993).

Watanabe *et al.* (1994a) report an increase in glucose levels in the adductor and foot muscles of *H. discus* at night, which it is assumed correlates with feeding and foraging behaviour. Similar increases in tissue glucose concentrations are observed in the adductor muscle and rim of the foot of juvenile *H. discus* during emersion (Watanabe *et al.* 1994b).

Tissue glucose has been observed to increase during hypoxia in other molluscs. Tidepool stranding and air emersion are also known to produce large and significant elevations in the haemolymph glucose titre of the sea hare *Aplysia dactylomela* from 25-35 μg mL^{-1} to 80 μg mL^{-1} (0.14 to 0.45 μmol mL^{-1}, Carefoot 1994). During 40h anoxia, the gastropod *Lymnaea stagnalis* displays a considerable stress response, including the production of large amounts of mucus and a maximal effort to facilitate gas exchange. Associated with this anoxia are a bradycardia (30 to 10 beats min^{-1}) and hyperglycaemia during the first 6 h, where haemolymph glucose increases from 40 to 674 μg mL^{-1} (0.22 to 3.7 μmol mL^{-1}, Livingstone and De Zwaan 1983). Increases in muscle glucose concentration from resting levels of 0.29 μmol g^{-1} are observed in the foot muscle of the bivalve *Cardium tuberculatum* following both exercise (0.42 μmol g^{-1}) and 10 h anoxia (1.12 μmol g^{-1}, Meinhardus-Hager *et al.* 1989).

These large increases in haemolymph and tissue glucose in *H. rubra* and other molluscs during vigorous muscle activity and hypoxia probably result from an increased mobilisation of carbohydrate stores to deal with increased demands in fuels. Other physiological challenges such as starvation, and salinity and temperature changes, are also known to elicit increases in haemolymph glucose titres (Carefoot 1991, 1994; Boarder *et al.* 2001). Glucose in molluscs can be transported out of the gut and across cellular membranes during hypoxia and anoxia (Livingstone and De Zwaan 1983). The delivery of glucose via the haemolymph tissues of *H. rubra* during hypoxia ensures their continued metabolism.

4.4.4 Recovery of abalone from hypoxia

Although probably not complete in this time, recovery of *H. rubra* for 2 h following exercise resulted in a decrease in muscle D-lactate and tauropine concentrations, with little effect on haemolymph concentrations. After 2 h of recovery in water, these muscle metabolite concentrations had approached control treatment (resting) levels. Similar

relatively rapid decreases in muscle metabolite levels observed during recovery have been noted for other marine molluscs. Recovery from exercise in the whelk *Buccinum undatum* is typified by a 50% decrease in columellar muscle octopine levels during the first 30 min, with a much slower reduction thereafter until control levels are reached 5 h later (Koormann and Grieshaber 1980). Following exercise, octopine in the pedal retractor of the whelk *Nassarius coronatus* returns to resting levels after 2 h recovery (Baldwin *et al.* 1981). Recovery from exercise by jumping in the cockle *Cardium tuberculatum* (Meinhardus-Hager and Gäde 1987) involves the rapid replenishment of phosphagen stores and reduction in octopine concentration, accompanied by a large and significant increase in O_2-consumption.

No increases in anaerobic metabolite concentrations were noted in *H. rubra* tissues during 2 h recovery from exercise. The use of anaerobic glycolysis to replenish phosphagen and ATP levels during recovery from exercise is well documented for other gastropods. Members of the Strombidae and Nassariidae (Baldwin *et al.* 1981; Baldwin and England 1982b; Gäde *et al.* 1984) accumulate octopine in the pedal retractor muscles during exercise, and strombine/alanopine or D-lactate during recovery.

While recovery from burst-muscle work has not been previously investigated in *Haliotis*, the recovery of *H. lamellosa* (Gäde 1988) *H. rufescens* (Tjeerdema *et al.* 1991b) and *H. iris* (Ryder *et al.* 1994) from environmental hypoxia has been monitored, and the results are discussed for comparison with the effects of exercise. Following 6 h of anoxic incubation of *H. lamellosa* (N_2 bubbled seawater), Gäde (1988) observed a relatively rapid decrease in foot muscle D-lactate concentration (50% of anoxia values after 3 h recovery), whereas D-lactate and tauropine in the adductor remained elevated after up to 13 h. Gäde (1988) observed no considerable increase in metabolite levels during recovery, and concludes that recovery does not involve an anaerobic component.

Recovery from 45-60 min air emersion in *H. rufescens* involved the replenishment of depleted arginine phosphate stores and a gradual return of intracellular pH and ATP to normal resting levels after 15 h (Tjeerdema *et al.* 1991b).

Ryder *et al.* (1994), in a study simulating transport of *H. iris* to mimic commercial practices, involved a more protracted time course. Recovery from 24 h air emersion in moist, cool conditions (polystyrene box with moist seaweed and ice packs) was monitored for up to 5 days. In central portions of the pedal musculature, D-lactate returned to resting levels within 48 h during recovery in water, whereas tissue pH and tauropine required 120 h to reach resting levels. In contrast to *H. lamellosa*, *H. iris* appears to employ anaerobic glycolysis in the initial period of recovery. Tauropine levels in the pedal musculature continue to rise for

the first 24 h of recovery, and this anaerobic energy supplement is thought to reflect the limited aerobic capacity of these muscles (Ryder *et al.* 1994). No obvious anaerobic component of recovery was observed for *H. rubra*. However, this may manifest if the duration or severity of hypoxia were to increase (*e.g.* during emersion), as for *H. iris*.

In none of these previous studies were haemolymph metabolite levels monitored during treatment or recovery. Gäde (1988) suggests that tissue metabolites are resynthesised *in situ* in *H. lamellosa*. Interestingly, during recovery from 6 h anoxia, succinate and D-lactate in the foot muscle of *H. lamellosa* reach control levels much quicker than in the adductor muscle, a pattern demonstrated also by *H. rubra*, at least during the first hour. This decrease or 'removal' of metabolites does not necessarily involve a physical displacement of the metabolites. The pattern observed between the adductor and foot muscles is not influenced by the concentration of the metabolite, as more D-lactate is made in the foot of both *H. lamellosa* and *H. rubra*. The pattern may result from a clearance of the metabolites into the haemolymph, thus reflecting a greater perfusion of the foot. The adductor muscle of abalone is believed to be poorly perfused, even at rest (Taylor *et al.* 2001; Just 2002). Chen (1996) could not identify either haemolymph vessels or sinuses in the shell adductor of *H. diversicolor*, and found it difficult to withdraw haemolymph from this muscle. Jorgensen *et al.* (1984) found the pedal musculature of *H. cracherodii* does not receive haemolymph in proportion to its weight.

During hypoxia, concentrations of D-lactate in *H. rubra* muscles approach, but do not attain, the K_m values of the reverse reaction as determined *in vitro* at pH 9.0 (K_m D-lactate 2.43 mmol L^{-1}; Chapter 3, Section 3.3.2). Under these current conditions, it is not likely therefore that the majority of the D-lactate is being oxidised to pyruvate in these muscles. However, the large decreases in muscle concentration as opposed to haemolymph concentrations does not entirely support this suggestion. Muscle D-lactate in the snail *Helix pomatia* easily attains concentrations that are compatible with the K_m values for D-lactate (Wieser and Wright 1978).

It seems to be a general assumption in the literature that anaerobic end products are metabolised *in situ* following hypoxia or anoxia. Clearly not the entire metabolite load is re-metabolised, as some proportion is observed in the haemolymph of *H. rubra*. During recovery in *H rubra*, tissue metabolite levels decrease, and whilst haemolymph tauropine levels remain fairly constant, a small rise in D-lactate levels occurs. This indicates a largely tissue-mediated recovery in metabolite levels, as a strict release of metabolites would be

accompanied by an increase in haemolymph concentration. The large volume of the haemolymph, or a lag in tissue metabolite release, may mask this release however. Given the higher muscle and haemolymph concentrations of metabolites as a result of air emersion, recovery following emersion may better illustrate the situation.

4.4.5 Effects of hypoxia on muscle and haemolymph pH

A negative correlation exists between the extent of hypoxia in *H. rubra* and the haemolymph pH. Haemolymph pH decreased from resting levels of 7.3 to as low as 6.8 during 12 h emersion. Abalone muscle and haemolymph pH decreases as a result of exercise, air emersion and repeated handling of abalone (removal of animals from water, physical handling for 15-30 sec and replacement; Table 4.3). This drop probably involves a combination of a respiratory and metabolic acidosis. The magnitude of pH change in abalone depends on whether internal hypoxia is induced by environmental or activity causes, with air emersion generally resulting in lower pH.

It appears that, with a few exceptions, resting pedal muscle and haemolymph pH is rather conservative across the genus, and that both exercise and air emersion result in a fall in pH. Values for *H. rubra* approximate those of other temperate Australasian abalone. The decrease in haemolymph pH following 12 h emersion in *H. rubra* is not as great as that for *H. iris* muscles following 24 h air emersion (Baldwin *et al.* 1992) or *H. rubra* pedal sole after 24-42 h air emersion (James and Olley 1970). Submerged *H. cracherodii* maintain a comparatively low adductor muscle pH of 6.71, which decreases to 6.56 after 1.5 h of emersion (Bowen and Bakhit 1976, as cited in Bowen 1984). Repeated handling of *H. iris* also results in a decrease in haemolymph pH, with no D- or L-lactate detected (Behrens *et al.* 2002). The haemolymph pH of control or undisturbed *H. iris* is unusually low however (7.02), although it is not altogether clear why this is so.

The extent to which anaerobic metabolism affects intracellular and haemolymph pH is probably affected more by the duration of hypoxia than the pH buffering capacity of abalone muscle, given the similarities in β capacities of pedal muscle from different species (Chapter 3, Section 3.4.5).

Haemolymph sampled from *H. rubra* after 12 h emersion, regardless of origin, displayed a range of oxygenated states, as indicated by a colourless, pale or blue colour. The same phenomenon was observed for *H. iris* haemolymph after 24 h air emersion (pers. obs). Without replenishment of O_2, *H. rubra* haemocyanin (Hc) probably would be deoxygenated to some extent after 12 h air emersion. Changes in haemolymph pH and metabolite load

influence the O_2-binding and carrying capacity of abalone Hc (Chapter 6), which may be reflected in the colour of the haemolymph.

Although *H. rubra* muscle pH was not measured, it may be assumed that muscle pH is equivalent to haemolymph pH in *H. rubra*, given results for other abalone.

Not only is a decrease in haemolymph pH observed in hypoxia-stressed abalone, but an apparent decrease in haemolymph volume is also observed in *H. rubra*. Air exposed *H. rubra* tend to lose "a liquor" that is first colourless then assumes the colour and composition of haemolymph (Olley and Thrower 1977). Similarly, for abalone held in air in commercial bins for up to 24 h, a 5-20% fluid loss (% weight) was observed, sufficient to cause substantial morbidity (Gorfine 2001). Gorfine (2001) suggests these volume losses are primarily haemolymph, and also mantle water and body fluid, loss.

4.4.6 Possible role of haemolymph and fate of metabolites during hypoxia in abalone

It is apparent that during hypoxia in abalone, haemolymph anaerobic metabolite concentrations may be equivalent to or even higher than muscle concentrations. The extent to which the circulatory system can accumulate D-lactate and tauropine, and possibly function as a store, can not be accurately determined for *H. rubra* or other abalone at this stage. However, extrapolations may be made based on the body composition results presented in Chapter 2. An average *H. rubra* of 200 g total weight including shell may have a haemolymph volume of about 100 mL, a volume that potentially could serve as a larger reservoir for acidic metabolites than the muscle mass. For example, the mean haemolymph D-lactate concentration following 12 h of air emersion is 0.94 μmol mL^{-1}. For a 200 g abalone, a total haemolymph D-lactate load is likely to be 94 μmol, assuming haemolymph volume is 50% weight/volume. Muscle D-lactate, at a mean of 1.08 μmol g^{-1}, would be in total about 76 μmol, given that adductor and foot may compromise 35% of total weight, and each muscle is approx. 50% of total pedal musculature (Chapter 2).

The role of the haemolymph as a reservoir, or as a mechanism for removal of anaerobic metabolites, has been investigated for pulmonate gastropods such as *Helix pomatia* and *Lymnaea stagnalis* which accumulate D-lactate to impressive levels (40-60 mmol L^{-1}, Wieser 1981; Kluytmans and Zandee 1983; Wijsman *et al.* 1985). Following up to 24 h air emersion, abalone haemolymph concentrations remain below 5 mmol L^{-1}. It may be that abalone haemolymph metabolite levels themselves, as well as haemolymph pH, reach a point where they are detrimental to other functions, such as O_2-delivery by Hc (see Chapter 6).

What is the importance or fate of D-lactate in the haemolymph of gastropods? Upon transfer from anaerobic to aerobic conditions, haemolymph lactate levels fall in both *Lymnaea stagnalis* and *Helix pomatia*, with a concomitant increase within the liver of *Helix pomatia* (Wieser 1981). These data suggest that anaerobic metabolites of one muscle may be fuel for another tissue. In pulmonates at least, there appears to be a lactate cycle functionally analogous to that of vertebrates (Everse and Kaplan 1973). That is, D-lactate produced in the foot muscle for example enters the haemolymph and is sequestered by the hepatopancreas or other non-muscular tissue to serve as a substrate for gluconeogenesis. The haemolymph D-lactate load of terrestrial and freshwater gastropods following hypoxia or anoxia may be further reduced by excretion in to the medium (von Brand *et al.* 1950, 1955; Wieser 1978, 1981; Kluytmans and Zandee 1983; Wijsman *et al.* 1985). Although considered, the examination of metabolite excretion was not undertaken in this study, as neither the tissue nor haemolymph metabolite concentration reached levels observed for pulmonates, and at haemolymph concentrations of only 0.5-1.5 mmol L^{-1} would be difficult to detect in the external environment.

If pyruvate reductase end products such as D-lactate and octopine are being sent to various tissues in pulmonate gastropods and cephalopods to be re-oxidised, it is not unreasonable to suggest this also may be the case for abalone. Whilst the maintenance of muscle cell homeostasis is of importance, abalone may benefit indirectly from the presence of metabolites in the haemolymph by their use as an alternative fuel at other sites in the body. A loss of carbon in the form of D-lactate may be recovered by recycling the metabolite. According to studies mentioned above, prime candidates for 'acceptor' or 'target' tissues are those that that display considerable enzymatic competence (*e.g.* hepatopancreas/digestive gland), or those that are metabolically demanding (*e.g.* cardiac and neural tissue, or radula muscle).

4.4.7 Respiratory and cardiovascular responses of abalone to hypoxia

In association with changes to muscle metabolism, environmental and functional hypoxia in abalone also invokes respiratory and cardiovascular responses. Submerged *H. rubra* maintain a steady heart rate until a critical level of 90-80 mm Hg PO_2 in the external water, below which heart rate decreases with decreasing PO_2 (Russell and Evans 1989). Nakanishi (1978) however suggests hypoxia, in the form of both decreased water PO_2 and air emersion, does not seriously affect heart rate of *H. discus hannai*. Heart rate increased only

1.1 times faster than normal at or below 50% O_2-saturation. Ainslie (1977) noted the regulation of O_2-uptake by *H. laevigata*, *H. roei* and *H. rubra* as PO_2 decreased, until concentrations of 2.8 ppm (PO_2 about 55 mmHg, 15°C). At lower O_2-concentrations (less than 2 ppm), abalone showed escape responses (twisting) and signs of distress, and lost their grip on the experimental surfaces (Ainslie 1977). *Haliotis diversicolor* was found to adjust its O_2-uptake according to decreasing seawater PO_2 (Jan and Chang 1983). Likewise, Boyd and Bourne (1995) observed an uptake of O_2 in both adult and juvenile *H. kamtschatkana* independent of falling PO_2, until a critical point of 12.7 kPa (approx. 95 mmHg).

Since most abalone tend to inhabit water subject to constant mixing, stresses of low O_2 or high temperature may be encountered rarely (although the field collection site often experienced periods of low water level and movement, and a thermocline was observed, particularly between 0.5 and 1 m, during warmer months). Hypoxic conditions may ensue in the respiratory/mantle cavity when the animals are clamped tightly to the substratum and ventilation is inhibited.

Upon emersion, *H. cracherodii* shows a bradycardia of at least 25%, with cardiac output decreasing substantially (Jorgensen *et al.* 1978). During emersion, the major source of O_2 is thought to be that dissolved in the mantle cavity fluid. Mantle fluid, estimated at approx. 10 mL for *H. fulgens* might contain up to 100 µl of dissolved O_2, sustaining typical immersed aerobic metabolism for only about 5 min (Pilson 1963, as cited in Bowen 1984).

Environmental conditions and physical activity may reduce the water PO_2 sufficiently to cause cardiovascular and respiratory responses in *H. rubra* as described by the authors above. Although not quantitatively determined in this study, both behavioural and O_2-consumption data give us some indication as to the onset of anaerobic metabolism in haliotids. Small *H. rubra* held in air quickly switch over to anaerobic metabolism, as indicated by reduced CO_2 production accompanied by decreased O_2-consumption, with larger abalone apparently taking longer to become anaerobic (James and Olley 1974).

Ainslie (1977), studying the respiration of *H. laevigata*, *H. roei* and *H. rubra*, concluded that increasing water temperature or depleting atmospheric O_2 results in both a severe reduction in the capacity of the circulatory system to deliver O_2 to abalone tissues, and a predicted decrease in aerobic respiration. However, the same investigation failed to detect an increase in aerobic respiration during recovery in seawater in these species following 1 h of anoxia (Ainslie 1977). It is known that the reduction of O_2-availability results in the production of anaerobic metabolites in abalone. How this affects O_2-consumption, transport and binding to Hc however is not entirely elucidated. Work on *H. iris* by Wells *et al.* (1998a)

and Behrens *et al.* (2002) however indicates a shifting of the O_2-dissociation curve for *H. iris* Hc when haemolymph pH is reduced, as occurs during anaerobiosis. This results in an increase in Hc O_2-affinity (see Chapter 6).

A depression of metabolic rate, as measured by O_2-consumption, by *H. rubra* following short periods of emersion was proposed by Edwards (1996). Holding abalone in damp conditions in air for 1.5 to 7 h produced a decreased rate of O_2-consumption when replaced in water (Edwards 1996). A degree of caution must be used in interpreting these results, given that the study was performed on the same small group of individuals and does not appear to have been verified subsequently. Nonetheless, in conjunction with work by Ainslie (1977), a period of emersion or anoxic incubation appears to influence O_2-consumption in recovering animals *i.e.* it remains at resting levels, or is even depressed.

In contrast, an actual increase in metabolic rate has been observed in *H. rubra* and *H. laevigata* following handling and transfer to a new environment (Edwards 1995). Upon transfer, abalone displayed an initial increase in O_2-consumption of up to 320% of a resting or settled rate. This initial increase however decreased over the first 2 days in the new environment, and was only 20% greater than resting after 3 days. It appears that a brief disturbance elicits a different physiological response in abalone than does prolonged emersion. This variance may relate to fundamental differences in metabolic regulation or depression, particularly over extended periods of hypoxia. Whether this rapid increase in metabolic rate following brief handling of abalone reflects an O_2-debt is not clear.

While an immediate repayment of an O_2-debt has not been conclusively demonstrated for abalone, the repayment of an O_2-debt has been shown for other molluscs, such as the cockle *Cardium tuberculatum* (Meinhardus-Hager and Gäde 1987). Oxygen consumption for this species increased up to 3.5-fold within 5 min of termination of anoxia or exercise.

It is known that high intramuscular pressures develop in the pedal musculature of abalone during muscle work (see Chapters 1 and 2), which results in increasing peripheral resistance in, or occlusion of, the pedal vessels and lacunar tissue spaces (LTS) in the pedal muscle. This essentially isolates the foot and adductor from the general circulation (Russell and Evans 1989). The pedal musculature of abalone theoretically can then be rendered hypoxic. Injection of labelled inulin (^{14}C-inulin) or fluorescent microspheres during clamping in *H. iris* indicates the adductor muscle is poorly perfused, or isolated, from the main circulation, and the relative haemolymph flow to the foot and adductor muscles is decreased (Taylor *et al.* 2001; Just 2002).

In addition, the proposed vascular shunt between the cephalic sinuses in *H. rubra* (Russel and Evans 1989) allows haemolymph to bypass and isolate the pedal muscle mass, conserving O_2 for more O_2-dependent tissues. The foot and adductor muscles may not be affected by this vascular shunt, as they still retain considerable capacity for anaerobic ATP production. The capacity for facultative anaerobiosis in these muscles, especially the adductor, may circumvent any problem in delivery of adequate O_2.

4.4.8 Overview of effects of hypoxia on pedal musculature and haemolymph

The major findings of this chapter suggest that *H. rubra* reacts similarly to other abalone when faced with periods of enhanced muscle work involving the pedal musculature or air emersion. Resting in water, a behaviour that appears to characterise abalone, does not involve the accumulation of D-lactate in *H. rubra* pedal muscle or haemolymph. Tauropine levels on the other hand suggest that clamping down, moderate locomotion or even postural maintenance in abalone involves an anaerobic component, with the adductor muscle responsible for development of much of the muscular force required for these functions.

In *H. rubra* as with other abalone species, both exercise and air emersion resulted in a switching over to anaerobic energy provision, particularly in the adductor and foot muscles. Whilst *H. rubra* D-lactate and tauropine concentrations appear to decrease relatively quickly during recovery following exercise, a more protracted time course is observed following air emersion in other species.

As has been noted for muscles and haemolymph of other abalone, *H. rubra* haemolymph pH drops as the extent of hypoxia increases. New findings indicating that levels of haemolymph metabolites such as D-lactate and glucose increase with hypoxia, opening the possibility that anaerobic metabolites in the haemolymph of abalone may be transported to other sites.

It was concluded from the data presented in Chapter 3 that the buccal and radula muscles of *H. rubra*, compared to the adductor and foot muscles, possess a higher aerobic scope and a limited capacity for facultative anaerobic metabolism. These differences exhibited by radula and pedal muscle would be expected to translate into contrasting responses to various physiological challenges experienced by *H. rubra*. The pedal musculature of *H. rubra* has been shown to derive energy anaerobically during both elevated sustained activity, and air emersion. However, little is known about the metabolism of other muscles during these hypoxic events.

While radula muscles may be affected by whole animal hypoxia, they are involved almost solely in the protraction and contraction of the odontophore and radula during feeding. Metabolism of prosobranch gastropod radula muscles is considered aerobic, a hypothesis based on the physiology of these muscles and the buccal cavity. These assumptions may be valid for muscles at rest, but what about during feeding? Working radula muscles, on the basis of their probably high aerobic scope, may show differences in metabolism and end product accumulation to exercising pedal muscles. With respect to the radula muscles, the facultatively anaerobic pedal muscles act as a good 'control'.

As the metabolism powering the movements of the radula had yet to be identified in abalone, examining the effects of natural feeding behaviour on radula muscles was the next extension of this study. The following chapter is concerned therefore with examining the effects of feeding and, as a comparison to the pedal musculature, whole-animal hypoxia on the metabolism of these muscles.

Table 4.1. Haemolymph pH and concentrations of D-lactate and tauropine in adductor and foot muscles (μmol g^{-1} wet wt muscle) and haemolymph (μmol mL^{-1}) of *H. rubra* at rest, and following exercise and 12 h air emersion.

Treatment	pH	Tissue/Haemolymph	Metabolite	
			D-lactate	Tauropine
Control	7.29 ± 0.16	Haemolymph aorta	0 ± 0	0 ± 0
		Haemolymph foot	0 ± 0	0 ± 0
		Adductor	0 ± 0	1.04 ± 0.19
		Foot	0 ± 0	0.29 ± 0.09
Exercise	7.21 ± 0.02	Haemolymph aorta	0.15 ± 0.09	0.06 ± 0.02
		Haemolymph foot	0.09 ± 0.06	0.03 ± 0.01
		Adductor	0.53 ± 0.17	3.24 ± 0.62
		Foot	0.84 ± 0.15	1.57 ± 0.62
Emersion	6.82 ± 0.02	Haemolymph aorta	1.09 ± 0.14	0.11 ± 0.01
		Haemolymph foot	0.78 ± 0.10	0.07 ± 0.01
		Adductor	0.83 ± 0.10	1.88 ± 0.25
		Foot	1.34 ± 0.16	0.73 ± 0.13

Significant differences between metabolite levels of different tissues and treatments are detailed in the text.

Values given as Mean ± S.E.M for N=12, except for haemolymph aorta where N=6.

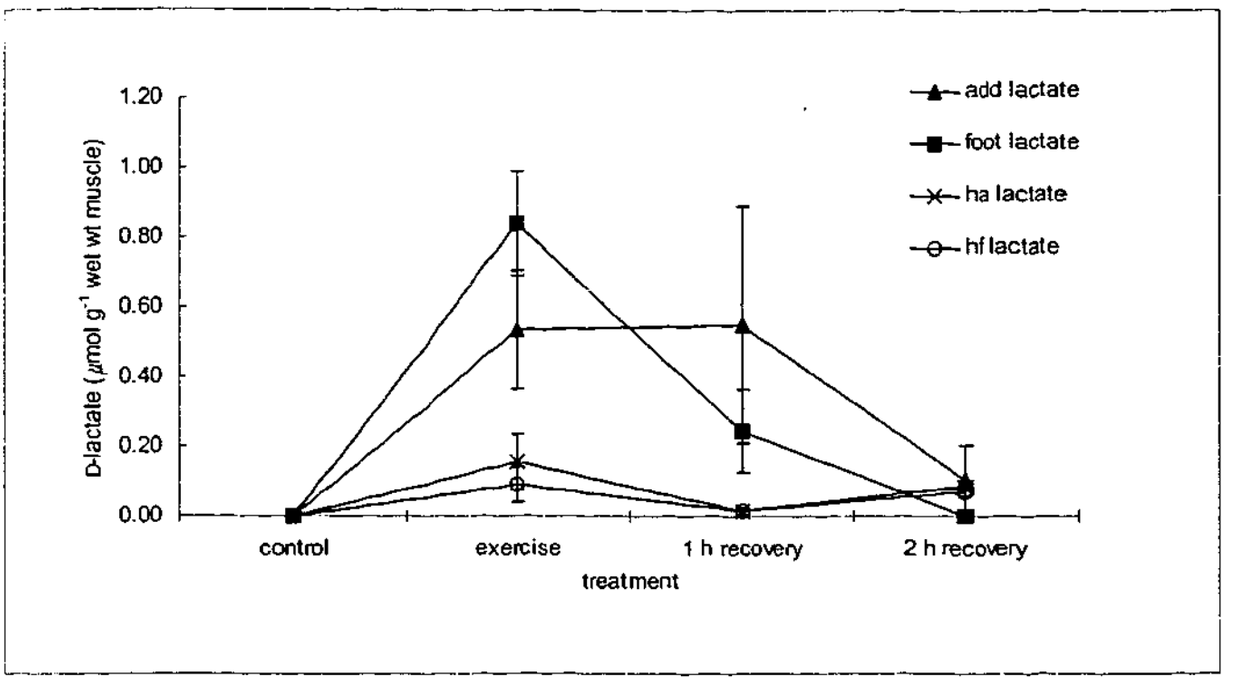
Table 4.2. Concentrations of D-lactate and tauropine in the adductor and foot muscles (μmol g^{-1} wet wt muscle) and haemolymph (μmol mL^{-1}) of *H. rubra* during 2 hours of recovery in water following exercise.

Treatment	Tissue/Haemolymph	Metabolite	
		D-lactate	Tauropine
1 hour recovery	Haemolymph aorta	0.02 ± 0.02	0.06 ± 0.03
	Haemolymph foot	0.01 ± 0.01	0.07 ± 0.02
	Adductor	0.55 ± 0.34	2.98 ± 0.32
	Foot	0.25 ± 0.12	0.87 ± 0.28
2 hour recovery	Haemolymph aorta	0.09 ± 0.01	0.03 ± 0.02
	Haemolymph foot	0.07 ± 0.01	0.11 ± 0.03
	Adductor	0.11 ± 0.11	1.23 ± 0.73
	Foot	0 ± 0	0.34 ± 0.23

Significant differences between metabolite levels of different tissues and treatments are detailed in the text.

Values given as Mean ± S.E.M for N=4.

(A)



(B)

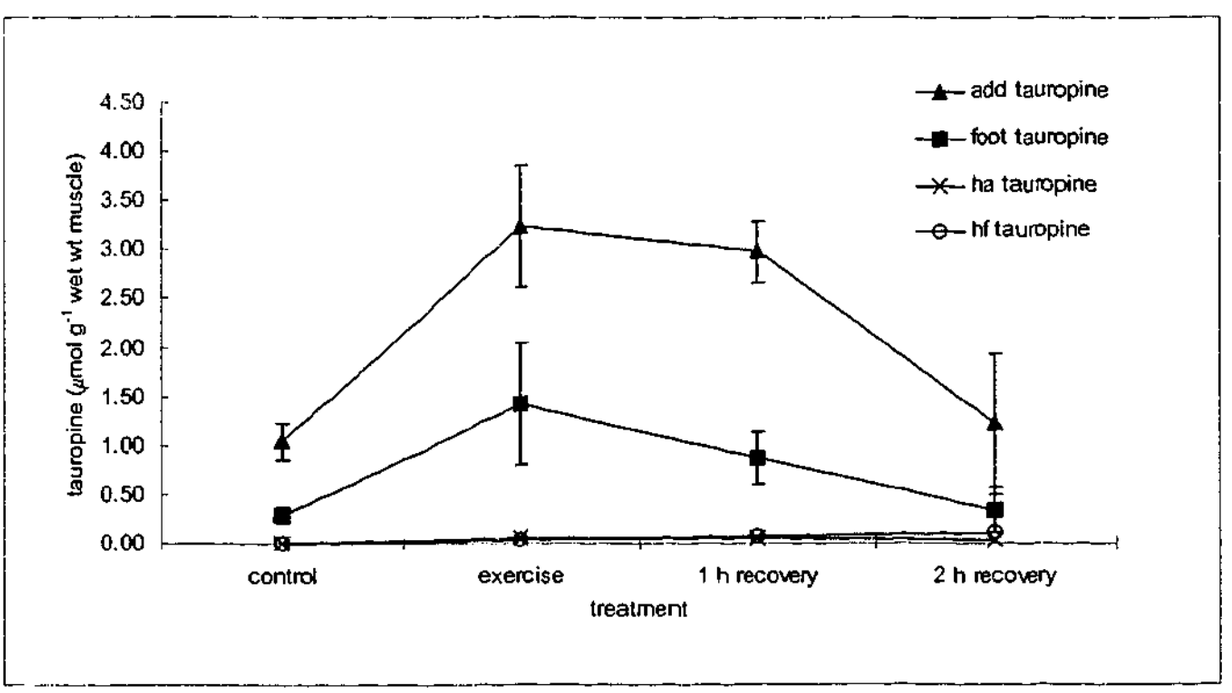


Figure 4.1. Concentration of (A) D-lactate and (B) tauropine in adductor muscle and foot muscle (μmol g^{-1} wet wt) and haemolymph (μmol mL^{-1}; ha = haemolymph aorta sample, hf = haemolymph foot sinus) of *H. rubra* at rest, following exercise and after 1 h and 2 h recovery in water. Values are mean ± S.E.M.

Table 4.3. Muscle and haemolymph pH of resting and hypoxic abalone

Species	Treatment	Sample	pH	Reference
H. iris	Resting	Pedal muscle	7.32	Baldwin *et al.* (1992)
	Exercise		7.12	
	Air emersion		6.64	
	Resting	Haemolymph	7.39	Wells *et al.* (1998)
	Exercise		6.51	
	Resting	Haemolymph	7.02	Behrens *et al.* (2002)
	Repeated handling		6.83	
H. laevigata	Resting	Haemolymph	7.32	Ainslie (1980b)
H. roei			7.37	
H. rubra			7.38	
	Resting	Haemolymph	7.30	Ainslie (1977)
	Resting	Haemolympn	7.30	This study
	Exercise		7.21	
	Emersion		6.82	
	Resting	Pedal muscle	7.35	James and Olley (1970)
	Emersion	Pedal sole	6.55	
		Pedal muscle	6.75	
	Resting	Pedal muscle	7.4	James and Olley (1971b)
H. rufescens	Resting	Pedal muscle	7.5	Tjeerdema *et al.* (1991b)
	Emersion		7.0	

CHAPTER FIVE

ENERGY METABOLISM OF *HALIOTIS RUBRA*: EFFECTS OF FEEDING, AND WHOLE ANIMAL EXERCISE AND EMERSION, ON RADULA MUSCLE METABOLISM

5.1 INTRODUCTION

The metabolic indices identified for *H. rubra* adductor and foot muscles (Chapter 3) correlate well with the response of the pedal musculature to whole animal hypoxia (Chapter 4). A predominantly anaerobic poise of these muscles translated into facultative anaerobic metabolism during exercise and emersion, resulting in the accumulation of the end products D-lactate and tauropine.

The radula muscles of *H. rubra* however display a more aerobic poise (Chapter 3) and, as a result of this poise and the associated continuous haemolymph supply to these muscles (Chapter 2), would be expected to perform differently to the pedal musculature during muscle work and other potentially hypoxic situations. This chapter aims to examine the effects of muscle work in the form of natural feeding, and whole animal hypoxia, on the radula muscle metabolism of *H. rubra*.

5.1.1 Physiology of *Haliotis rubra* and gastropod radula muscles

The radula muscles of abalone constitute an important part of the animal-environment interface and are intimately associated with the cephalic haemolymph sinuses (CAS). Blood flow to these muscles is more direct and presumably more continuous than to the pedal musculature (Chapter 2). In addition, given the widening of the aorta into the CAS, the haemolymph bathing the muscles is relatively highly oxygenated.

As discussed in Chapter 3, the metabolic capabilities of gastropod radula muscles may be quite high, with some containing high cytochrome levels, high glycolytic and Krebs cycle enzyme activities and large numbers of mitochondria. In addition, these muscles may display a high O_2-consumption *in vitro*. These features indicate that such muscles, which tend to be mechanically active over long periods of time, probably operate aerobically (Ball and Meyerhoff 1940; Fänge and Matisson 1958; Alp *et al.* 1976; Zammit and Newsholme 1976a).

This assumption however is confused by the extraordinarily high activities of pyruvate reductase enzymes in some radula muscles (Ellington and Foreman 1981; Ellington 1982), and the presence of anaerobic metabolites (D-lactate and opines) in these muscles after

artificial stimulation or environmental anoxia *in vitro* (Wiseman and Ellington 1987; Eberlee and Storey 1988). Additionally, the radula muscles of limpets and littorinids display extremely high activities of the phosphagen-hydrolysing enzyme arginine kinase (Zammit and Newsholme 1976a; Livingstone *et al.* 1983).

The metabolic indices of *H. rubra* radula muscles suggest a predominantly aerobic poise. Although comparable data are available for some gastropod species, the effect of sustained muscle work on the metabolism of the muscle itself has never been investigated during natural feeding behaviour. This is especially true for the Haliotidae.

A number of studies on isolated gastropod radula muscles have attempted to replicate the natural metabolic function of these muscles. However, *in vitro* studies may provide the researcher with a false sense of muscle capacity and function. Theoretically, it should be possible to measure any anaerobic component of muscle work during actual feeding trials. Whilst numerous feeding preference trials have been conducted for *H. rubra*, no study to date appears to have investigated the metabolism of the radula muscles themselves. To examine the metabolism of *H. rubra* radula muscles *in vivo* during normal function, it was decided to subject them to natural continuous work in the form of feeding.

In order to conduct experiments examining the effects of feeding on abalone radula muscle metabolism, consideration must first be given to examining the natural feeding ecology and behaviour of *H. rubra*, both by observation and trial. This would highlight, for example, possible preferred diets, behaviour and appropriate experimental and sampling techniques. Therefore, before describing the feeding experiments involved in detail, the feeding ecology of *H. rubra* will be considered.

A further aspect of *H. rubra* and radula muscle metabolism to consider is the effects of hypoxia. The effects of hypoxia, which elicits facultative anaerobiosis in the pedal musculature of *H. rubra*, have not previously been documented for abalone radula muscles. Furthermore, the radula muscle metabolism during rest or natural feeding may differ from that during the altered energy demands required as a result of a reduction in available O_2. The use of haemolymph O_2 by the large exercising pedal muscles, or a reduction in environmental O_2, may severely affect the radula muscles with their predominantly aerobic poise. It was therefore of interest to also examine and compare the metabolic response of *H. rubra* radula muscles to whole animal hypoxia in the form of pedal muscle exercise and whole animal air emersion.

5.1.2 General feeding ecology of *Haliotis rubra*

Owing to an increased interest in the general ecology and commercial value over the last three decades, the feeding habits and diets of abalone have gained importance as a research subject. As briefly addressed in Chapter 2, it is generally agreed that Australasian abalone display a preference for rhodophytes (red algae), and reject most species of phaeophytes (brown algae), according to the possibilities present in the natural habitat (*H. iris* Poore 1972; *H. laevigata*, *H. roei* and *H. rubra* Shepherd 1973a, 1975). Numerous studies have investigated parameters influencing food selection and processing in *H. rubra*. Results suggest that chemosensory attractiveness and intake are strongly directed by nutritional quality (*e.g.* calorific and nitrogen content, Day and Fleming 1992; Fleming 1995a, 1995b), phenolic levels (Foale and Day 1992; Shepherd and Steinberg 1992), and also algal toughness (McShane *et al.* 1994).

Starvation in abalone often induces a tendency to feed on less preferred algae (Wells and Keesing 1989; Foale and Day 1992), and an overall decreased metabolic rate may result in a slower digestion of these algae (Fleming 1991, as cited in Foale and Day 1992).

Studies have demonstrated a preference for the red alga *Jeannerettia lobata* by *H. rubra* from Port Phillip Bay, Victoria, although the actual diet of *H. rubra* varies according to location and algal species availability. Victorian oceanic abalone populations are often associated with stands of Cray-weed (*Phyllospora comosa*) and, despite the phenolic levels of Australian phaeophytes (Steinberg 1989), abalone are known to ingest this and other brown algae of the genera *Ecklonia*, *Sargassum* and *Cystophora* (Fleming 1995a, 1995b; McShane 1999).

Abalone use a combination of tactile and chemosensory stimuli to detect suspended and attached seaweed in their environment (Fleming 1995a; Allen *et al.* 2001). The majority of algae ingested by abalone is in the form of drift algae, particularly after sunset. Although known to graze upon epilithic species, abalone display distinct drift feeding behaviour. Abalone are often orientated vertically on rock or wall faces in areas of water movement that guarantees the presence of drift algae. In this position abalone are observed to grasp and consume passing algae. They do so by extending their tentacles and anterior foot lobes, which detect and retrieve passing algae with suprising dexterity (Shepherd 1973a; Allen *et al.* 2001; pers. obs)

Adult *H. rubra* are also known to graze opportunistically on diatom and micro-algal growth. Home scars and patches of substratum cleared of epibiota have been reported under resting animals (Shepherd 1973a, 1975; pers. obs).

5.1.3 Muscle and radula function during feeding in *Haliotis*

The effects of feeding are better interpreted if the mechanics and muscle function during feeding are understood. While briefly summarised in Chapters 1 and 2, the action of the radula muscles and method of feeding in abalone is described in more detail as follows:

The sensitive cephalic tentacles and papillae on the lips detect and identify the substratum or algae, while the foot and lips provide fixation for the working of the buccal mass. As feeding commences, the lips spread flat against the algal surface, the mouth opens and the buccal apparatus is protracted by the contraction of the odontophore and radula protractor muscles. The radula is thrust out simultaneously owing to its association with the underlying membrane. The radular membrane moves down and back over the anterior end of the odontophore. Upon passing over the protruding tip (the bending plane) the posteriorly directed and flat radular teeth are erected vertically and anteriorly. The tip of the odontophore and radula are pressed against the substratum and the radular membrane is retracted, with a slight depression of the odontophore. As a result, erected teeth now collapse forward, scraping and brushing material into the midline and through the mouth into buccal cavity (Chapter 2, Plate 2.8). Food particles are then moved back through the buccal cavity and into the oesophagus.

The effective feeding stroke of abalone radulae is on the return pull of the apparatus under the influence of the retractor muscles, in particular the median unpaired retractor (Crofts 1929; Fretter and Graham 1962; Owen 1966; Kohn 1983). Whilst the protrusion of the gastropod radula and odontophore is mediated neurally and by muscular contraction (Drushel *et al.* 1998), Russell and Evans (1989) suggest that the protrusion of the radula and odontophore complex in *H. rubra* may be augmented by an increase in blood pressure in the CAS.

Abalone possess a rhipidoglossate radula (Gr. *rhipidos*: fan) (Chapter 2, Plate 2.9), which are considered the most primitive and are characteristic of lower Archaeogastropoda. The polydont rhipidoglossan radulae bear a large central rachidian tooth and large numbers of teeth in each transverse row. Gastropods possessing a rhipidoglossan radula are thought to be limited in the range of food resources available to them. Steneck and Watling (1982) describe rhipidoglossan radulae as a broom, incapable of grazing on tough substrata due to the lack of hardened teeth, and are constrained to consume macroalgae with soft rather than leathery thalli (McShane *et al.* 1994). Additionally, the radula muscles of rhipidoglossan grazers are thought not to be very robust. As opposed to smaller grazers however, the large size of abalone and their more robust radulas may allow these gastropods to remove tough algal cortical cells (Steneck and Watling 1982).

It is possible that feeding upon large pieces of potentially tough algae may require a slightly different orientation of the radula and odontophore against the algal surface as opposed to graxing upon the substratum. Moreover, the mechanics of radula movement and muscle contraction may differ between browsing and grazing, with macro- and microalgae requiring different amounts of muscular effort. It was of interest therefore to examine possible differences in radula muscle metabolism between modes of feeding: browsing on macroalgae and grazing on microalgae.

5.1.3 Effects of hypoxia on *Haliotis rubra* radula muscles

Whereas the effects of hypoxia on abalone pedal musculature is well documented, and does not appear to differ among species, little work has examined the metabolism of other muscles or visceral tissues in these gastropods. This is surprising, considering the data available on the regulation of enzymes and accumulation of metabolites in various tissues of other gastropods and bivalves during anoxia (Eberlee and Storey 1988; Michaelidis and Storey 1990; Carvajal *et al.* 1990; Meinhardus-Hager and Gäde 1992). Differences in the activation of glycolytic enzymes during anoxia, and the depletion of fuels and accumulation of anaerobic end products following anoxia, have been observed for radula and foot muscles of the whelk *Busycon canaliculatum* (Plaxton and Storey 1985; Storey 1988; Storey *et al.* 1990).

The activity and distribution of pyruvate reductase and other glycolytic enzymes has been mapped for abalone (Chapter 3), but often not the role of these enzymes in aerobic or anaerobic energy provision. This chapter aims to address this shortcoming somewhat, with the comparison of effects of whole animal hypoxia on the radula muscles of *H. rubra*. Radula muscle, on the basis of its predominantly aerobic poise, small contribution to animal weight and anatomical orientation, may not respond to a decrease in O_2 in the same manner, or to the same extent, as pedal muscle. The exposure of the radula muscles to whole animal exercise and air emersion serves as an additional comparison not only to the specific effects of radula muscle work, but to the effects of hypoxia on the pedal musculature of *H. rubra*.

As outlined in the Chapter 1, the radula muscles of abalone perform an essential role for the growth, health, survival and, ultimately, natural and commercial value of *H. rubra*. These important aspects rely upon the function and efficacy of the radula and buccal muscles. Information collated on abalone radula muscles so far suggests these muscles most probably

function aerobically, although it is remarkable that the physiology or metabolism of these important muscles has never been investigated.

Do abalone utilise anaerobic metabolism to support sustained radula muscle work? Given that abalone are known to feed continuously for relatively long periods, any involvement of anaerobic metabolism should be evident by following the accumulation of pyruvate reductase end products and depletion of arginine phosphate during feeding. Whilst obvious differences exist in behaviour and acquisition of food between grazing and drift feeding or macro-algal 'browsing' abalone, it is not known whether radula muscle metabolism differs between these feeding strategies.

The metabolic indices of *H. rubra* radula muscles suggest they have a predominantly aerobic poise. It is the aim of this chapter to test these indices and to investigate the effects of, primarily, sustained muscle work during natural feeding behaviour and effects of whole animal hypoxia on radula muscle metabolism in *H. rubra*.

5.2 MATERIALS AND METHODS

5.2.1 *Haliotis rubra* feeding trials

The determination of metabolic responses to natural feeding in *H. rubra* required an understanding of the feeding ecology and behaviour of the animal. In order to replicate natural behaviour and conditions in the laboratory, consideration was given to the following factors: how *H. rubra* feeds; the probable and actual diet of *H. rubra*; and what constitutes natural feeding of *H. rubra*. This was primarily achieved by observations in the wild, as described in Chapter 2, and observations and trials in the laboratory. These observations and trials on algal preference and feeding behaviour in the laboratory are discussed below.

Algal preferences of *Haliotis rubra*

Prior to the commencement of experiments, *H. rubra* were presented with a range of algal species to determine the most appropriate for feeding experiments. Although brief hand-feeding trials in the field and laboratory were performed and described in Chapter 2, it was necessary to present abalone used for the specific feeding experiments with a range of algae likely to be encountered in their natural environment, in order to determine the most preferred species. Animals were collected and maintained as previously described in Chapter 2. The following algae were collected as a representative range of the flora from the Mornington study site: *Ulva australis* (Chlorophyta), and *Cystophora moniliformis*, *Ecklonia radiata* and *Sargassum* sp. (Phaeophyta). *Jeannerettia lobata* (Rhodophyta) was collected from Flinders Pier, a separate semi-oceanic site (38°28.5'S, 145°1.8'E: Figure 2.1, Chapter 2).

The tendency of *H. rubra* to feed on drift algae was exploited by offering animals by hand loose pieces of algae upon which to feed. Individual blades of algae were presented to abalone on either side of the cephalic region, and gently swept across the cephalic and epipodial tentacles. This method has been used in previous feeding experiments of *H. rubra* (Fleming 1995a). Small blades of each algal species were individually presented in this manner to a total of 12 captive abalone, and each animal's response was recorded. Feeding preference trials were carried out over two days, in the evening when abalone begin natural foraging behaviours. A 10-15 min 'rest' period was allowed following the presentation of each separate species of algae.

Based on this short preliminary trial, abalone showed preference for the green algae *Ulva australis*, and this was subsequently used during feeding observations and trials.

General feeding behaviour (observations and trials)

Prior to commencing experiments to examine effects of feeding on radula muscle metabolism, it was necessary to determine not only the algal preference but also abalone feeding behaviour. From these observations, an appropriate standardised experimental method for feeding and sampling animals could be developed. These observations and feeding behaviours are detailed below.

Animals were collected and maintained as described previously, along with quantities of *Ulva australis*. During the period of feeding trials, fresh *U. australis* was collected regularly. The lighting system of the aquarium room was adjusted to coincide with the seasons (light:dark ranging from 10:14 to 14:10, sunset at approx. 1900 h, sunrise at approx 0700 h). Animals were observed under red light over much of the dark period, for both preliminary studies and trials. Previous observations suggested red light did not affect abalone behaviour, whereas white light did. Observations and trials were carried out from 1900 to 0200 h, or 0500 to 0800 h, which covered both ends of the activity and feeding period. Preliminary observations were made over a period of 2 weeks prior to experiments commencing, and these observations were considered in order to determine appropriate experimental design.

Generally, animals did not become active until at least 1.5 to 2 hours after sunset, although occasionally activity increased soon after sunset. For the purpose of this study, animals feeding on macro algae in the form of drift are referred to as "browsing", to differentiate from animals "grazing" on micro-algal growth on the surfaces of the experimental aquaria. Typically, browsing animals assume a position along a vertical face (in this instance the aquaria walls), with the anterior of the shell, mantle and foot lobes raised. In this state, animals appeared active and were responsive to the presence of hand delivered 'drift algae'.

Feeding began when the animal responded to contact by, surprisingly rapidly, extending cephalic tentacles and anterior lobes of the foot. This, in conjunction with the raising of the anterior of the animal, served to trap and hold the algae directly under the working mouth. These observations concur with those of Shepherd (1973a), and Allen *et al.* (2001) for *H. iris*. The algae could be seen moving and periodically disappearing under the animal, and the head would be raised and moving rhythmically as the animals browsed.

The response of *H. rubra* to drift algae varied considerably over the period of observation. Occasionally animals would grab and pin the blade of algae down, but refuse to feed. Of these animals, a number initiated feeding only after some time, and still others

would eventually release the algae without feeding. As a test, the protruding blade would be gently tugged. Animals either freely released the algae or maintained a firm hold. A number even performed the capture behaviour again to pull back the algae.

Browsing abalone were also observed to accept a second piece of algae whilst feeding on the first. Feeding was temporarily halted in order to trap the new algae, and usually resumed after a period of quiescence, which may indicate a repositioning of the algae under the mouth.

Not all animals reacted favourably to drift algae. Some would retract sensory structures, and even exhibit withdrawal. Still other animals, after what appeared a brief examination by tentacles and sometimes lips, the animal would reject and move away from the algae.

These responses to drift algae were exhibited by individuals that had been previously fed, or starved.

Whilst browsing animals were presented with drift algae, grazing animals were allowed to graze freely on the epilithic microalgae. This required the culture of a microalgal film on the aquarium surfaces. Prior to the collection and treatment of abalone, the main aquarium tank was not disturbed for a period of 4-5 weeks, then kept under constant light conditions for approx. 3 weeks to enhance algal growth. This resulted in the increased stimulation of microalgae on the floor and walls of the aquarium. The detritus from other organisms in the system and degrading phytoplankton probably contributed to the biota, especially on the aquarium floor. The dark film of microalgae was approx. 1.0 mm thick. No quantitative measurement of the composition of this film was made.

Typically, animals that grazed displayed rhythmical head and buccal mass movements. Cephalic tentacles and eyestalks would also move as a result of the protrusion of the odontophore and radula and the depression of the mouth upon the substratum. Animals often fed in a lateral zigzag motion, sweeping their head and anterior foot lobes across the aquaria walls or floor, which resulted in characteristic radula scrape marks or 'feeding tracks'. Abalone were quiescent and did not appear to graze during daylight observations.

Feeding experiments for detecting metabolite changes

Having observed and become familiar with feeding behaviours, abalone were then subjected to either control (no feeding) or feeding treatments. Feeding animals were further separated into browsing (animals to be hand fed macroalgae) and grazing (animals allowed to graze the microalgae in the aquarium) groups. Control animals were placed in a lidded tank

(approx. 800 × 500 × 150 mm), previously scrubbed clean with freshwater to remove all microalgae, and supplied with flowing seawater and aeration. This isolation tank was sitting in the main aquarium, and as such was subject to the same water temperature and conditions. Animals were placed in this tank and sampled 24 hrs later. This time period was chosen as it restricted animals during the normal feeding time (dark) and allowed at least 24 h clearance of any possible residual metabolites in the haemolymph or radula muscles from previous muscle activity.

In order to control for feeding duration, a feeding period of 15 min was selected. This figure primarily was based on the observations of behaviour made prior to these feeding trials. Given the variability of feeding responses in some individuals, 15 min represented the upper end of a reproducible time period common to all animals used for feeding trials.

Individual browsing animals were quickly and gently removed from a lidded tank (see above) where they had been starved for up to 3 days, and allowed to acclimate for up to 1h to main aquarium conditions. Animals were observed to ensure they did not commence grazing feeding behaviour. Animals were then presented with the alga *Ulva australis* and observed carefully for the duration of feeding (15 min), which was judged to have begun upon first contact with the algae. If the animal stopped feeding before 15 min, the trial was aborted. Likewise, if the animal refused to feed, it was left undisturbed for at least 30 min. If the animal received the algae, but did not feed, the trial was aborted also. This occurred frequently, when the animal would simply hold the algae under itself, and not feed (pre and post weight of algae identical). The dry blotted weight of algae offered to the browsing animals was recorded, as was any remaining at the end of the feeding period. A similar size and weight blade of algae was allowed to sit in the water for 15 min, to control for loss of weight due to non-abalone effects. No change in control algae weight was observed over any 15 min feeding period.

While browsing abalone were presented with macroalgae, grazing animals were allowed to feed on the microalgae cultures on the aquarium surfaces (see above). Grazing animals were handled in a similar matter to browsing animals, except observation periods often lasted far longer than the 15 min feeding period. Abalone were removed from the isolation tank and allowed to acclimate to main tank conditions. Animals were observed carefully as they negotiated the main tank and fed. Feeding was deemed to have commenced when the rhythmic movement of the head and cephalic tentacles were first noticed. Feeding was evidenced by the regular feeding tracks on the microalgae on the aquarium surfaces.

In addition to the obvious cessation of feeding, excessive movement (locomotion) terminated grazing feeding experiments.

5.2.2 Whole animal exercise and air emersion in *Haliotis rubra*

Haliotis rubra were subjected to whole animal exercise by righting after inversion and 12 h air emersion as described previously in Chapter 4 (Section 4.2.1).

5.2.3 Muscle and haemolymph sampling

Whilst familiarity with the anatomy of *H. rubra* had been gained during experiments outlined in earlier chapters on morphology and metabolic profiles, the efficient accessing and treatment of haemolymph and radula muscles had to be further developed. This was done by dissection, sampling and processing trials, which are addressed in the Discussion (Section 5.4.1). The methods eventually used for removing and processing haemolymph and radula muscle samples is described below.

Feeding

After 15 min of feeding, or rest for controls, animals were quickly removed from the aquarium, blotted and weighed. For browsing animals, any algae remaining was weighed to determine weight ingested during feeding. Haemolymph samples (0.9 ml) were immediately taken from the cephalic arterial sinus (CAS) with a 1 mL syringe and fine gauge needle and injected into eppendorf tubes on ice containing 0.1 mL of 6 mol L^{-1} Perchloric acid (PCA). The CAS is best accessed from the ventral surface of the animal, and is located in the midline (identified by a cleavage) at the juncture of the head ('throat') and foot, posterior to the mouth. Penetration is relatively shallow, as oesophageal, neural and muscular tissue lie deeper. CAS samples were taken in conjunction with radula muscles samples as it was considered advantageous to withdraw haemolymph for metabolite analysis in close proximity to the muscle.

The buccal mass (anterior oesophagus, odontophore cartilages, radula, all radular and buccal muscles, jaws, neural tissue, and buccal and salivary glands) was rapidly dissected out on ice. From this, the radular and buccal musculature was excised as quickly as possible and transferred immediately to 6 mL of ice-cold 0.6 mol L^{-1} PCA to arrest metabolism.

The whole animal handling procedure took about 5-7 mins, and was performed as quickly as possible. Every attempt was made to keep manipulation to a minimum, and to maintain a favourable temperature.

Whole animal exercise and air emersion in *Haliotis rubra*

Radula muscles and haemolymph were sampled from 6 abalone for each of exercise and air emersion treatments. Control or resting animals for feeding experiments also served as controls for hypoxia experiments. Following treatment, CAS haemolymph and radula muscle samples were taken as described above for feeding treatment animals.

5.2.4 Tissue extracts and metabolite assays

Following dissection, preparation of muscle and haemolymph PCA extracts were carried out as described previously (Chapter 4, Section 4.2.4). As a result of the dissection technique employed for sampling radula muscle, tissue processing was carried out immediately, as opposed to the storage in liquid N_2 or at -80°C as for adductor and foot samples.

Muscle and haemolymph metabolite assays for D-lactate and tauropine from all animals, and muscle arginine phosphate assays of radula muscle extracts from feeding treatment animals, were as previously described (Chapters 4 and 3, respectively). In addition the level of blood glucose was determined, as described in Chapter 4 (Section 4.2.5), to assess whether feeding, like hypoxia, elevated blood glucose titres in *H. rubra*. As with the pedal musculature of *H. rubra*, radula muscles samples were also assayed for L-lactate.

5.2.5 Data analyses

Statistical analyses were undertaken using both MS Excel and Systat statistical packages. Following checks for normality, analyses of variance (ANOVA) were performed to examine for significance in metabolite levels between control versus feeding treatments, control versus hypoxia treatments, and between treatments. Regression analyses were performed to determine any relationship between amount of algae ingested and levels of metabolites for browsing abalone. Significant differences were accepted at $P<0.05$.

5.3 RESULTS

5.3.1 Algal preferences of *Haliotis rubra*

The results of the feeding preference trial are given in Table 5.1. When presented with algae, *H. rubra* often responded and appeared to "taste-test" the algae, but either retracted from or discarded the sample. Of those animals that did not retract or refuse the sample, four accepted the red alga *J. lobata*, but none were observed to feed. When presented with *Ulva australis*, 8 animals accepted and 6 fed. Two animals responded to and accepted *Ecklonia radiata*, but only one fed. No positive responses were initiated by *Cystophora moniliformis* or *Sargassum* sp. Animals had also been induced to feed in the field by hand-feeding *U. australis* (Chapter 2).

In this study, *H. rubra* displayed a preference for *U. australis*, which was subsequently used as the food source during feeding experiments.

5.3.2 Feeding behaviour of *Haliotis rubra*

Drift browsing abalone were relatively still during feeding, with the exception of the movements of the anterior foot lobes and head, and occasional re-positioning on the aquaria wall. Grazing abalone however often moved or swivelled slowly back and forth across the floor or wall whilst feeding. It was not possible to consider feeding separate from these small movements, and it is probably more appropriate to consider grazing to include a small whole animal movement component.

Actual feeding by grazing abalone was more difficult to determine than browsing. Feeding movements are more subtle, and difficult to ascertain if the animal is not positioned favourably to the observer. Often, quiescence was mistaken for feeding, and vice-versa, and it was not until the animal moved that radula scrape marks (or lack of) were revealed. Moreover, grazing animals often fed for short periods (1 or 2 minutes) before stopping, a behaviour displayed repeatedly.

Browsing abalone consumed, on average, 0.10 ± 0.03 g algae during the 15 minute feeding period. The amount of algae consumed during the 15 minute period varied considerably. No significant relationship was observed between size of animal (weight) and weight of algae ingested ($r^2 = 0.394$, $P>0.05$). Upon dissection of a number of browsing animals, fragments of ingested *Ulva australis* were found in the buccal mass and on the radula itself, and occasionally in the oesophagus.

5.3.3 Effects of feeding on radula muscle and haemolymph metabolites

Control animals showed low concentrations of D-lactate and tauropine (0.29 and 0.13 μmol g^{-1} wet wt muscle, respectively) in the radula muscles, and these metabolites were barely detected in the haemolymph (Table 5.2). There was no significant accumulation of either D-lactate or tauropine in the radula muscles or in the haemolymph following browsing or grazing (ANOVA, $P>0.05$). In both resting and feeding abalone, significantly more D-lactate was found in the radula muscle than in the haemolymph (ANOVA, $P<0.05$). However, there were no significant differences in tauropine levels between muscle and blood (Table 5.2). Muscle arginine phosphate reserves were not significantly different from control values following feeding. No significant differences in D-lactate, tauropine or arginine phosphate concentrations were observed between muscles from browsing and grazing abalone (ANOVA, $P>0.05$).

No significant relationship was observed between the weight of algae consumed and levels of D-lactate or tauropine accumulated in radula muscle following browsing ($r^2 = 0.003$ and 0.019 respectively, $P>0.05$). Likewise, the amount of arginine phosphate was not related to the weight of algae ingested during browsing ($r^2 = 0.09$, $P>0.05$).

Control (resting) blood glucose for *H. rubra* was 0.11 ± 0.02 μmol ml^{-1} (Table 5.2). Blood glucose did not increase significantly following browsing (0.14 ± 0.03 μmol ml^{-1}) or grazing (0.12 ± 0.04 μmol ml^{-1}).

5.3.4 Effects of whole animal exercise and air emersion on radula muscle and haemolymph metabolites

The levels of D-lactate and tauropine in the radula muscles and haemolymph of resting animals, and those exposed to hypoxic conditions, are given in Table 5.3. The repeated righting of an abalone after inversion did not result in significant increases in concentration of either D-lactate or tauropine in the radula muscles, nor of tauropine in haemolymph sampled from the CAS (Table 5.3). In contrast, D-lactate concentrations were significantly higher in the CAS after exercise in *H. rubra*.

As seen following exercise, concentrations of D-lactate and tauropine are not significantly elevated in the radula muscles following 12 h air emersion (ANOVA, $P>0.05$). Likewise, there was no significant increase in haemolymph tauropine following air emersion. Air emersion however resulted in a large and significant increase in haemolymph D-lactate from 0.01 to 0.84 μmol mL^{-1} (Table 5.3).

Haemolymph:radula muscle ratios were not calculated as for pedal musculature (Chapter 4), based on the observations that radula muscle does not significantly accumulate anaerobic metabolites during hypoxia, and that most of the haemolymph metabolites are therefore thought to have arisen from other tissues, presumably the foot and adductor muscles.

L-lactate was not detected in the radula muscles of control or post-feeding animals, or following whole animal exercise and air emersion.

5.4 DISCUSSION

The aim of this chapter was to examine *H. rubra* radula muscle metabolism during feeding, and whole animal exercise and air emersion. The feeding and hypoxic treatments, and their effects on muscle metabolism, are addressed in this Discussion, beginning with a consideration of some of the methodological aspects. The algal preferences and feeding ecology of *H. rubra* are considered next. The effects of feeding on radula muscle metabolism of *H. rubra* and possible role of the haemolymph during feeding are then considered. The effects of whole animal hypoxia on radula muscle metabolism are then examined. The data are approached in this manner to include discussions of other gastropods and their physiological responses.

5.4.1 Methodology

The procedure for dissecting radula muscles and processing these samples described in the Methods (Section 5.2.3) was determined to be most appropriate following numerous trials.

Concentrations of D-lactate and tauropine for muscles handled as above were considerably lower and more stable than those from initial trials where radula muscles were simply dissected, minced and then placed in 0.6 mol L^{-1} PCA (D-lactate concentration up to 3.5 μmol g^{-1} wet wt). It was assumed that the high and variable metabolite levels from these initial trials were due to the prolonged handling of the radula muscles during dissection.

Alternatively, the snap freezing of the radula muscles and associated structures was examined as a possible procedure. However, further handling of muscles was required upon partial or full thaw to successfully separate them from other tissues. Usually, muscle yield was considerably lower following freeze/thaw.

The immediate arrest of muscle function by immersion in 0.6 mol L^{-1} PCA was also investigated. The denaturation of most abalone tissue by PCA is obvious by a colour change to a dull grey-white or cream, which makes distinguishing radula muscle from membranes, salivary glands, neural structures and buccal walls extremely difficult.

5.4.2 Algal preference and feeding behaviour in *Haliotis rubra*

Feeding trials in the laboratory indicate *H. rubra* readily accepts algae by hand, and *H. rubra* collected from Mornington showed a distinct preference for the green alga *Ulva australis*. This preliminary work on algal preference in *H. rubra* correlates well with observations made in the wild (Chapter 2) and previous studies. In studies investigating food selection and processing by *H. rubra*, the abalone displays a strong preference for the red

algae *Jeannerettia lobata*, followed closely by the chlorophyte *Ulva* sp. (Foale and Day 1992, Fleming 1995a, b, McShane *et al.* 1994). *Ulva lactuca* is employed as a diet for the commercially reared abalone *H. lamellosa*, *H. discus hannai*, *H. iris* and *H. roei* (Shpigel *et al.* 1999; Boarder and Shpigel 2001; Lamare and Wing 2001).

The pattern of diet preference exhibited by *H. rubra* in this instance probably reflects the representative flora and natural diet from the study site. Red algae represent only a small fraction of macroalgae from the study site, and *J. lobata* was not identified from the site (pers. obs). *Jeannerettia lobata* collected for trials was obtained from a semi-oceanic site (Flinders Pier). It is possible that *J. lobata* is alien to *H. rubra*, although some may appear in the area as drift. *Jeannerettia lobata* was used in this study in order to compare results to previous studies on *H. rubra*, despite the possible unfamiliarity of the algae to animals from Mornington. The failure of *H. rubra* in the initial trials to respond to or feed on the phaeophytes *Cystophora moniliformis* and *Sargassum* sp. is not unusual (Shepherd and Steinberg 1992). Phaeophytes are often reported as least favoured by temperate Australian abalone, possibly due to toughness and phenol content (Shepherd and Steinberg 1992). The only individual observed to ingest the brown alga *Ecklonia radiata* was collected from Tortoise Head, a semi-exposed site exhibiting a generally greater macro-algal diversity than the main study site (Chapter 2, Figure 2.1; pers. obs).

Interestingly, it appeared that the smaller animals displayed a preference towards grazing. Smaller abalone often refused 'drift' algae, whereas larger individuals accepted it more readily. The difference may reflect the distribution of *H. rubra* in the wild. Shepherd (1973a, 1975) noted that juvenile and smaller animals were more frequently observed in crevices and caves, which offer better protection against predators. In these environments, animals may not encounter drift algae.

The selection criteria and observations of feeding animals make it unlikely that non-feeding individuals were sampled. Nevertheless, it is possible that periods of non-feeding (particularly with grazing animals), or periods of feeding mistaken for non-feeding, may have been overlooked. In future, audio recordings of radula scraping during animal feeding may serve as a better tool (Kitting 1979), or perhaps inducing animals to graze upon waxed surfaces (Forrest *et al.* 2001), which would record feeding marks.

5.4.3 Effects of feeding on radula muscle metabolism in *Haliotis rubra*

Feeding failed to invoke a significant activation of anaerobic glycolysis in the radula muscles of *H. rubra*. The concentrations of the anaerobic end products D-lactate and

tauropine in both muscle and haemolymph following feeding are identical to those of non-fed individuals. It appears that anaerobic glycolysis does not contribute significantly to radula muscle energy provision for feeding in *H. rubra*. Blood glucose levels were not higher in fed individuals compared to resting abalone.

The muscular contractions associated with feeding were not powered either by anaerobic glycolysis or the hydrolysis of arginine phosphate. Concentrations of arginine phosphate were similar between control and fed individuals. This suggests the frequency and/or force of the contractions are sufficiently supported by aerobic metabolism. This conclusion correlates well with the aerobic poise of the radula muscles, as suggested by the metabolic indices of high myoglobin (Mb) concentration, low pH buffering capacity, low pyruvate reductase enzyme activities and high activities of the aerobic enzymes HK, CS and HOAD. Carbon from glucose and glycogen appears to be channelled through the normal glycolytic, Krebs cycle and mitochondrial pathways of aerobic metabolism, instead of accumulating as the anaerobic end products D-lactate or tauropine. A high pH buffering capacity therefore is not essential.

This aerobic metabolism is mediated by the supply of O_2 via the cephalic sinuses, the delivery of which to the mitochondria is probably ensured by the high concentration of Mb. The position of the radula muscles in the CAS ensures a regular supply of haemolymph, and with it, O_2 and fuels. The difficulty in deoxygenating *H. rubra* radula muscle Mb (Chapter 3, Section 3.4.1) suggests that *H. rubra* Mb has a high affinity for O_2, similar to that of the abalone *Sulculus diversicolor* (Chapter 3). No estimate is available on the density of mitochondria in *H. rubra* radula muscles, although recall from Chapter 3 that gastropod radula muscles appear to contain a large abundance of mitochondria. High activities of mitochondrial enzymes suggest mitochondria are important in the radula muscles.

No increase in haemolymph glucose was seen after 15 min of feeding in *H. rubra*. Whilst the endogenous glucose and glycogen in these muscles may be sufficient to maintain muscle work, the release of glycogen from other sites (*e.g.* pedal muscle) may further supply the radula muscles with blood-borne fuel during feeding. An increase in glucose levels in the adductor and foot muscles of *H. discus* at night was reported by Watanabe *et al.* (1994a). This increase is assumed to be correlated with the increase in activity displayed by abalone at night, associated with feeding and foraging behaviour.

Feeding experiments on the sea hare *Aplysia dactylomela* (Carefoot 1991, 1994) revealed that handling and sampling of haemolymph did not significantly affect haemolymph glucose titres and that haemolymph glucose titres remained constant up to 6 days of

starvation. Haemolymph glucose titres in *H. kamtschatkana* were very similar to resting concentrations after 2-3 days starvation (Carefoot *et al.* 1993). The haemolymph glucose concentrations for *H. rubra* are therefore not likely to reflect any effects of isolation and starvation prior to experiments commencing, or handling.

Qualitative comparisons may be made between adductor and radula muscle contractions. Contractions of the adductor muscle were fuelled by anaerobic glycolysis, while those of the radula muscles were not. *Haliotis rubra* exercised for, on average, 40 min during which time 9 successful righting manoeuvres and up to twice as many unsuccessful manoeuvres were performed (Chapter 4). Feeding involved an unquantified number of contractions. These contractions however were most probably consistent for the feeding period (15 min) and, given the observations on animal head and tentacle movement, occurred every 1-5 seconds (pers. obs). Using 5 seconds as a guide, this would equate to at least 180 complete protraction and retraction cycles of the radula and odontophore during 15 min of feeding.

Given that no differences were observed in metabolite accumulation or depletion between radula muscles of grazing and feeding animals, the metabolism of radula muscle work is considered inherently the same between the two feeding methods. Identical metabolite profiles following the two feeding treatments suggest any differences in feeding method are primarily behavioural. In both grazing and browsing the radula would be brought to work against a surface. Drift algae may not constitute a tougher diet than micro-algal growth. Feeding preferences, rapid assimilation rates and digestibility of *Ulva sp.* (Foale and Day 1992) suggest abalone have little trouble feeding upon this soft chlorophyte. The manipulation of macro-algae by cephalic tentacles, lips and anterior foot lobes during drift algae feeding is not considered to contribute significantly to metabolite levels in the radula muscles or haemolymph in browsing animals, although the movements are surprisingly rapid and dexterous. However, feeding on the more leathery thalli of larger brown algae may require a more concentrated feeding effort, and this might be reflected in the metabolite accumulation of the muscles. Toughness of algae is a primary factor in the feeding preferences of *H. rubra* and other abalone (McShane *et al.* 1994).

With the exception of the careful handling and removal from the isolation tank, little whole body movement was observed during feeding by most browsing animals. Having orientated themselves vertically on fissures or rock faces, usually in areas of water movement

sufficient to deliver quantities of drift, abalone prefer to remain still and wait for algae (Shepherd 1973a, 1975). It is unlikely therefore that the pool of metabolites, particularly in the haemolymph, is compromised by input from other muscle work. The handling of the animals themselves from the isolation tank does not appear to have influenced muscle or haemolymph metabolite levels. The levels of D-lactate in control radula muscles from these feeding experiments are not significantly different to feeding levels.

Unlike browsing on drift algae, grazing on microalgae involves a tacit movement of at least the head of the animal. Grazing abalone usually moved forward slowly during feeding, and occasionally rotated up to 180° from their original position during the feeding bout. With work on *H. kamtschatkana*, Donovan *et al.* (1999) established a considerable anaerobic component to locomotion (see Chapter 4). Whilst anaerobically-fuelled locomotion may bias haemolymph metabolite levels, it is hard to envisage how radula muscle levels could be influenced.

During the feeding observation periods, *H. rubra* was observed to move regularly, but with the exception of the movements described above, rarely during actual feeding. The selection procedure for treatment animals prevented any influence of excessive movement. Although *H. rubra* have been observed to crawl relatively quickly, attempts made in the field and aquaria to induce locomotion almost always resulted in the clamping and withdrawal of the animal, with escape occasionally following this response in the wild (see Chapter 2).

It was originally considered of interest to examine the effects of foraging behaviour on muscle metabolism in *H. rubra*. Unfortunately, it was difficult to both control and quantify natural locomotory behaviour in *H. rubra* at night. Despite this, a number of observations were made on foraging *H. rubra*. One individual was recorded at crawling speeds of 11 cm in 36 seconds (1.66 body lengths min^{-1}). A second animal exhibited speeds ranging from 0.75 body lengths min^{-1} to approximately 1.33 body lengths min^{-1}, intermittently over an hour period. This same animal was estimated to have covered over 5 m during the first part of an observation period (approx. 3 h). These animals were exhibiting natural foraging and homing behaviour. Large distances covered by abalone during nocturnal activities, and homing behaviours, are not uncommon in captivity (Nakamura and Vazquez Archdale 2001).

5.4.4 Effects of feeding on radula muscle metabolism in other gastropods

Few studies have been made of the physiology or biochemistry of radula muscle work in *Haliotis*, and none on feeding specifically. However, work on whelk radula muscle preparations contracting *in vitro* suggests these muscles differ markedly from *H. rubra* in having a high capacity for anaerobic metabolism. Ellington and Foreman (1981) and

Ellington (1982) found high activities of OpDHs (up to 500 i.u. g^{-1} wet wt) in the radula retractor of the whelk *Busycon contrarium*. Following potassium-induced contractures, alanopine-strombine, alanine and succinate accumulated significantly, while aspartate was depleted. In contrast, no significant changes in D-lactate or arginine phosphate levels were observed, leading Ellington (1982) to conclude that D-lactate constitutes a minor end product of contraction of *B. contrarium* radula retractor muscle (Table 5.4).

Further work on *Busycon contrarium* however by Wiseman and Ellington (1987) suggested otherwise. Isolated radula protractor muscles were subjected to electrically-induced isotonic contractions for up to 15 min. Arginine phosphate levels declined with muscular activity, and even though D-LDH activity was lowest of all pyruvate reductase enzymes, D-lactate was the dominant glycolytic end product accumulated (Wiseman and Ellington 1987). More modest levels of strombine, octopine and alanopine were recorded. Whereas none of the opines were released in to the incubation medium by the muscle preparations, a significant amount of D-lactate was found in the medium (Table 5.4). During the last 5 min of contraction, more D-lactate was released than accumulated in the muscle (Wiseman and Ellington 1987). A similar pattern was observed for the radula protractor muscles of the closely related whelk *Busycon canaliculatum* (Wiseman *et al.* 1989). For these isolated radula muscle preparations, up to 75% of the total amount of D-lactate may enter the incubation medium, at a rate that may exceed 3 $\mu mol\ min^{-1}\ g^{-1}$ (Wiseman and Ellington 1987; Wiseman *et al.* 1989).

Studies on the contractile ability of gastropod radula muscles generally fail to examine the function during natural behaviour. Extrapolation of these *in vitro* studies therefore should proceed with caution. The studies on whelk muscle primarily are concerned with examining under what conditions, and to what extent, the pyruvate reductase enzymes observed are utilised, rather than effects of feeding *per se*. Prior to and during electrical stimulation, protractor muscles of the whelks *Busycon contrarium* (Wiseman and Ellington 1987) and *B. canaliculatum* (Wiseman *et al.* 1989) were subjected to anoxia (exposed to nitrogen). Wiseman *et al.* (1989) went so far as the addition of 10 mmol L^{-1} sodium nitrite, in order to strip Mb-bound O_2. It is therefore difficult to discriminate between the effects of functional hypoxia (radula contraction) from environmental hypoxia or anoxia. The constant dark red colour of *B. canaliculatum* radula muscle, and difficulty in totally stripping radula retractor Mb of O_2 (Wiseman *et al.* 1989) suggest a high affinity of whelk Mb for O_2. *Haliotis rubra* working muscle was presumably supplied with oxygenated haemolymph during feeding and an anoxic state was not likely to have been conferred. The considerable difficulty in rendering *B. canaliculatum* radula muscle fully anoxic suggests, in association with general

radula muscle characteristics, that work during normal feeding events in whelks is likely to be supported aerobically.

Although the metabolic indices of *H. rubra* radula muscle correlate here with the observed responses to feeding, no definite relationship is readily observed between gastropod feeding mode and the activities of pyruvate reductase and glycolytic enzymes, or Mb concentrations, in gastropod radula muscles (Chapter 3). Whelks typically use the edge of the shell to either chip valve edges and/or wedge the partially open valves of bivalves, thereupon inserting the proboscis and tearing flesh away in strips (Magalhaes 1948; Paine 1962; Peterson 1982). Compared to the short snout and compact buccal region of abalone, the proboscis and thus the radula protractor and retractor muscles of whelks are relatively massive. The distal position of the working surfaces, and the importance of the retractors in the return power stroke of the working radula may account for the impressive enzyme activities in these animals. The rapid acquisition of food is of importance to predators and scavengers taking their food in competition with other animals. This considerable capacity for anaerobic metabolism may also support feeding bouts by these gastropods at low tides. Whelks in the northern Hemisphere are known to forage and feed on exposed mud flats, a hypoxic environment that is also exploited with the aid of haemocyanin (Hc) O_2-binding characteristics (Chapter 6).

This raises the question as to whether abalone have the capacity to feed under an hypoxic environment. The metabolic and physiological profiles of radula muscle suggest not, although Russel and Evans (1989) believe feeding when clamped down is possible, due to the possible protrusion of the odontophore apparatus by increased CAS blood pressure. Abalone are not known to feed while emersed. The author is not aware of studies examining this question, although live animals shipped commercially are often accompanied by moist algae (Fleming 2000; Freeman 2001; Gorfine 2001). This arrangement however is more likely to keep the animals and atmosphere moist and hydrated rather than act as a food source.

One can also question whether the 15 min feeding regime was sufficient to elicit and illustrate a normal metabolic response to work in the radula muscles. As mentioned previously (Chapter 3), the Mb content of gastropod radula muscle is relatively high, and molluscan Mbs typically have low P_{50} values. A 15 min period of constant contraction may not exhaust *H. rubra* radula muscle O_2 stores, elicit anaerobic metabolism or be of long enough duration to detect any changes in metabolism. Also, this time frame may not be long enough to disrupt or exhaust haemolymph O_2 and fuel supply. However, with the variable response of abalone, 15 min was at the upper end of a reproducible time period. This

behaviour may reflect the adjustment of feeding periods according to muscle aerobic capacity. That is, animals may stop feeding before the muscles become anaerobic.

Contractile activity of isolated whelk *Busycon contrarium* radula muscle *in vitro* was maintained for the first 5 min, after which the contractile force declined down to approximately 50% of initial (Wiseman and Ellington 1987). Glycolytic rates in *B. contrarium* are low in the early phases of contractile activity, and energy production is largely aerobic during this period (Wiseman and Ellington, 1987). The decrease in contractile force, which may be attributed to fatigue, is associated with a rise in pyruvate and pyruvate reductase end products and suggests a transition from aerobic to anaerobic processes. In the natural feeding situation however, it is probable that gastropod radula muscles do not suffer fatigue as readily, if at all.

5.4.5 Role of haemolymph during feeding

The extensive nature of the anterior aorta and the CAS has led to suggestions of their possible involvement in protraction of cephalic structures of abalone, like the tentacles (Bourne *et al.* 1990). Likewise, the close anatomical relationship between the feeding apparatus and the CAS also suggests that the circulatory system may serve a hydrostatic function. In examining *H. rubra*, Russell and Evans (1989) propose that "an increase in blood pressure in this region would be expected to exert a force that could assist radula protrusion". Such an arrangement would be evident in increased cardiac outputs or blood pressures during feeding in *H. rubra*. This relationship has been observed for the terrestrial slug *Deroceras reticulatum*. A significant increase in heart rate during feeding was found for this gastropod, a phenomenon which Duval (1983) ascribes to the need for an increase in pressure required to protrude the odontophore apparatus. Similarly, heart rate, blood pressure and blood flow in the cephalic artery of the sea hare *Aplysia californica* increased during a stimulated state prior to the commencement of feeding (Koch and Koester 1982).

Whilst *H. rubra* may supplement muscular contractions with increases in blood pressure to protract the odontophore during feeding, the increase in cephalic blood flow and pressure during feeding in gastropods may also, or perhaps singularly, mediate an increased delivery of O_2 and fuels to the respiring muscles. The association of the radula muscles and CAS presumably would benefit feeding in *H. rubra*. Russell and Evans (1989) realised that a large amount of time is invested in feeding in *H. rubra*, given the large amounts of low nutritive quality algae required. Consequently, the energy demand of the muscles involved in radula protrusion and retraction would be relatively high, and the bathing of the feeding

apparatus with the CAS would adequately facilitate a supply of both O_2 and blood-borne fuels to these muscles.

5.4.6 Effects of whole animal hypoxia on radula muscles

Radula muscle work is powered aerobically and, unlike the pedal musculature, *H. rubra* radula muscles in non-feeding animals do not appear to resort to anaerobic glycolysis during whole animal exercise or air emersion. The lack of significant concentrations of D-lactate or tauropine in the radula muscles of *H. rubra* is not surprising for whole animal exercise at least, as exercise, which utilises the adductor and posterior portion of the foot, should not necessarily involve feeding musculature.

Resting levels of D-lactate from *H. rubra* radula muscles are very similar to those from the radula retractor muscles of the whelk *Busycon contrarium* (0.26 μmol g^{-1}. Ellington 1982). *In vitro* incubation of *B. contrarium* radula retractor muscle for 3 h in anoxic seawater produced a small but significant increase in D-lactate (0.47 μmol g^{-1}). The concentrations of alanopine-strombine, alanine and succinate also increased significantly in this muscle (Ellington 1982). Whole animal anoxia and *in vitro* anoxic incubation of radula muscles of *B. canaliculatum* results in a decrease in radula muscle pH and phosphagen reserves, and no accumulation of opine end products (Eberlee and Storey 1988, Brooks and Storey 1989, Combs and Ellington 1995, Storey *et al.* 1990). The pattern of total end product accumulation and rate of clearance of these metabolites following whole animal anoxia (air emersion) and recovery was similar in the foot and radula muscles of *B. canaliculatum* (Eberlee and Storey 1988). *Haliotis rubra* foot and radula muscles, despite similar activities of D-lactate dehydrogenase, show different responses to hypoxia and patterns of end product accumulation.

While no increase in haemolymph anaerobic metabolites were observed during feeding in *H. rubra*, hypoxia resulted in large and significant increases in these metabolites. During the current experiments on effects of hypoxia on *H. rubra* muscles, a total of three haemolymph sampling sites were chosen to determine if there was any large difference in metabolite level due to sampling sites. There was no significant difference in mean metabolite concentrations among haemolymph sampled from these sites (pedal sinus and aorta Chapter 4, CAS this chapter). Behrens *et al.* (2002) were also able to measure haemolymph metabolite concentrations of *H. iris* from 3 sampling sites, although not simultaneously, with similar metabolite concentrations from all three sites.

Under conditions where the massive pedal musculature becomes hypoxic *i.e.* using facultative anaerobic glycolysis, it is clear that these same conditions are not resulting in hypoxic radula muscles. What are some of the possible reasons for this?

The kinetics of D-LDH, as examined in Chapter 3, suggest that the *in vivo* function of the foot and radula muscle enzymes should be similar. This does not however necessarily translate into a similar role *in vivo*. The low pyruvate reductase enzyme activities and higher activities of aerobic enzymes in the radula muscles (Chapter 3) probably ensure the muscles exploit aerobic circumstances. The metabolic rate of these muscles may not be high at rest, and hence anaerobic metabolism may not be required to supplement energy requirements.

Sustained aerobic metabolism of the radula muscles during periods of whole animal exercise in water and air emersion, just as during feeding, may also be mediated by a number of physiological and anatomical adaptations. That is, O_2 may still be available to the radula muscles. Considering the position of the odontophore complex in the CAS, a supply of haemolymph is guaranteed given cardiac function is maintained. The proposed 'shunt' *i.e.* diversion of haemolymph from the pedal circulation under high muscular pressure that may be realised during exercise or clamping, would reserve O_2 for more dependent tissues (see Chapters 2 and 6). An increase in haemolymph pressure, particularly at the CAS and CVS would ensue. Although the effects of hypoxia on the Hc and haemolymph O_2-delivery is as yet unknown (see Chapter 6), the presence of Mb in the radula muscles may also ensure a supply of O_2, especially if a higher Mb O_2-affinity favours O_2-offloading to these muscles. If this were so, then hypoxia affecting the adductor and pedal musculature may not elicit an anaerobic response in the radula muscles.

The above suggestions are leant credence by observations on *H. rubra* stressed by increased water temperatures. According to Drew *et al.* (2001), one obvious sign of stress in *H. rubra* was a swelling of the soft tissue around the back of the head, causing it to protrude. This may reflect a pooling of haemolymph in the cephalic sinuses. Air exposed *H. rubra* in this study were noted to expose the mantle, foot and epipodium, which appeared 'puffy'.

The same factors that predispose radula muscles to aerobic metabolism may also predispose these muscles to using haemolymph-borne D-lactate as a fuel to supplement metabolism or resynthesise pyruvate, and the levels of D-lactate seen in the radula muscles following hypoxic treatment may have been taken up from the haemolymph. Recall however that while foot and radula muscle D-LDH enzymes show almost identical H-type characteristics (Chapter 3), there is clear evidence that the reaction is able to proceed in the direction of lactate production. The potential of the radula muscles to take up or utilise D-lactate as a fuel may be more appropriately studied by introducing and following the

distribution of labelled D-lactate (*e.g.* ^{14}C lactate), or following levels of D-lactate during recovery (Chapter 4).

5.4.7 Overview of radula muscle metabolism during feeding and hypoxia in *Haliotis rubra*

This current study is believed to be the first to describe the metabolic poise of abalone radula muscles, and the response of these muscles to natural feeding, and whole animal exercise and air emersion. Natural feeding behaviour in *H. rubra* does not appear to involve anaerobic glycolysis, and arginine phosphate does not appear to be hydrolysed to power feeding movements in these muscles. Carbohydrates are metabolised aerobically, with no anaerobic end product accumulation. The radula muscles similarly do not show a significant anaerobic response to whole animal exercise or air emersion.

The position of the radula-odontophore apparatus in an arterial haemolymph sinus, higher activities of aerobic enzymes and relatively high Mb concentrations probably ensure that *H. rubra* radula muscle function during work and low O_2 is powered aerobically. The energy demands of these muscles appear to be capably met by the low flux but high and efficient ATP yield of aerobic metabolism. It is most important that feeding in abalone is an efficient process, given the important role the radula muscles play in abalone biology and the feeding effort required to ingest large amounts of low-nutritive algae. Abalone may afford to spend considerable amounts of time feeding, as their usual nocturnal feeding habits ensure protection from many predators.

The position of the radula muscles in the CAS probably ensures a constant supply of haemolymph, and thus O_2 and potential fuels, to the working musculature. While estimates of actual blood flows and rates of O_2-delivery to these muscles were not determined for *H. rubra*, an alternative method for gauging the ability of the haemolymph to carry O_2 is to determine the amount, and characteristics, of the respiratory protein haemocyanin. These aspects are considered in Chapter 6.

Table 5.1. Algal preferences of *H. rubra* collected from Mornington. The number of animals showing no response, accepting but not feeding, and accepting and feeding are shown for each algal species presented.

Response	*C. moniliformis*	*Sargassum* sp	*E. radiata*	*J. lobata*	*U. australis*
No response	12	12	10	8	4
Accept only	0	0	1	4	8
Accept + Feed	0	0	1	0	6

Table 5.2. Concentrations of D-lactate, tauropine, arginine phosphate and glucose in radula muscle (μmol g^{-1} wet wt muscle) and haemolymph (μmol ml^{-1}) of *H. rubra* at rest and following continuous feeding for 15 min.

Treatment	Tissue	D-lactate	Tauropine	Arginine-P	Glucose
Control	Radula muscle	0.29 ± 0.08	0.13 ± 0.06	10.55 ± 1.10	
	Haemolymph	0.01± 0.01	0.05 ± 0.01		0.11 ± 0.02
Feeding					
Graze	Radula muscle	0.35 ± 0.08	0 ± 0	9.08 ± 1.25	
	Haemolymph	0.14 ± 0.01	0.05 ± 0.01		0.12 ± 0.04
Browse	Radula muscle	0.32 ± 0.07	0.18 ± 0.13	10.41 ± 3.16	
	Haemolymph	0.01 ± 0.00	0.02 ± 0.01		0.14 ± 0.03

Arginine-P = Arginine phosphate

Significant differences between metabolite levels of different tissues and treatments are detailed in the text.

Values given as Mean ± S.E.M for N=8 for control, N=6 for grazing and N=7 for browsing.

Table 5.3. Concentrations of D-lactate and tauropine in radula muscle (μmol g^{-1} wet wt muscle) and haemolymph (μmol mL^{-1}) of *H. rubra* at rest, and following whole animal exercise and 12 h air emersion.

Treatment	Tissue	Metabolite	
		D-lactate	Tauropine
Control	Radula muscle	0.29 ± 0.08	0.13 ± 0.06
	Haemolymph	0.01 ± 0.01	0.05 ± 0.01
Exercise	Radula muscle	0.26 ± 0.09	0.23 ± 0.03
	Haemolymph	0.11 ± 0.02	0.05 ± 0.02
Emersion	Radula muscle	0.41 ± 0.10	0.10 ± 0.07
	Haemolymph	0.84 ± 0.14	0.09 ± 0.03

Significant differences between metabolite levels of different tissues and treatments are detailed in the text.

Values given as Mean ± S.E.M for N=6.

Table 5.4. Anaerobic metabolite concentrations in *in vivo* and *in vitro* contracting radula muscles (μmol g^{-1} wet wt muscle), haemolymph (μmol ml^{-1}) and incubation medium (μmol ml^{-1}) of marine prosobranchs.

Species	D-lactate	Alanopine	Octopine	Arginine-P	%Lact	Reference
H. rubra						This study
control rad	0.3			10.55		
control haemolymph	0.01					
feed rad	0.34			9.75		
feed blood	0.01				97.14	
B. canaliculatum						Wiseman *et al.* (1989)
control rad				6.42		
control media						
contract rad	9.14	10.55	5.54	2.02		
contract media	27.21				25.15	
B. contrarium						Wiseman and Ellington (1987)
control rad	0.75	0	0.46	10.69		
control medium	0					
contract rad	9	5.5	4.72	6.98		
contract medium	26				25.71	

Arginine-P = Arginine Phosphate

%Lact = % total D-lactate pool remaining in radula muscle after treatment

CHAPTER SIX

THE HAEMOLYMPH OXYGEN TRANSPORT SYSTEM OF *HALIOTIS RUBRA*

6.1 INTRODUCTION

There are clear differences between the pedal muscles and radula muscles of *H. rubra*, as seen from the biochemical profiles (Chapter 3) and responses to work and hypoxia (Chapters 4 and 5). *Haliotis rubra* pedal musculature possesses a limited aerobic scope, while the radula muscles appear to function aerobically not only during sustained work associated with feeding, but also during whole animal exercise and hypoxia. While specific differences at the cellular level to support aerobic and anaerobic metabolism have been identified, consideration is now given to the role of the haemolymph in O_2-delivery. The amount of O_2 and manner in which it is delivered to *H. rubra* muscles may be influenced by changes in haemolymph flow, the amount of the O_2-binding respiratory protein haemocyanin (Hc), and the conditions associated with uptake and release of O_2 from the haemolymph.

The general cardiovascular system of *Haliotis* was addressed in Chapter 2, and is revisited here in more specific detail to illustrate the potential differences in haemolymph supply between the muscles of *H. rubra*. In association with the cardiovascular anatomy, it was important to consider other functional aspects of the haemolymph O_2-delivery system in *H. rubra*. These include the amount of Hc present which will influence the oxygenation of the haemolymph and the O_2-binding characteristics of *H. rubra* Hc that determine O_2-uptake at the gills and delivery to the muscles.

6.1.1 Haemolymph supply to pedal and radula muscles of *Haliotis rubra*

The cardiovascular anatomy and haemolymph supply to the tissues of *H. rubra* is represented in Figure 2.2 (Chapter 2) and, for the radula and pedal muscles specifically, Figure 6.1 (this Chapter). According to Voltzow (1990), the adductor and foot muscles of abalone receive haemolymph from separate branches of the anterior aorta, and there appears to be no mixing of the haemolymph between the two muscles. Crofts (1929) identified a shell (adductor) muscle artery for *H. tuberculata* that arises from the anterior aorta, and separate venous drainage, which empties into the posterior pedal venous sinus. A distinct shell adductor artery was detected by Russell and Evans (1989) for *H. rubra*. The vessel originates as a fine branch off the anterior aorta, prior to the CAS. However, Bourne *et al.* (1990) are of

the opinion that the posterior pedal arteries of *H. kamtschatkana* supply both the foot and shell muscles.

Pedal arteries were easily identified in *H. rubra* (Chapter 2), and the morphology of the cephalic region indicates, in conjunction with haemolymph samples taken from the immediate area of the odontophore apparatus, the presence of cephalic sinuses (Figures 2.2 and 6.1). Regardless of the possible separate perfusions of the portions of the pedal musculature, the adductor and foot muscles do not enjoy the direct haemolymph supply afforded the radula muscles by their position adjacent to the cephalic sinuses. Discounting the O_2 supplied to the cardiac muscle and radula sac posteriorly, the haemolymph entering the CAS via the anterior aorta may be presumed to be relatively highly oxygenated. Assuming this is correct, the radula muscles in effect receive "first-bite" of the oxygenated haemolymph from the aorta. This direct haemolymph supply also means these muscles also are exposed to blood-borne fuels, an important requirement for sustained aerobic metabolism.

As considered in Chapter 2, haemolymph in abalone appears not to be distributed in an organ weight-specific manner, but more probably in relation to tissue metabolic rate (Coulson *et al.* 1977; Jorgensen *et al.* 1984). The radula muscles of *H. cracherodii* for example show a high O_2-uptake, second only to the kidneys (Churchwell 1972, as cited in Jorgensen *et al.* 1984), but constitute only around 0.8% of whole wet body weight. In contrast, the foot muscle of *H. cracherodii* may account for about two-thirds of whole wet body weight, but at rest receives only 27% of the cardiac output (Jorgensen *et al.* 1984). Likewise the foot of *H. iris*, comprising around 40% of total tissue weight, receives 19% of the relative haemolymph perfusion (Just 2002). Conclusive data is not available on the perfusion of the radula muscles of abalone. The low perfusion (1% of cardiac output) of *H. cracherodii* radula muscles (Jorgensen *et al.* 1984) is thought to be underestimated, reflecting a failure of the method used to determine haemolymph distribution (Russell and Evans 1989).

Taylor *et al.* (2001) and Just (2002) also found the adductor muscles of *H. iris* to be relatively poorly perfused, as did Chen (1996), who experienced considerable difficulty in withdrawing haemolymph from the adductor muscle of *H. diversicolor*. These same studies have also documented a change in the distribution of cardiac output in abalone during episodes of stress. The flow of haemolymph to the foot and adductor muscles of *H. iris* is reduced during periods of muscular contraction (clamping) and hypoxia (emersion) (Taylor *et al.* 2001; Just 2002). The presence of valves between the pedal and cephalic sinuses in abalone (Russell and Evans 1989) may contribute to the isolation of the pedal musculature

from the general circulatory system during instances of [illegible]ed resistance or potential haemolymph loss, further reducing O_2-delivery.

It is obvious then that different muscles of abalone are exposed to different haemolymph flows. Whilst tissue-specific increases in perfusion may occur according to normal function (*e.g.* digestion of food, locomotion, feeding), challenges to abalone physiology such as air exposure and muscular contraction (Chapter 4) may drastically influence the distribution of haemolymph.

6.1.2 Haemocyanin and its role in oxygen transport

Haemocyanin is the most important respiratory pigment of the Mollusca with regard to the number of species which possess it (Ghiretti 1966). Haemocyanins are copper containing proteins of high molecular weight (around 9×10^6 Da, Ellerton and Lankovsky 1983; Ellerton *et al.* 1983), in which the metal is bound directly to the protein molecule in the form of a copper-protein complex (van Holde and Miller 1995). The Hc of *H. iris* exists primarily as a didecamer of approximately 8 MDa with a characteristic hollow cylindrical quaternary structure. Subunits of approximately 400 kDa are folded into functional units, each reversibly binding O_2 (Behrens *et al.* 2002). Rhogocytes (pore cells) in the connective tissue of abalone have been identified as sites of Hc biosynthesis (Albrecht *et al.* 2001).

The presence of dinuclear copper sites confers on Hc the ability to bind O_2 reversibly, allowing it to function as a haemolymph respiratory protein (Brix 1983; Brouwer 1992). During the oxygenation of Hc, one molecule of O_2 binds stoichiometrically with two atoms of copper (van Holde *et al.* 1992; van Holde and Miller 1995).

The body of data concerning Hc and its physiological role in the Phylum Mollusca is extensive. Numerous reviews deal with the structure and physical properties of the protein, its distribution and more thoroughly its respiratory function and physiological performance (Ghiretti 1966; Nickerson and van Holde 1971; Bonaventura and Bonaventura 1980, 1983; Mangum 1980, 1983a, 1992, 1997; Brix 1983; Ellerton *et al.* 1983; Bridges and Morris 1989; Brouwer 1992; van Holde *et al.* 1992; van Holde and Miller 1995; Terwilliger 1998; Bridges 2001).

The amount of respiratory protein circulated in the haemolymph of abalone can provide a simple estimate of the haemolymph capacity to transport O_2. Abalone Hc concentrations however display high inter- and intra-specific variability, which has even raised some doubts over the importance of Hc in O_2-delivery in abalone. Variation in Hc concentration of up to 900-fold was noted for *H. corrugata*, *H. cracherodii*, and *H. fulgens* (Pilson 1965). Similarly, an extensive field survey of *H. laevigata*, *H. roei* and *H. rubra* by

Ainslie (1977, 1980a) showed considerable disparity within and between species. Neither author could establish correlations with environmental (latitude, geography, season) or physiological (feeding and reproductive status, weight, sex) parameters.

The variability seen in abalone Hc concentrations partly may relate to the methods of analysis, as various methods used to quantify Hc may yield different results. A common method of determining haemolymph Hc concentration is to quantify haemolymph protein, based on the observations that, for *Haliotis*, essentially all haemolymph protein is Hc (Pilson 1965; Ainslie 1977; Taylor 1993). Other quantitative methods include determining the optical absorbance of oxyhaemocyanin (HcO_2), the copper content of the haemolymph, or the amount of O_2 bound (and hence O_2-carrying capacity). Spectrophotometric Molar extinction coefficients based on the relationships between these parameters may be determined, and are of considerable practical value in calculating many features of Hc containing haemolymphs. As a result, Hc concentrations are available as various estimates based on optical absorbance, copper content, protein or bound O_2.

However, the conditions under which these coefficients and parameters are determined are critical: identical pH environments, Hc history, denaturation, experimental treatment and corrections for optical scattering should all be considered. While results from various methods may differ in terms of the unit of measurement, they may also vary in absolute terms.

During the course of this investigation, it became obvious that the range of Hc concentrations given in the literature, regardless of method used to determine concentration, reflects the inherent variability of this respiratory protein. One of the aims of this chapter therefore in examining Hc levels in *H. rubra* is to investigate these problems in determining Hc concentration by comparing a range of commonly used methods.

Oxygen-binding properties of *Haliotis* haemocyanin

The availability of O_2 to tissues, and the total O_2-carrying capacity of abalone haemolymph, is also dependent on the manner in which Hc binds and releases O_2. The respiratory functions of molluscan Hcs are controlled by a number of allosteric mechanisms. Heterotropic interactions manifest when the binding of an effector molecule changes reactivity at the active site of the protein, affecting the affinity of the molecule for O_2. The O_2-binding properties of molluscan haemolymph can be expressed by P_{50}, the PO_2 at half saturation, which describes the O_2-affinity of the Hc. A higher P_{50} value indicates a lower O_2-affinity. The functional properties of molluscan Hcs are controlled, like those of arthropod Hc, by modulators such as pH, CO_2, anions, cations and temperature (van Holde *et al.* 1992; van Holde and Miller 1995; Mangum 1997; Bridges 2001).

Homotropic interactions concern the effect of O_2-binding at one active site on the Hc molecule on the reactivity of sites in other subunits, the extent of which is referred to as cooperativity. Hcs exhibiting cooperative binding display sigmoidal O_2-binding curves, allowing large changes in saturation over a narrower gas partial pressure range. Cooperativity can be expressed by the Hill coefficient n_{50}, with n values of one indicating a hyperbolic curve with no interaction between subunits. Values of n greater than one signify greater subunit interaction and sigmoidicity as n increases.

During the course of this study it was observed that whole animal hypoxia resulted in a decrease in pH and an accumulation of D-lactate in the haemolymph of *H. rubra* (Chapter 4). In contrast to the normal or negative Bohr effect ($\Delta \log P_{50}/\Delta pH$) shown by vertebrate globins and the Hc of arthropods, the reverse or positive Bohr shift of many marine gastropod Hcs results in an *increase* in O_2-affinity with falling pH. For most species this occurs below about pH 7.9 (Brix *et al.* 1979; Mangum and Lykkeboe 1979; Mangum 1980; Brix 1983), and is replaced by a small normal shift above this pH, which approximates *in vivo* haemolymph pH in many marine molluscs (Mangum and Schick 1972).

Since it was shown that Australian abalone Hcs possess a reverse Bohr shift (Ainslie 1977, 1980b), a reverse Bohr shift has been identified for a number of other abalone species, including *H. iris* and *H. australis* (Wells *et al.* 1998a; Behrens *et al.* 2002). Whether the reverse Bohr shift has any respiratory significance is debatable, although *in vivo* pH data suggests a physiological operating range and possible function for this effect. However, even very recently, the reverse Bohr shift was considered not to impair O_2-transport, because it is "largely limited to a non physiological pH range in aquatic species" (Mangum 1998). This is somewhat surprising considering data on *H. iris* (Wells *et al.* 1998a) supports much earlier findings (Ainslie 1977, 1980b) that the *in vivo* haemolymph pH of abalone lies well within the reverse Bohr shift range exhibited by these gastropods.

Metabolic end products, namely L-lactate and urate, are known to influence O_2-binding by arthropod Hcs (Truchot 1980, 1992; Lallier and Truchot 1989; Spicer and McMahon 1991; Zeis *et al.* 1992). L-lactate exhibits a modulatory effect of an increase in Hc O_2-affinity, mediated by binding to Hc itself. This increase in affinity may offset the normal Bohr shift experienced at a lower *in vivo* pH in crustaceans. It has been suggested however that organic effectors such as D-lactate do not significantly affect O_2-binding of molluscan Hcs (Mangum 1983a, 1983b, 1992, 1997).

To date, the only data available on the Hc of Australian abalone are the studies of Ainslie (1977, 1980a, 1980b). These investigations concentrate on the level of Hc in abalone populations, the O_2-binding of Hc, and the O_2-consumption of *H. laevigata*, *H. roei* and *H. rubra*. Haemocyanin was estimated spectrophotometrically using protein analyses to determine absorption coefficients. Whilst these studies are extensive, it is apparent that variability in Hc levels in gastropods may not only be due to inter- and intra-species variability, but variability in the methods of analysis as well. The current study on *H. rubra* Hc extends aspects of Ainslie's early work by examining multiple methods of Hc analysis, and by also examining the effects of pH as a metabolic acidosis (not respiratory acidosis), and D-lactate, on the O_2-binding properties. The effects of a metabolic acidosis or D-lactate load on Hc function are important to abalone, as O_2-binding potentially may be altered during hypoxia in these animals.

During the course of this work, the author was involved with a parallel study of a similar nature on Hc function of *H. iris* (Behrens *et al.* 2002). That data serves as a key comparative reference for the current investigation on *H. rubra*. The study of haemolymph physiology of Australian abalone has been largely ignored since the comprehensive study on Hc function by Ainslie (1977, 1980a, 1980b). A renewed interest in this field should be seen as intrinsic to other metabolic or physiological investigations of the genus.

The aims of this chapter therefore are to examine the O_2-transport characteristics of *H. rubra* haemolymph and Hc. It was considered of interest to determine Hc concentrations concurrently using a number of different commonly employed techniques. Often concentration is based on measurements of one or two determinants, and it is not common that haemolymph characteristics are examined by multiple methods at the same time. Due to the stoichiometric relationships between copper and bound O_2, both these parameters may be used to determine Hc concentration. Haemolymph protein, copper, optical absorbance and bound O_2 were determined in order to estimate haemolymph Hc concentration and haemolymph O_2-capacity.

An initial series of *H. rubra* haemolymph protein analyses suggested that the Hc concentration for *H. rubra* was approx. twice that recorded previously for the same species (Ainslie 1977, 1980a). The animals from which the haemolymph had been sampled for analysis had been held in captivity for some period (up to 8 weeks), during which time they had experienced reduced water flow and mixing. As a result, Hc concentrations of wild *H. rubra* were also determined to investigate any possible effects of captivity on *H. rubra* Hc

levels. In association with determining the Hc concentration of *H. rubra* haemolymph using multiple techniques, consideration was given to physiological and physical processes that may influence Hc concentration.

Furthermore, it was of interest to investigate the general O_2-binding properties of *H. rubra* Hc. A metabolic acidosis and metabolite load was observed in the haemolymph of *H. rubra* during hypoxia (Chapter 4), and it is possible to mimic these changes *in vitro* to examine any effects these changes in pH and metabolites may have on O_2-binding of Hc. In doing so, the importance of the reverse Bohr shift and organic effectors on *H. rubra* O_2-binding is examined.

Finally, in conjunction with the literature and given the variability and binding properties of Hc, the role of this respiratory protein in haemolymph O_2-delivery in abalone is considered.

6.2 MATERIALS AND METHODS

6.2.1 Determination of haemocyanin concentration

Given the equipment required to determine the copper content, O_2-carrying capacity and O_2-binding characteristics of *H. rubra* Hc, most of this work was performed in an appropriately equipped laboratory at the Department of Zoology, University of Canterbury, Christchurch, New Zealand, under the direction of Associate Professor H. Harry Taylor. Prior to these experiments however, a short investigation into *H. rubra* haemolymph protein levels was undertaken at Monash University.

Abalone were captured and held according to the earlier protocol (Chapter 2). Abalone were removed from the aquaria, blotted, and haemolymph was taken from the medial pedal sinus or the cephalic arterial sinus (CAS) using a 23 or 19 gauge needle and a 1 or 3 mL syringe without chemical additives. Sub-samples of the haemolymph were dispensed into centrifuge tubes, and centrifuged for 10 min at 13000 × *g* and 4°C to remove the small number of haemocytes.

Since practically all protein in haliotid haemolymph is Hc, concentrations were estimated using a standard biuret protein assay (Dawson *et al.* 1969). Following centrifugation, haemolymph supernatants were diluted 1:4 (haemolymph:biuret) with fresh biuret reagent (Dawson *et al.* 1969), and haemolymph protein assays performed as described previously (Chapter 3). Haemolymph protein concentrations were determined from a standard curve (bovine serum albumin, BSA), and duplicate measurements were made for each individual haemolymph sample.

Biuret protein assays were also performed on haemolymph collected from abalone in the wild. Abalone were carefully collected from the field site, and haemolymph samples taken *in situ* as for captive animals. Haemolymph samples were divided amongst eppendorf tubes on ice. Animals had their shells marked for future reference and were then returned to the site of removal. For each animal, the whole process was undertaken in less than 2 minutes. Samples were transported on ice back to the laboratory, where biuret assays were immediately performed as described previously.

The following analyses on *H. rubra* Hc were performed in the Department of Zoology, University of Canterbury, Christchurch, on samples collected at Monash University. Haemolymph samples from individual *H. rubra* were collected from the CAS or pedal sinus as above, centrifuged at 10000 × *g* for 30 mins at 4°C, and stored in 45 mL plastic sample

tubes at –80°C. Tubes were insulated and transported by air to New Zealand. Stock samples were then thawed and appropriate aliquots taken and re-frozen for each of the following analyses: Hc concentration (protein, copper content, O_2-content and spectrophotometry), and O_2-binding curves (OBCs).

Haemolymph protein

Frozen haemolymph samples were thawed, and prepared and assayed for protein using the biuret method as described previously.

Copper content

Analysis of copper content of *H. rubra* haemolymph was performed using graphite furnace Atomic Absorption Spectroscopy (AAS; GBC Avanta Sigma). Haemolymph samples of 100 µL were digested overnight at 50°C in 1 mL of conc. HNO_3. Sub-samples (10 µL) were further diluted 100 fold with doubly distilled water and 20 µL samples were analysed by graphite furnace AAS at a wavelength of 324.7 nm using deuterium background correction. Samples were dried in the furnace at 150°C, then ashed at 800°C and atomised at 2200°C in an atmosphere of nitrogen. Standards (0-50 µg L^{-1} Cu) were prepared in 1% HNO_3 and values corrected using reagent blanks and spikes of known Cu concentration. Sample blanks that contained all of the reagents in the same volume as used in processing the samples were carried through the entire sample digestion scheme, and used to count sample values. All results were expressed as ppm (µg L^{-1} Cu). Results were transformed into mmol L^{-1} by dividing by Mw of Copper (63.55). Copper analyses were run a total of four times for each sample (2 trials, each with duplicates).

Oxygen content

Oxygen content determinations of haemolymph samples were performed using an "Oxycon" O_2-content analyser (a galvanic O_2 cell designed and built by the University of Tasmania, Grubb and Mills 1981). The unit was provided with a constant flow of O_2-Free Nitrogen (OFN; <10 ppm O_2), supplied by a Cameron Gas Mixing Flowmeter, as a reference and flushing gas. A glass chamber was fitted onto the unit, containing 1 mL O_2 scrubber solution, and bubbled with OFN. The composition of the scrubber solution is as follows: 6 g L^{-1} Potassium ferricyanide, 3 g L^{-1} Potassium cyanide, 15 g L^{-1} Tri-sodium phosphate (hydrated), 5 g L^{-1} Di-sodium orthophosphate (anhydrous), 1 mg L^{-1} Triton X® and 1 mL L^{-1} Antifoam A (Sigma Chemicals).

As nitrogen is bubbled through the chamber, any O_2 present is liberated by the scrubber solution, blown off and calculated (mmol L^{-1}) by the unit according to temperature and barometric pressure. The Oxycon was calibrated on 5 µl samples of room air, introduced into the glass scrubber chamber via a 10 µl Gastight 1701 syringe (Hamilton®, USA) adjusted to 5 µl with a fitted Chaney adaptor.

A 1 mL sample of whole haemolymph was placed in a tonometer chamber and spun and mixed by a Cameron DEQ1 Dual Equilibrator tonometer (see below) under atmospheric O_2 (compressed air 250 mL min^{-1} flow) and CO_2 (0.5 mL min^{-1} flow) for 20 minutes at 15°C to ensure full oxygenation of Hc. The Oxycon was equilibrated as described previously with 5 µl samples of air, and 50 µl samples of tonometered haemolymph was injected into the chamber. Oxygen content (mmol L^{-1}) was calculated on duplicate measurements. Final whole haemolymph-bound O_2 (mmol L^{-1}) was calculated by subtracting the dissolved fraction (unbound; determined by solubility coefficient of sea water at 15°C and 150 mmHg), and correcting for equilibration. Values were also converted into vols% (vol O_2: vol haemolymph) to allow comparison with literature. Since 1 mole of gas occupies 22.4 L at STP, 1 mmol L^{-1} = 2.24 mL(STP) 100 mL^{-1} = 2.24 vol %.

Spectrophotometry

The optical absorbance of *H. rubra* Hc was estimated spectrophotometrically from peak absorbance of HcO_2 at 346 nm. Gastropod Hc expresses an absorbance peak at this wavelength (Nickerson and Van Holde 1971). This absorbance peak was verified for *H. rubra* Hc by scanning the absorption spectrum of haemolymph samples from 300 to 400 nm. Samples of 0.1 mL haemolymph were diluted 1:10 v:v with 50 mmol L^{-1} glycine 10 mmol L^{-1} EDTA buffer, pH 8.8 (buffer based on Nickerson and van Holde 1971; Ellerton and Lankovsky 1983; Ellerton *et al.* 1983 and Behrens 1999), shaken vigorously to ensure oxygenation and left for 5 min to equilibrate with air. Samples were measured at 346 nm against a buffer blank, and Hc concentrations expressed as the absorbance of undiluted haemolymph at a light path length of 1 cm.

The assay relies on the full oxygenation and dissociation of the Hc. At the assay pH however the absorbance reading may be influenced by a scattering of the light beam due to interfering dissociated particles. To correct for scattering, absorbance readings were run on the same samples following deoxygenation by cyanide addition and the differential absorbance was obtained by subtracting the de-oxy absorbance (Behrens 1999).

Molar extinction coefficients for *H. rubra* Hc were calculated from absorbance against bound O_2-content (HcO_2), and absorbance against copper content, for both scattering-corrected and -uncorrected samples (Brown and Terwilliger 1998). Coefficients are expressed as absorbance units/(mmol L^{-1}) for 1 cm light path (E_{346} mM^{-1} cm^{-1}).

The concentration of *H. rubra* Hc (as µmol mL^{-1} functional units) was calculated from the variables measured. Haemolymph protein, in mg mL^{-1}, was converted to µmol mL^{-1} by dividing by the molecular weight of a Hc functional unit (53,000 Da, Ellerton *et al.* 1983), and multiplying by 1000. Haemocyanin concentrations were also calculated by converting absorbance values to µmol mL^{-1} using the extinction coefficients derived from HcO_2 and copper analyses. Bound O_2 is a direct measurement of Hc concentration as µmol mL^{-1}.

6.2.2 Oxygen binding curves

Oxygen binding curves (OBCs) were constructed for *H. rubra* haemolymph to investigate the characteristics of haemolymph O_2-binding, namely P_{50} (O_2-affinity) and n_{50} (cooperativity of binding), and effects of both pH and D-lactate on these parameters.

Haliotis rubra haemolymph solutions were prepared by adding to whole thawed haemolymph stock 1 mol L^{-1} Bis-Tris or Tris-HCl buffers to a final concentration of 0.1 mol L^{-1}. Bis-Tris and Tris-HCl buffers have been used previously in comparable abalone Hc studies (Wells 1998a; Behrens *et al.* 2002). The PO_2, O_2-content and pH were measured simultaneously on buffered *H. rubra* haemolymph solutions to construct OBCs.

Humidified compressed air, O_2-Free Nitrogen (OFN) and CO_2 were supplied to a Cameron DEQ1 Dual Equilibrator tonometer by a Cameron Gas Mixing Flowmeter, with a total flow rate of 250.5 mL min^{-1}. PO_2 was measured using a miniature O_2 electrode (Microelectrodes Inc.). The electrode was calibrated with atmospheric O_2 (room air) and adjusted if necessary, and zeroed using a zero O_2 solution (approx 10 mg mL^{-1} sodium sulphite in 10 mmol L^{-1} disodium tetraborate). The electrode was coupled to a Strathkelvin 781b O_2 analyser. pH was measured with a miniature electrode (Microelectrodes Inc.) and Radiometer PHM64 meter (Radiometer, Copenhagen), calibrated with BDH Colourkey Buffers. Both electrodes were housed in a capillary microcell (Cameron Instruments) mounted above the tonometer, thermostatted at 15°C with a circulating Haake D1 water bath.

After calibration of electrodes, 5 mL of buffered haemolymph was introduced into one of the tonometer chambers, thermostatically controlled at 15°C as above. The tonometers were set spinning, and the whole system flushed with zero O_2 gas mixture (250 mL min^{-1} OFN, 0.5 mL min^{-1} CO_2) until the O_2 electrode recorded zero O_2 (approx. 1 h). CO_2 was

delivered at a constant flow of 0.5 mL/min, equivalent to 1.52 mmHg. The tonometers were stopped, and a 50 µL haemolymph sample withdrawn (Gastight syringe, Hamilton, USA) and introduced into the "Oxycon" (see above) for immediate O_2-content analysis.

Meanwhile, a 0.5-1 mL sub-sample of haemolymph from the tonometer was carefully drawn through the thermostatted chamber accommodating the electrodes, and 3-5 mins allowed for PO_2 and pH readings to stabilise. The haemolymph sample was then returned to the tonometer chamber, and the mixing resumed. The electrodes were rinsed with distilled water flushed with the same gas mixture flowing to the haemolymph.

Following this initial zero O_2 reading, compressed air as the source of O_2 was introduced into the system at the expense of an equal flow of OFN (extra CO_2 was considered to have negligible effects). Oxygen (air) flows were increased by stepwise additions (2, 5 or 10 mL min^{-1} flow) until full saturation (250 mL min^{-1} compressed air), and 8-10 min mixing was allowed between measurements to ensure full equilibration of the haemolymph with the gas mixture (deemed appropriate from previous tests). The procedure continued until two satisfactory full-saturation values were obtained (at atmospheric O_2), and the haemolymph sample refrozen for further analyses, if needed.

Effects of pH on *Haliotis rubra* Hc oxygen-binding

The effects of metabolic acidosis on *H. rubra* Hc O_2-binding were investigated by running OBCs at various haemolymph pHs. After careful consideration of the reported physiological pH range of haliotids (Chapter 4), Hc solutions of varying pH were prepared by adding to whole haemolymph stock 1 mol L^{-1} Bis-Tris (pH 6.3 and pH 6.9) or Tris-HCl (pH 7.5) buffers to a final concentration of 0.1 mol L^{-1}. Given that these buffers possess negative temperature coefficients, actual pH of buffered haemolymph samples run at 15°C was expected to deviate from stock buffers made up at room temperature (20-25°C).

Following dilution with the appropriate buffer, haemolymph samples were introduced into the tonometer and an OBC run as described above. Oxygen binding curves were run at each pH (6.3, 6.9 and 7.5) for three or four separate haemolymph samples, with duplicates run for most samples.

Effects of D-lactate on *Haliotis rubra* Hc oxygen-binding

Considering D-lactate had been observed in the haemolymph of *H. rubra* during and following hypoxia (Chapter 4), the possible effects of D-lactate on haemolymph O_2-binding were examined. Given that hypoxia and anaerobic metabolism are associated with a decrease in pH in abalone, the effects of D-lactate were examined only at the low and intermediate pH

treatments (pH 6.3 and 6.9). A D-lactate concentration of 5 mmol L^{-1} was used to simulate an anaerobic metabolite load in the haemolymph. In order to control pH fluctuations, a neutral salt (lithium D-lactate) was used.

Oxygen binding curves were run for haemolymph solutions buffered with Bis-Tris pH 6.3 and 6.9 buffer. Haemolymph solutions were brought back to zero PO_2, and a second OBC was immediately run on the same samples following the addition of aliquots of 250 mmol L^{-1} D-lactate solution (made up in appropriate pH Bis-Tris buffer) to a final concentration of 5 mmol L^{-1} (corrected for volume). Four OBCs prior to and after addition of D-lactate were run at each pH.

6.2.3 Data analyses

The concentration of *H. rubra* Hc (as µmol mL^{-1} functional units, where 1 Hc functional unit = 1 O_2 = 2 Cu = 53,000 Da Mwt) was calculated from the variables measured. Values for Hc concentration were transformed to meet assumptions of the test and compared statistically (ANOVA) using MS Excel. Significance was accepted at $P<0.05$.

Oxygen binding curve data were analysed using MS Excel spreadsheets, and curves were generated for bound, unbound and total O_2-content versus PO_2. Data was transformed into log PO_2 and % saturation, from which Hill plots were generated for each curve. For each OBC, four or five equilibrium steps between 20 and 80% saturation were used to construct Hill plots of {log[S/(1-S)] *vs* log PO_2} where S is the fractional O_2-saturation and PO_2 the O_2-tension. From the Hill Plot regression equation, the P_{50} (O_2 partial pressure at half saturation) and n_{50} (the slope of the plot at half saturation that indicates cooperativity of O_2-binding) values were calculated. From the raw data, Bohr factors ($\varphi = \Delta \log P_{50}/\Delta pH$) were calculated for each sample following a change in pH or D-lactate addition.

Mean P_{50} and n_{50} values were determined for each pH treatment and lactate treatment, and OBCs graphed. Values for P_{50} and n_{50} were checked for normality and compared statistically (ANOVA) using MS Excel and Systat statistical packages. Significance was accepted at $P<0.05$.

6.3 RESULTS

6.3.1 Haemocyanin concentration of *Haliotis rubra* haemolymph

The mean (± S.E.M) haemolymph protein concentration of *H. rubra* is 9.57 ± 0.50 mg mL^{-1} (Table 6.1). Animals that had been kept in captivity for up to 8 weeks showed no significant difference in Hc concentration compared to animals sampled in the wild (ANOVA, $P>0.05$). One individual had been sampled twice (as noted by marked shell on recapture 9 days later) and yielded a second Hc concentration of 5.84 mg mL^{-1}, lower than the original value of 7.48 mg mL^{-1} (no statistical analysis performed due to single sample).

Protein determinations performed on subsequent experimental abalone randomly during the course of the study resulted in similar concentrations, even for individuals held in aquaria for up to 5 months (Table 6.1).

The data for all variables measured (haemolymph protein, copper, bound O_2 (HcO_2) and absorbance) are given in Table 6.2. Generally, a positive relationship exists between all variables measured for all samples. Haemolymph samples with high protein (*e.g.* sample 3) show a greater HcO_2 fraction, copper content and absorbance, with the exception of sample 4 (high copper) and sample 6 (low HcO_2). The means of the data from the six samples show considerable variance.

The normalised linear relationship amongst all variables for all samples is depicted in Figure 6.2. Against differential absorbance (corrected for scattering), HcO_2 and copper maintain a significant linear relationship (r^2 = 0.99, $P<0.05$ for HcO_2). The only real exception is the concentration of copper (mmol L^{-1}) in sample 4, which is considerably elevated. Duplicate copper analyses on this sample yielded identical results. Protein levels however do not appear to be linearly related in this instance to any of the other variables.

The regression of raw values against HcO_2 on a common abscissa is depicted in Figure 6.3. From each of the relationships, the amount of HcO_2 may be estimated from any of the other measured variables. As indicated with the normalised plot, most parameters are linearly related, indicating some confluence with the different experimental estimations.

Haemocyanin extinction coefficients based on absorbance are given in Table 6.3. With the exception of the samples 4 and 6 as mentioned previously, extinction coefficients (expressed as Absorbance units/(mmol L^{-1}) for 1 cm light path) are similar. The mean ratio of HcO_2 to copper coefficients is 2.52, which is close to the stoichiometric binding ratio of two copper atoms to one molecule of O_2. When calculated excluding samples 4 and 6, the mean ratio is 2.11 ± 0.11.

The concentration of *H. rubra* Hc, given as μmol mL^{-1} functional units, is shown in Table 6.4. Although the mean values show considerable variability, all values are around 0.15-0.19 μmol mL^{-1}, and are not significantly different from each other (ANOVA, $P>0.05$). Estimates based on protein analysis give the highest value (0.19 mmol L^{-1}), and the lowest value is achieved by estimating concentration from absorbance using the extinction coefficient derived from bound O_2 (0.14 mmol L^{-1}, Table 6.4).

6.3.2 Oxygen equilibrium curves of *Haliotis rubra* haemocyanin

Effects of pH

The effects of pH on O_2-affinity and cooperativity of *H. rubra* Hc are given in Table 6.5. Values of P_{50}, n_{50}, and Bohr factors (φ) are given for accumulated or 'summary' data (results of a single curve for all data of the same treatment), and 'average' data which represents the mean ± S.E.M of the individual curves (N = 7 or 4).

Both O_2-affinity and binding cooperativity are pH sensitive. *Haliotis rubra* Hc shows high O_2-affinity (P_{50}=4.75 mmHg) and negative cooperativity (n_{50}=0.71) at low pH (6.46). Increasing pH to 7.12 did not significantly affect affinity or cooperativity. An increase in pH to 7.74 significantly increased both P_{50} (13.77 mmHg) and n_{50} (1.81; ANOVA, $P<0.05$). At pH 7.74, *H. rubra* Hc exhibits marked positive cooperativity, reflected also in the more sigmoidal nature of the binding curve (Figure 6.4).

Decreasing pH resulted in a positive Bohr shift. As pH rises the data show a moderate Bohr effect (φ = +0.334 for pH 6.46 to 7.12, and +0.46 for pH 7.12 to 7.74, respectively).

Effects of D-lactate

The effects of 5 mmol L^{-1} D-lactate on Hc O_2-binding are also given in Table 6.5. At both pH 6.3 and 6.9, 5 mmol L^{-1} D-lactate does not significantly decrease O_2-affinity or binding cooperativity (ANOVA, $P>0.05$). The addition of a neutral D-lactate salt did not alter sample pH (observed pH changes due to addition of fresh buffer). The addition of 5 mmol L^{-1} D-lactate however results in a negative Bohr shift (φ=-3.29 for pH 6.3 D-lactate and -1.12 for pH 6.9 D-lactate, respectively). The specific effect of D-lactate, ($\Delta\log P_{50}/\Delta\log$ mmol L^{-1} effector, Graham *et al.* 1983), results in values of 0.28 for pH 6.3 treatment and 0.1 for pH 6.9 treatment. This effect of lactate appears to be pH dependent.

Typical OBCs and Hill plots for each pH and lactate treatment are given in Figures 6.4 to 6.7. The sigmoidicity of normal pH curves, and of both control and lactate curves are

given in the single Hill plots for these treatments (Figures 6.5 and 6.7 respectively). Curves for all initial, control and lactate treatment samples are estimated as theoretical, calculated from the regression equation generated by the Hill plot. As indicated by the n_{50} values, Hc O_2-binding at low pH displays negative cooperativity, with cooperativity increasing dramatically at higher pH. The reverse or positive Bohr effect is indicated by the left shift of the curves and decrease in P_{50} as pH decreases (Figure 6.4).

When viewed qualitatively, the addition of D-lactate at both pH 6.3 and 6.9 results in a small right-shift of the OBCs (Figure 6.6). There is a slight trend for a right and upward shift in the curves as pH increases and D-lactate is added. Generally however, the curves for *H. rubra* haemolymph at of control and lactate for both pH treatments appear similar.

Care must be exercised in interpreting these results. Based on summary data, D-lactate appears to exert an effect on Hc cooperativity (Table 6.5). This is not the case when mean data are considered. Overall, whereas similar results are achieved regardless of calculation method, an obvious advantage of statistical comparison exists when using mean data. Also, graphical summaries of summary OBCs are calculated from the Hill plot regression function. Applying this equation to the data set to predict % saturation assumes a constant n_{50}, or slope.

6.4 DISCUSSION

The aims of this chapter were to determine Hc concentration by investigating various methods for estimating the Hc content of *H. rubra* haemolymph, and to examine the effects of changes in haemolymph pH and D-lactate concentration on the O_2-binding of *H. rubra* Hc. In order to determine Hc concentration, haemolymph protein, copper, bound O_2 and absorbance were measured. Oxygen binding curves were then constructed for *H. rubra* haemolymph samples at varying pH and following the addition of D-lactate, with the changes in P_{50} and n_{50} examined. In the following discussion, the variability in Hc concentrations of abalone and gastropod haemolymph and methods used to determine them are discussed, followed by an examination of the O_2-capacity of abalone haemolymphs. The effects of pH and D-lactate on Hc O_2-binding properties are then considered. This section then concludes with an examination of the role of Hc in abalone. Initially however, consideration is given to some of the experimental methods and the conditions under which these experiments were conducted.

6.4.1 Methodology

Haemolymph samples

With the exception of the initial captive and wild abalone Hc investigation, all haemolymph determinations were performed on samples of haemolymph that had been stored frozen. This was unavoidable as live *H. rubra* could not be transported to New Zealand, and the equipment necessary for the experiments was not available at Monash University. It is possible however that the repeated freeze-thaw of *H. rubra* haemolymph had affected the characteristics or structure of *H. rubra* Hc. Morris (1988) suggests that if the native properties of Hc are at issue, then the effect of freezing must be carefully determined. Generally, the cooperativity of O_2-binding for Hc is altered or reduced following freezing, with negligible effects on P_{50}. This feature, suggested to be due to structural changes, has been demonstrated for amphipod and decapod crustacean Hc (Spicer and McMahon 1991; Morris 1988). In contrast, it appears freezing has no observable effect on gastropod Hc O_2-affinity or binding (Petrovich *et al.* 1990 for *Busycon canaliculatum*). Recent studies on *H. iris* Hc were performed on transported frozen samples with little mention of possible freezing effects (Wells *et al.* 1998a, Behrens *et al.* 2002). Every attempt was made to maintain haemolymph freeze-thaw and handling to a minimum during the course of these studies, to reduce any influence. Unfortunately, any effect of freezing was not quantified in this study.

Oxygen binding curves

Haemolymph samples were buffered with Bis-Tris or Tris-HCl buffers prior to analysis of effects of pH and D-lactate on O_2-binding. The buffer pH range covered haemolymph pH values reported for *H. australis*, *H. iris* and *H. rubra* (Ainslie 1977, 1980b; Wells *et al.* 1998a; Chapter 4). Although Ainslie (1977) cautions on potential changes in *Haliotis* haemolymph characteristics associated with buffering, as opposed to using whole haemolymph, haemolymph samples for this study were buffered prior to O_2-binding studies for a number of reasons. Firstly to allow direct comparison with recent results on *Haliotis* reported in the literature, and secondly to closely control haemolymph pH. The use of pH buffer solutions and constant PCO_2 (1.52 mmHg) had the further advantages of simulating a metabolic acidosis, and negating any possible pH effects of haemolymph and animal history. This was seen as more appropriate and direct than altering haemolymph pH with PCO_2 (Ainslie 1977, 1980b). A CO_2 level of 1.52 mmHg was chosen as it approximated the mid-range *in vivo* PCO_2 of a marine invertebrate (H. H. Taylor University of Canterbury, pers. comm), and is not too dissimilar from abalone arterial partial pressures (PCO_2 1.2 mmHg, Ainslie 1977).

Haemolymph concentrations of D-lactate in *H. rubra* following 12 hrs emersion may reach 1.35 mmol L^{-1} (Chapter 4), and as high as 2.68 mmol L^{-1} after 24 hrs emersion in *H. iris* (Behrens *et al.* 2002). Previous attempts to investigate the influence of organic effectors on haliotid haemolymph employed D- and L-lactate concentrations of up to 10 mmol L^{-1} (Behrens 1999; Behrens *et al.* 2002). In light of this data, 5 mmol L^{-1} was accepted as a reasonable load of metabolite to impose on the haemolymph. Higher concentrations approach un-physiological levels (pers. obs). In addition, a neutral salt (lithium D-lactate) was preferred, to avoid pH fluctuation.

During all work on *H. rubra* Hc O_2-binding, temperature was held at 15°C. This compares with conditions reported in the literature and the temperature of the holding aquaria at Monash at the time of haemolymph collection. The effects of temperature were kept to a minimum, as ambient temperature is known to affect abalone Hc O_2-binding characteristics. Ainslie (1977, 1980b) demonstrated a decrease in O_2-affinity of abalone Hc following an abrupt temperature increase from 20°C to 25°C, accompanied by a decrease in arterial O_2-saturation, with the net result of decreasing the amount of O_2 delivered to the tissues. Haemocyanin O_2-affinity is reduced with an increase in temperature in both *H. australis* and

H. iris (Wells *et al.* 1998a). Similarly, Burnett *et al.* (1988) identified a temperature sensitivity of *H. corrugata* P_{50}, with sensitivity decreasing as temperature rose.

6.4.2 Haemolymph haemocyanin concentrations in *Haliotis*

The haemolymph protein concentration for *H. rubra* is around 9.6 to 10.3 mg mL^{-1}, or 0.19 µmol mL^{-1}. Care must be exercised in comparing haemolymph protein results (and extinction coefficients) from the literature, as values may be based on different assays (*e.g.* dry weight, Nickerson and van Holde 1971; Bio-rad reagent, Taylor 1993). Taylor (1993) indicates that the Bio-rad assay tends towards non-linearity above absorbances of 0.6 OD units. Many studies however appear to utilise the biuret procedure.

When converted to functional units, the Hc concentration of *H. rubra* haemolymph is around 0.17 µmol mL^{-1}, regardless of the method of calculation. Although protein analysis results in the highest estimates of Hc concentration, a more linear relationship is seen between absorbance and HcO_2, and absorbance and copper (Figures 6.2 and 6.3). The high variability seen in the mean Hc concentrations may mask any differences between the values or methods used, and a larger sample size may distinguish any significant differences.

The fact that no significant differences were observed between the concentrations of Hc (in µmol mL^{-1} functional units) when calculated using different methods suggests that, if performed with care and on the same samples, reasonable estimates of Hc may be obtained. There appears therefore some confluence between methods, and the most appropriate method for determining Hc concentration may depend largely on the available resources. The most expedient methods are the determination of haemolymph protein and absorbance. Recent studies have derived Hc extinction coefficients using copper analyses, such as that by Behrens *et al.* (2002) to calculate *H. iris* HcO_2 functional units (E_{346} mM^{-1} cm^{-1}=11.42 ± 0.17). The results of the current study, using corrected data and excluding sample 4 (elevated copper) are similar: E_{346} mM^{-1} cm^{-1}=10.72 ± 0.72; and 12.62 ± 0.93 for all results, uncorrected. For extinction coefficients derived from absorbance and copper or HcO_2 determinations, correction for scattering is important (Brown and Terwilliger 1998), although raw uncorrected values may be considered more practical, especially when comparing to most other literature.

An elevated copper level was noted for haemolymph sample 4, the most likely explanation being an elevated non-Hc free copper. The copper concentrations in the haemolymph and other tissues of *Haliotis* are known to be variable (Marks 1938; Pilson 1965; Bryan *et al.* 1977; Hyne *et al.* 1992). This present anomaly may reflect an elevated

environmental heavy metal concentration from the field site, or a contamination of the holding aquaria in which the animals were maintained.

Copper is generally distributed uniformly in the muscles and viscera of *H. rubra*, although a higher concentration is noted in the haemolymph (Hyne *et al.* 1992). Of this, 99% is bound to high molecular weight proteins in the haemolymph serum. Interestingly, copper has been found bound to the amino acid taurine, a major haemolymph amino acid in bivalve and gastropods (Simkiss and Mason 1983), and a substrate for tauropine production in abalone. While muscle taurine concentrations are known for *H. lamellosa* (up to 243 μmol g^{-1}, Gäde 1987), no data are available on other abalone haemolymph concentrations.

Compared to most other abalone, *H. rubra* from this study displays relatively high concentrations of haemolymph protein, higher than reported previously for this species. The mean Hc concentrations of a range of abalone are shown in Table 6.6. Harris and Burke (2001), with work on the respiratory physiology of juvenile *H. rubra* and *H. laevigata*, observed very low Hc concentrations in the haemolymph of these abalone (0.49 and 0.65 mg mL^{-1} respectively, Table 6.6). Their data are confused however, as these authors also state *H. laevigata* haemolymph possesses an average Hc concentration of 7.71 mg mL^{-1}.

Work by Pilson (1965) on Californian *Haliotis* species indicates up to a 900-fold variation in Hc concentration (measured as copper and protein nitrogen) for *H. corrugata*, which could not be related to weight, sex, reproductive activity, nutritional state, depth of occurrence or season. Likewise, Ainslie (1977, 1980a) excludes environmental temperature and feeding behaviour as bearing influence on Hc concentrations of *H. rubra*, *H. laevigata* and *H. roei*. Sampling history of individuals from which multiple samples were drawn over a year did not bias Hc determinations (Ainslie 1977, 1980a). However, the consistently higher haemolymph Hc concentrations and O_2-carrying capacity exhibited by *H. roei* than for *H. rubra* or *H. laevigata* (Table 6.6) is thought to reflect the more active lifestyle and intertidal distribution of this species (Shepherd 1973a; Ainslie 1977, 1980a, 1980b).

Although direct measurements of weight or sex were not made during the present study, the discrepancy observed between the present results and those of Ainslie (1977, 1980a) is presumed to be a reflection of the naturally high variation among populations. Sampling for the haemolymph used in the current study was performed over a space of 3 months (late Jan to early April), a period which is also encompassed in the scope of Ainslie's study. Seasonal variation of Hc (as copper) in the haemolymph and tissues of the whelk *Busycon canaliculatum* has been observed rising with the onset of feeding in spring and early summer (Betzer and Pilson 1974).

Haliotids appear to possess relatively lower Hc concentrations than other marine gastropods. Haemocyanin concentration for *Busycon canaliculatum* has been reported at 24 mg mL^{-1} (DePhillips *et al.* 1970) and 35 mg mL^{-1} (Petrovich *et al.* 1990). Variation for this species is also noted however, with Mangum and Lykkeboe (1979) reporting Hc as low as 3×10^{-4} mg mL^{-1}. Natural variation in the Hc concentration is also observed for crustaceans (Truchot 1992; Mangum 1997; Spicer and Baden 2000).

Factors affecting haemocyanin concentration in *Haliotis*

No effect of holding *H. rubra* in aquarium conditions on Hc concentration was observed. Any effects, should they occur, are likely to manifest themselves over a longer time period, or under more severe conditions. The extent of hypoxia was not actually determined in the aquaria, and even though water flow and mixing were reduced during the period that the animals were held, it is possible that the tank system did not actually pose an hypoxic stress. Haemocyanin synthesis has been induced by prolonged hypoxia in crustaceans however (Taylor and Anstiss 1999). After 2 weeks of moderate hypoxia (40% water O_2-saturation), haemolymph Hc concentration doubled in brown shrimps, *Crangon crangon* (Hagerman 1986). Following one week of hypoxia (PO_2 = 30 mmHg), the concentration of Hc in the haemolymph of the whelk *Buccinum undatum* was not altered, although it had acquired a reverse Root effect (Brix 1982, 1983). Likewise, copper and protein concentrations of the gastropod *Lymnaea stagnalis* haemolymph were not altered during 24 h anoxia (Wijsman *et al.* 1985). No equivalent data are available for abalone, although Hc concentrations are known to increase in *H. iris*, in the short term, as a result of handling (Ragg *et al.* 2000). This increase in concentration is most likely an effect of a decrease in haemolymph volume and thus haemoconcentration, rather than *de novo* synthesis.

Interestingly, haemolymph Hc concentrations of juvenile *H. rubra* and *H. laevigata* are reduced in the presence of nitrite both *in vivo* and *in vitro* (Harris and Burke 2001). Haemolymph Hc levels in *H. rubra*, as determined by absorbance, dropped by more than 50% after 3 days exposure to a high sodium nitrite environment. Nitrite is a serious toxin in many aquaculture systems, and serves not only to deoxygenate Hc, but also may react further with the protein to form metHc. As a result, nitrite reduces both the amount of functional Hc and the carrying capacity of Hc in the haemolymph of *H. rubra* (Harris and Burke 2001).

It has been hypothesised that abalone inhabiting sheltered waters might exhibit physiological differences to those inhabiting exposed oceanic environments (Wells *et al.* 1998b). In *H. iris*, no difference was observed in haemolymph Hc concentrations between

individuals from a sheltered bay environment and those from a site routinely exposed to heavy wave action (Wells *et al.* 1998b). It is not certain that *H. rubra* from Mornington possess relatively high concentrations of the respiratory protein as a result of a sheltered shallow environment.

Interestingly however, geographical differences in O_2-affinity of gastropod Hc are well documented. A reverse Root shift observed in the boreal whelk *Buccinum undatum* was not present in inhabitants of warmer waters (Brix 1983). Similarly, O_2-affinity of Hc from warm water *B. canaliculatum* was considerably higher than for *B. undatum* (Brix *et al.* 1979; Mangum and Lykkeboe 1979; Mangum 1992; Mangum 1997). This hypothesis of latitudinal differences in O_2-affinity is also supported by data on haliotids. *Haliotis cracherodii*, exhibiting a more northern distribution along the North American Pacific coast, displays a lower O_2-affinity than *H. corrugata* and *H. fulgens* (Holste 1972 as cited in Mangum 1992; Burnett *et al.* 1988).

Perhaps the elevated Hc concentration of *H. rubra* reflects the high water temperatures (as high as 30°C, Harris *et al.* 1996; Drew *et al.* 2001) known to occur in the shallows of Port Phillip Bay. Wells *et al.* (1998a) suggest the temperature effects on *H. iris* O_2-binding (decreasing affinity) limit the northern (warmer) distribution of this species. This may also hold true for *H. rubra*, and it would prove interesting to compare temperate abalone to *H. assinina*, a tropical Indo-Pacific species.

Given that, so far, no reasonable hypothesis has been suggested for the observed variation in haliotid Hc levels the current study adds to the existing body of work and prompts further investigations.

6.4.3 Oxygen carrying capacity of haemocyanin

According to Truchot (1992), O_2-carrying capacity of haemolymph is usually defined as "the maximum amount of O_2 (dissolved + bound) that can be contained in one unit volume of the air-equilibrated sample when the pigment is fully saturated." In the current study on *H. rubra*, carrying capacity relates directly to *bound* O_2. The O_2-carrying capacity of *H. rubra* Hc is substantially lower than that of other abalone species (Table 6.7). Determination of O_2-carrying capacity (Oxycon) assumed full saturation of *H. rubra* haemolymph samples (tonomotered under atmospheric PO_2, and given time to equilibrate). While it is not entirely impossible that saturation was not achieved, it may be possible that excessive manipulation or conditions under which the Hc was stored may contribute to an altered O_2-capacity.

The amount of O_2 bound by *H. kamtschatkana* Hc may be as high as 2.77 vols% (Boyd and Bourne 1995). Capacities of the haemolymphs of Californian abalone *H. cracherodii*, *H. corrugata*, and *H. fulgens* are quite similar to that determined for *H. rubra* (Table 6.7). Oxygen-binding capacities for other marine gastropods are equally variable. Brix *et al.* (1979) determined the O_2-capacity of *Buccinum undatum* Hc at about 0.45 vols% at pH 7.3, whereas Mangum and Polites (1980) recorded O_2-capacities of 1.2 to 3.7 vols% for *Busycon canaliculatum*.

The average O_2-carrying capacity of 0.33 vols% (0.44 vols% for best 4 measurements) for *H. rubra* falls short of the dissolved or dissociated fraction of O_2 at atmospheric or ambient PO_2 of 0.544 vols% (haemolymph dissolved fraction calculated as for seawater at 15°C and 150 mmHg PO_2, with solubility coefficient of 1.622, Dejours 1981). This indicates a reduced carrying capacity of the samples (possible effects of handling, freezing, denaturation), or that full oxygenation had not been achieved. Haemocyanin therefore appears to carry less O_2 than is dissolved in the haemolymph. The amount actually delivered to the tissues would be expected to be less than this, especially if the reverse Bohr shift were to persist. *Haliotis iris* Hc has an average O_2-carrying capacity of 0.522 vols% (Taylor 1993), approximating the O_2 content of seawater under the same conditions.

Using data on general body measurements for *H. rubra* obtained in Chapter 2, total body Hc and O_2 parameters for *H. rubra* may be calculated. As summarised in Chapter 2, abalone have an approximate haemolymph volume of 40-50% wet wt. Lower values of 25-30% were recorded for *H. rubra* by bleeding. If we assume that the haemolymph volume of *H. rubra* is around 50% wet weight, as has been suggested for *H. iris* and *H. asinina*, then a 300 g abalone with a shell weight of 30% (Chapter 2) possesses a haemolymph volume of around 105 mL.

The mean haemolymph protein concentration for *H. rubra* is 9.63 mg mL^{-1} (Table 6.1). Considering the molecular weight (Mwt) of a Hc functional unit at 53,000 Da (1 O_2 = 53,000 Mwt protein), *H. rubra* Hc concentration would be 0.1817 µmol mL haemolymph. This value is very similar to the mean Hc concentration calculated for *H. rubra* (Table 6. 4). A Hc concentration of 0.1817 µmol mL would bind 0.1817 µmol O_2 per mL haemolymph, or a total of 19.08 (19.1) µmol O_2 for a 300 g animal.

If 1 mole O_2 is equivalent to 22.4 L, then 19.1 µmol O_2 equates to 427 µL O_2 bound to Hc. That is, the haemolymph HcO_2 capacity is 427 µL O_2 per 300 g animal (0.43 mL, or around 0.40 vols%). This compares with the vols% values calculated for *H. rubra* in this study (0.33-0.44 vols%).

Quite a different carrying capacity was determined for *H. rubra* Hc by Ainslie (1977, 1980b). According to the relationship between the protein concentration and O_2-carrying capacity of abalone haemolymph (Figure 2 Ainslie 1980b), a *H. rubra* haemolymph sample of 9.63 mg mL^{-1} has an O_2-carrying capacity of approx. 1.6 vols%, considerably higher than the results of the current study.

Given the variation in both Hc concentrations and O_2-carrying capacities, how does one establish a relationship between the two? Although a relationship exists between haemolymph protein and carrying capacity (Ainslie 1977, 1980b), it appears that at higher intrinsic Hc concentrations, proportionally less O_2 is bound. In addition, the regression of the parameters fails to extrapolate to zero, indicating a proportion of protein that does not bind O_2 (Ainslie 1977), referred to as apohaemocyanin (Mangum 1992). Mangum (1992) suggests that this phenomenon is an artefact of scattering, inherent in most spectrophotometric analyses.

6.4.4 Oxygen affinity and binding characteristics of haemocyanin

Effects of pH

Compared with previous studies on temperate abalone, *H. rubra* Hc in this instance displays a high O_2-affinity. Half saturation values at 20°C, and arterial PCO_2 (but unspecified pH) for *H. rubra*, *H. laevigata* and *H. roei* are 28, 26 and 24 mmHg, respectively (Ainslie 1977, 1980b), higher than P_{50} values recorded for *H. rubra* in this study (Table 6.5). Similarly, P_{50} values for buffered *H. australis* and *H. iris* Hc are high (approx. 20 mmHg at pH 7.5, Wells *et al.* 1998a). Further work on *H. iris* Hc (Behrens 1999; Behrens *et al.* 2002) indicates a much lower P_{50} for native buffered haemolymph (P_{50}=3.9 mmHg at pH 6.9, and 11.6 at pH 7.7, respectively). These values parallel those of *H. rubra* in this study.

As has been demonstrated for other marine gastropods and abalone to date, *H. rubra* Hc displays a positive or reversed Bohr shift. In the functional pH range of 7.3 to 6.8 (Chapter 4), decreasing haemolymph pH as a result of hypoxia increases Hc O_2-affinity, with a general reduction in cooperativity. This Bohr shift has been identified previously for *H. rubra*, *H. laevigata* and *H. roei*, at both 20°C and 25°C (Ainslie 1977, 1980b). The greatest increase in affinity was noted as PCO_2 was increased from 0 to 3 mmHg, which it is assumed indicates a relatively abrupt decrease in pH (arterial PCO_2 1.2 mmHg for all species, Ainslie 1977, 1980b). The magnitude of the Bohr shift decreased at higher partial pressures of CO_2. The reverse Bohr effect appears to be greater for *H. rubra* than for *H. laevigata* and *H. roei*.

The Bohr factor (φ) for *H. australis* and *H. iris* remains around +0.6 to +0.7 (Wells *et al.* 1998a, Behrens *et al.* 2002), particularly between pH 6.5-7.5. The corresponding values for *H. rubra* were somewhat lower (Table 6.5). As with *H. rubra*, a high O_2-affinity (P_{50}=10 mmHg) and non-cooperative binding (n_{50} approx. 1.0) was found for the Hc of *H. kamtschatkana* (Boyd and Bourne 1995). However, a very large reverse Bohr effect persisted (φ=+2.2) in this species. A reverse Bohr factor was quantified for buffered haemolymphs of *H. rubra*, *H. laevigata* and *H. roei* (Ainslie 1977), with φ = +0.45, +0.32 and +0.30, respectively. The Bohr factor for *H. rubra* is identical to that identified in this study. Similar coefficients have been noted for *H. corrugata* at 15°C (φ=+0.35, Burnett *et al.* 1988).

Just as a reverse Bohr effect persists with haliotid haemolymph, so does an apparent pH dependence of cooperativity. Oxygen-binding usually exhibits positive or neutral cooperativity ($n_{50} \approx 1$) in most abalone species. Cooperativity of binding increases with a decrease in PCO_2 in *H. rubra*, *H. laevigata* and *H. roei* (Ainslie 1977, 1980b). Likewise, increasing positive cooperativity was noted for *H. australis* and *H. iris* Hc between pH range 6.5 and 9.0 (Wells *et al.* 1998a). Burnett *et al.* (1988) also identified a pH dependence of cooperativity for *H. corrugata* Hc, increasing with temperature. Oxygen curves for *H. iris* tended toward sigmoidicity as pH increased (n_{50} increasing from 1.29 to 1.54 as pH increased 6.96 to 7.69, Behrens *et al.* 2002). It appears that a decrease in sub-unit interaction during O_2-binding of abalone Hc occurs as haemolymph pH falls, modifying the shape of the curve from a sigmoidal to a hyperbolic manner. The haemolymph of the gastropod *Helix pomatia* displays a sigmoid dissociation curve at pH 8.2 and a hyperbolic curve at pH 7.0 (Ghiretti 1966).

The role of a reverse Bohr shift has been demonstrated in the whelks *Busycon canaliculatum* (Mangum and Lykkeboe 1979) and *Buccinum undatum* (Brix *et al.* 1979; Brix 1982). With a drop in ambient PO_2 (150 to 11 mmHg), *in vivo* haemolymph pH of *B. undatum* falls from 8.1 to 7.5. Exposure to hypoxic conditions, for example during late summer stagnant low tides, increases the O_2-affinity of the haemolymph due to the Bohr shift, favouring loading of O_2. Similarly, changes in salinity and temperature, both experienced by *B. canaliculatum* over its geographic range, influence affinity and the strength of the reverse Bohr shift. In conjunction, the large reverse Root effect typical of these gastropods ensures that haemolymph O_2-saturation is maintained and protected at low pH (Brix *et al.* 1979; Brix and Torensma 1981; Brix 1982), by increasing the effective levels of O_2 in the haemolymph without altering the Hc concentration (Brix 1983).

A reverse Root effect might also be expected for abalone haemolymph. This shift would see a decrease in the amount of O_2 bound at higher pH, assuming full saturation.

Consequently, the carrying capacity of the haemolymph would be expected to decrease. A reverse Root shift, suggested for *H. iris* (Wells *et al.* 1998a), could not wholly be determined for *H. rubra*.

A reverse Root effect would be observed as a decrease in the amount of O_2 bound by fully oxygenated Hc at high pH. The variability in the present data render it difficult to determine. Also, assuming the air-saturated values for tonomotered haemolymph as 100% bound (done to construct the curves), automatically eliminates the Root effect. The apparatus used to determine O_2-content (Oxycon) relies on liberation of all O_2 from the sample to determine concentration. This of course includes the O_2 in solution. It was not considered practical to use pure O_2 in preference to air to achieve full saturation, as the dissolved fraction at high PO_2 would have been large and 'swamped' the bound fraction (H.H.Taylor University of Canterbury, pers. comm). This problem can be avoided by determining HcO_2 spectrophotometrically, which does not register dissolved O_2.

Despite buffered haemolymph displaying a linear relationship between P_{50} and pH between pH 6.8 to 8.4, and a reverse Bohr effect, Ainslie (1977) found that even the small amount of buffer added to adjust pH caused an increase on O_2-affinities (Ainslie, 1977). At 20°C, the P_{50} of freshly drawn *H. rubra* haemolymph at pH 7.38 was 44.7 mmHg, compared to 25.0 mmHg of an equivalent sample buffered to pH 7.38. Even though the above determination was performed on only a single sample, Ainslie (1977) raises some speculation on the validity of using buffered samples, emphasising care in interpreting results with buffered Hc. Despite this, studies using buffered haemolymph are considered more expedient and stable, as described above. Comparisons of results therefore should indicate the treatment of the Hc.

Effects of D-lactate

The addition of 5 mmol L^{-1} D-lactate to *H. rubra* Hc resulted in a right-shift in the binding curve and a negative (normal) Bohr factor, although this shift did not significantly affect the O_2-binding properties of the respiratory protein. The reason for this reversal in the Bohr factor (from reverse to normal) is most probably the addition of a neutral pH salt: the pHs of the lactate treatment haemolymph samples were virtually identical to control treatments, and any change in P_{50} without a change in pH ($\Delta\log P_{50}/\Delta pH$) is translated into a large Bohr factor. The addition of an acidic D-lactate salt would serve to mimic a metabolic acidosis more closely, but with a possibly uncontrollable pH fluctuation. It is possible that any effects of the organic acid on abalone Hc *in vitro* manifest themselves purely in the form of pH perturbations (dissociation), as opposed to allosteric modification by binding. The

experiments of this study specifically isolate the effects of D-lactate, with no associated change in pH.

The modulatory effects of L-lactate on arthropod Hc are well known. L-lactate raises the O_2-affinity of crustaceans (left-shift of OBC), without changing the Bohr effect (*i.e.* effect of lactate independent of pH, Truchot 1980; Graham *et al.* 1983). Truchot (1980) suggests the *in vitro* changes of O_2-affinity are probably a specific effect of lactate alone. The role of this lactate effect opposing the normal Bohr shift is demonstrated by haemolymph buffered to low pH with added lactate showing equal O_2-affinities to haemolymph of physiologically low pH and endogenous lactate. The decrease in *Callinectes sapidus* Hc O_2-affinity following exercise is minimised by the 14-fold increase in haemolymph lactate (Booth *et al.* 1982). The terrestrial crab *Gecarcoides natalis* is unique among crustaceans in that increasing haemolymph L-lactate concentrations reduce Hc O_2-affinity beyond that predicted by the Bohr shift alone (Adamczewska and Morris 1998), increasing availability to the tissues.

Haliotis iris Hc shows only minor depressant effects on O_2-affinity and cooperativity by 5 or 10 mmol L^{-1} D- or L-lactate, with no difference exhibited by the two isoforms (Behrens *et al.* 2002). It is thought that insensitivity to both D- and L-lactate reflects an inability of *H. iris* Hc binding sites to distinguish between these organic ions (Behrens *et al.* 2002). Haemocyanin from the whelk *Busycon contrarium* was reported to be insensitive to both D- and L-lactate (Mangum 1983b, 1992). A reverse Bohr shift is present in a buffer system resembling haemolymph of anaerobic *Helix pomatia* (Weiser 1981). After up to 45 h anoxia, D-lactate reaches levels in *H. pomatia* haemolymph of 60 mmol L^{-1}, and O_2-affinity of the Hc is increased. The relative contribution of this elevated level of D-lactate to the Bohr shift can not be ascertained however.

D-lactate is also a major anaerobic end product in the horseshoe crab *Limulus polyphemus* (Carlsson and Gäde 1985, 1986). In *L. polyphemus*, the net result of D-lactate production and release into the haemolymph is an increase in O_2-affinity, due not to an allosteric action of the metabolite but to its effect on haemolymph pH and the attendant reverse Bohr shift (Mangum 1983a).

Mangum (1983b), in a review of lactate effects, proffers that the adaptive correlates of lactate sensitivity are beneficial only where a large normal Bohr shift and an acidotic response to hypoxia exist. The whelk *Busycon contrarium* apparently lacks both (Mangum 1983a, b). The lactate response of crustaceans is basically a mechanism that reduces or alleviates the otherwise maladaptive consequences of a large normal Bohr shift accompanying metabolic acidosis (Mangum 1983a, b, Truchot 1992). The response is unable to totally reverse the

Bohr shift, but it reduces the decrease in O_2-affinity brought about by the acidosis. In contrast, it appears gastropod Hcs either have a large reverse Bohr shift or very little pH dependence at all within the physiological range, and anaerobic metabolism either fails to cause an acidosis or the acidosis raises O_2-affinity, a far more effective mechanism of dealing with hypoxia (Mangum 1992, 1997).

The insensitivity of molluscan Hc to organic effectors is not restricted to gastropods. Terwilliger and Terwilliger (1987) observed that succinate, D-lactate, alanine, propionate and acetate, all molluscan metabolic end products, show little or no effect on Hc O_2-binding of Hc of the chiton *Katharina tunicata*. At 15°C and pH 7.4, the addition of 20 mmol L^{-1} D-lactate failed to raise the P_{50} of *K. tunicata* Hc.

Generally, it is agreed that molluscan Hc is insensitive to organic effectors such as lactate. To date, little convincing data suggests otherwise. The present data do not entirely satisfy this generalisation, and it is important to follow up this study with a more controlled and detailed account of the specific actions of organic effectors, including other metabolites such as tauropine, on *H. rubra* haemolymph. Although not significant, there is a possible positive effect of the lactate (or lithium) ion that increases P_{50}. If lactate in abalone were released as lactic acid, this possible lactate effect could counteract the pH effect, in an opposite manner to that seen in crustaceans. Based on a low number of trials for *H. rubra* however, conclusions on D-lactate effects must be tentative.

6.4.5 Role of haemolymph and haemocyanin in abalone O_2-delivery

Reverse Bohr shift

The current data unequivocally demonstrates a reverse Bohr shift in *H. rubra* Hc, supporting earlier findings on abalone. Measurements of pH of resting and hypoxic-stressed animals indicate the shift may operate during normal physiological function in *H. rubra*. What is not known for the current data set are the equivalent PO_2 and PCO_2 values. In constructing OBCs on buffered haemolymph, Ainslie (1977) obtained P_{50} values corresponding to arterial haemolymph at pH values between 7.55 and 7.60. Curves with P_{50} values corresponding to curves of venous (renal sinus) haemolymph were obtained at pH values between 6.85 and 7.02. These values are supported by intermediate pedal sinus pH recordings of 7.30 for *H. rubra*, identical to those of the current study (Chapter 4).

A reverse Bohr shift functionally impairs the offloading of O_2. A decrease in cooperativity would exacerbate the condition. As a result, a considerable venous O_2-tension may develop. In this respect, an O_2 reserve may form. A reverse Root shift, independent of hypoxia or temperature, is observed for *H. iris* by Wells *et al.* (1998a). No convincing

hypothesis has been presented for its significance, although Wells *et al.* (1998a) infer a role in ensuring an increased O_2-carrying capacity as pH drops. Although fully saturated only below pH 7.0, *H. iris* Hc is already 90% saturated at resting pH (7.39).

The attributing of a significant role for the Bohr shift to abalone biology requires some measurement of *in vivo* pH and O_2-tension. The *in vivo* pH for a number of abalone are given in Chapter 4 (Table 4.2). It does not appear that haliotid haemolymph expresses a bi-phasic response to pH, as is observed in other marine gastropods (Mangum and Lykkeboe 1979). However, P_{50} values for *H. laevigata*, *H. roei*, and *H. rubra* Hc appear to plateau at pH 8.3-8.4 (Ainslie 1977). At higher pH, which must be considered highly un-physiological, a normal Bohr shift may appear. More likely though is the risk of Hc dissociation (Nickerson and van Holde 1971; Ellerton and Lankovsky 1983; Ellerton *et al.* 1983). So far, *in vitro* and *in vivo* determinations of abalone haemolymph pH, PO_2 and PCO_2 all suggest that the Bohr shift and cooperativity decline operates under physiological conditions (Ainslie 1977, 1980b; Wells *et al.* 1998a; Behrens *et al.* 2002). For those animals that experience hypoxic or hypercapnic ambient conditions, or a metabolic or respiratory acidosis, the left-shift of the O_2-equilibrium curve theoretically provides a large venous O_2 reserve. This offers some protection against internal hypoxia, and may provide O_2 for more dependent organs. If encountering an acidic environment in the pedal musculature, haemolymph lacking the reverse Bohr shift may offload a significantly greater proportion of bound O_2, rendering sensitive tissues downstream of the pedal muscles, such as the renal organs, virtually anoxic. In addition, a normal Bohr shift would impair loading at the ctenidia interface. The reverse Bohr and, if present, Root shifts allow a considerable venous O_2-content. At critical water PO_2 of 30 mmHg, the haemolymph of the whelk *Buccinum undatum* may contain up to 0.44 vols% O_2. For *Busycon canaliculatum* and *Buccinum undatum*, a safeguarding of internal PO_2 for the nephridia has been proposed (Mangum 1980; Mangum and Polites 1980; Polites and Mangum 1980; Brix 1982).

For *Buccinum undatum*, the reversed Bohr and Root shifts allow to animal to maintain a near normal O_2-uptake and transport down to ambient water tensions of 25 mmHg, which appear seasonally in the habitat (Brix *et al.* 1979). On occasion that O_2-tensions drop to near zero, snails can survive for several days by shifting to anaerobic metabolism (Brix *et al.* 1979). For species encountering environmental or functional hypoxia, the reverse Bohr shift serves as an adaptation, avoiding low internal PO_2 and maintaining a large venous reserve. Whilst this confers an increased binding of potentially limiting O_2 in the media, a decrease in offloading at the tissues would seem somewhat of a paradox. What benefits are there to withholding O_2 from metabolising tissues, especially those experiencing functional hypoxia?

For these tissues to access bound O_2, O_2-tension is required to fall to very low levels. As Behrens *et al.* (2002) suggest, rather than impair unloading, the increased affinity and decreased cooperativity may shift unloading to lower critical levels, where anaerobiosis may supplement anaerobiosis. Secondly, the large venous reserve of O_2 may function as a haemolymph O_2 store, a view expressed repeatedly for abalone (Wells *et al.* 1998a; Behrens *et al.* 2002).

Pedal and radula muscle oxygenation in *Haliotis rubra*

The current study highlights the variability of Hc concentrations in abalone, and suggests that while the reverse Bohr shift occurs in a physiological pH range, D-lactate in the haemolymph may not significantly affect Hc function. How do these results, combined with the cardiovascular physiology, affect muscle oxygenation in *H. rubra*?

At rest, with haemolymph pH at 7.3 (Chapter 4), the P_{50} of *H. rubra* Hc would be around 11-12 mmHg, at 15°C. Ainslie (1977, 1980b) estimates *H. rubra* P_{50} at 25-28 mmHg, at 20°C and normoxic pH and CO_2. These affinities however may not greatly influence the oxygenation of abalone radula muscles. The affinity of *Sulculus diversicolor* radula muscle myoglobin (Mb) is much higher than Hc values, at 3.8 mmHg (Suzuki *et al.* 1996; Suzuki and Imai 1997; Chapter 3). As a consequence, Mb becomes saturated at a low PO_2, maintaining a low O_2-tension and high O_2-gradient (Manwell 1958). Providing there is a favourable haemolymph supply, O_2-delivery to the radula muscles should not be compromised.

During hypoxia as haemolymph pH decreases and the reverse Bohr shift results in an increased Hc O_2-affinity, the haemolymph retains O_2 and may function as a store for O_2-dependent tissues. At a physiological pH of 6.8, the P_{50} of *H. rubra* Hc decreases to around 6.4 mmHg (assuming decrease is linearly related to pH), and it is possible that Mb still maintains a higher affinity than Hc. The metabolic response of the pedal musculature to hypoxic treatments is probably a combination of a decrease in ambient O_2-availability, decreased Hc O_2-offloading as a result of the Bohr effect, and a possible reduction in haemolymph flow. This scenario, in association with an increased energy demand, would contribute to the accumulation of anaerobic metabolites in *H. rubra* tissues.

Even though tissue-specific cardiac output and haemolymph distribution data are not available for *H. rubra*, metabolite results suggest radula muscles receive sufficient O_2 to remain aerobic during whole animal hypoxia and work during feeding. In addition to the high O_2-affinity of Mb, which is lacking in adductor and foot muscles, *H. rubra* radula muscles are favourably positioned in the cephalic sinuses, which further ensures constant oxygenation of these muscles (Figure 6.1). However, Section 6.1 (this chapter) highlights differences

between estimated volumes and rates of haemolymph flow to tissues. Jorgensen *et al.* (1984) and Just (2002) estimate a tissue-specific cardiac output to the radula muscles and the head of *H. cracherodii* and *H. iris*, respectively, at only between 1-3%, compared to much higher values for the pedal musculature. Russell and Evans (1989) suggest the failure of the technique used by Jorgensen *et al.* (1984) results in an underestimate of haemolymph flow to the head and radula muscles in abalone.

Unless the anterior aorta is occluded or the cephalic region of abalone is severely compressed, haemolymph flow to the CAS and thus the radula muscles probably remains fairly reliable. Although not fully identified for any one species of abalone, recall the presence of valves partitioning the cephalic sinuses and the pedal circulation. Although resolution of cephalic and pedal sinus valves was not achieved for *H. rubra* during dissections in Chapter 2, observations of haemolymph pooling behind the head in *H. rubra* (Drew *et al.* 2001), and the decrease in adductor and foot haemolymph flow during emersion and clamping for *H. iris* (Taylor *et al.* 2001; Just 2002), suggest that there may be a by-passing of the pedal circulation in abalone under certain physiological challenges. In addition, haemolymph was more difficult to obtain from the pedal sinuses of *H. rubra* that had been disturbed or exposed to air (Chapters 2 and 4), further supporting any suggestion that haemolymph and O_2 may be directed toward more O_2-dependent organs in abalone. If these assumptions are correct, then the radula muscles may be assured an O_2 supply, despite the variable concentration of Hc. In terms of the pedal musculature then, O_2-delivery by Hc may be of little significance. The distribution of haemolymph potentially may be of more importance than the O_2-binding characteristics or Hc concentration.

The current data suggest that the O_2-carrying capacity of *H. rubra* haemolymph is considerably lower than that of other haliotids and gastropods. At atmospheric PO_2, the haemolymph dissolved O_2 fraction is equal to or larger than that carried by Hc (this study, Taylor 1993), which challenges the physiological relevance of Hc. Clearly, at atmospheric conditions, Hc does not increase the O_2-carrying capacity of *H. rubra* haemolymph. However, the PO_2 of haemolymph *in vivo* would be lower than atmospheric. According to Ainslie (1977, 1980b), the mean arterial and venous PO_2 at 20°C for *H. rubra* are 38.8 mmHg and 11.5 mmHg, respectively. The mean PO_2 of the pedal sinus is intermediate to the above range, at 21.6 mmHg. The PO_2 of haemolymph from the CAS of resting *H. iris* is similar to that reported for *H. rubra* (20.3 mmHg, Behrens *et al.* 2002).

A typical working O_2-binding curve for *H. rubra* Hc at pH 6.9 is given in Figure 6.8. According to this curve, the amount of O_2 bound to Hc is greater than that dissolved in the

haemolymph, up to a PO_2 around 50 mmHg,. Even at arterial partial pressures (up to 38 mmHg, Ainslie 1977, 1980b), the bound fraction is greater than the dissolved component. Under these circumstances, the O_2-binding of Hc would assume a greater importance in the transport of O_2.

Oxygen-carrying capacities of other Australian temperate species are considerably higher than those of *H. rubra* (Table 6.7). The amount of O_2 actually *delivered* to the tissues was determined by Ainslie (1980b) for *H. laevigata*, *H. roei* and *H. rubra* as the arterio-venous difference, controlling for the dissolved fraction at the given PO_2. For a typical *H. rubra*, the whole haemolymph at 20°C delivers 0.56 vols% to the tissues, with Hc contributing by far the greatest part of this total (about 86%, Ainslie 1977, 1980b). The importance of the protein in delivering O_2 is similar for *H. laevigata* (0.62 vols%; Hc 87% of total) and *H. roei* (0.90 vols%; Hc 91% of total). Even with an increase in temperature from 20°C to 25°C and a net decrease in amount of O_2 delivered for all species, over 80% is delivered by the protein (Ainslie 1977, 1980b).

Despite these studies suggesting that Hc delivers a significant proportion of O_2 to the tissues abalone, large variations in Hc concentrations must influence the importance of Hc O_2-delivery. While the relative contribution of Hc to O_2-delivery may not alter, the amount may be seriously compromised when *H. rubra* Hc concentrations vary 10 fold (1.0 to 9.9 mg mL^{-1}, Ainslie 1980a). Even larger variations are observed for other species (Table 6.6). Given the variability of Hc concentrations, some authors have virtually disclaimed a respiratory function for gastropod Hc. Pilson (1965) concluded that the large, apparently random variation was not compatible with the idea that Hc acts as either a respiratory pigment, or as a storage. Such large discrepancies in concentration are not consistent with a precise O_2-delivery role. Similarly, a large variation in *B. canaliculatum* haemolymph copper lead Betzer and Pilson (1974) to conclude that considerable doubt exists over the primary physiological function of Hc.

Haemolymph O_2 characteristics of *Haliotis* appear more variable than the biochemical profiles or responses to hypoxia observed for the genus. Variable Hc concentrations and a low O_2-capacity, in conjunction with a reverse Bohr shift and interruptions in haemolymph flow that may occur, suggests Hc O_2-delivery may not play an important role in energy metabolism in *H. rubra* pedal musculature during exercise or air exposure in *Haliotis*.

6.4.6 Overview of the haemolymph O_2-transport system of *Haliotis rubra*

Examination of the literature highlights the inter- and intra-specific variation of Hc and respiratory characteristics of gastropod haemolymph. One of the aims of this study was

to correlate fundamentally identical results derived from a diversity of techniques. These results generally agree, although the most appropriate methods are probably those based on absorbance, and derived from a large data set. As noted by Truchot (1992), since Hc is the major haemolymph protein, protein concentration, copper concentration and bound O_2 can all be considered valuable quantitative indices of not only Hc, but O_2-capacity as well.

The use of various methods of estimating Hc concentration achieved similar results in terms of μmol mL^{-1}. Obviously, results in the form of optical absorbance, copper, O_2 or protein content require transformation into a common unit to allow comparison. A strong linear relationship exists between estimates of haemolymph absorbance, bound O_2 and copper, and the results using the extinction coefficients derived from these analyses generally agree. The amount of O_2 bound or carried by *H. rubra* Hc is lower than previously determined for this species, and is at the lower end of the range reported for abalone. This may reflect some effect of freezing or excessive handling of the haemolymph samples.

As with the biochemical profiles of *H. rubra* muscles (Chapter 3), identical experimental techniques and conditions in the current study allow comparison of results both between haemolymph samples, and between methods. Extrapolating data from other studies on gastropod Hc, such as when using extinction coefficients or P_{50} values, introduces the problem of differences in experimental conditions or technique.

Similar to many other marine gastropods, a reverse Bohr effect is observed in the functional pH range for *H. rubra*, although the significance of the effect is unclear. As pH drops, both the O_2-binding and unloading of *H. rubra* Hc is impaired, which may partially account for the accumulation of anaerobic metabolites in muscle (Chapter 4). The position of the radula muscles in the cephalic sinus may protect these muscles from a severe hypoxia which, in association with a potentially high Mb O_2-affinity, may explain the lack of anaerobic metabolites in this muscle. D-lactate, which appears in the haemolymph following hypoxia, apparently demonstrates little importance as a modulator in haliotids. However, a more extensive examination of this phenomenon is suggested. The reverse Bohr effect may play a role in maintaining a large venous O_2 reserve during hypoxia in *H. rubra*, as it does for other marine gastropods.

Haemocyanin levels remain a variable entity amongst abalone and gastropod populations, for reasons yet to be quantified. This variability may influence the importance of Hc in O_2-delivery in abalone.

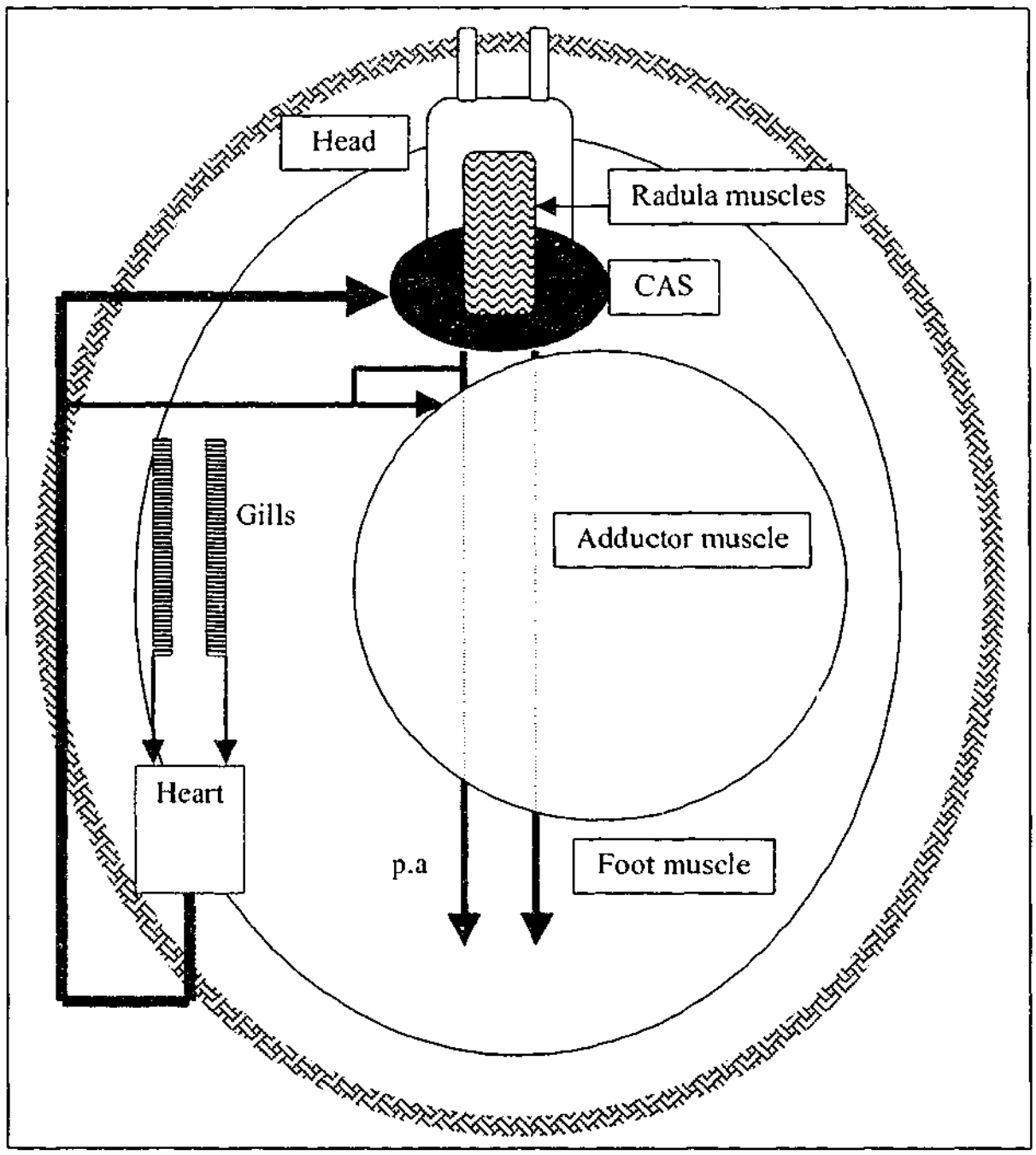


Figure 6.1. Simplified representation of the arterial haemolymph supply to the radula and pedal musculature of *H. rubra*. Haemolymph flow is indicated by direction of arrows. Heavy lines indicate the common arterial trunk and anterior aorta. The radula muscles lie within the CAS. The foot muscle is perfused by the paired pedal arteries (p.a; shown dashed as they pass ventral to the adductor muscle), and the adductor muscle is perfused by a branch of the anterior aorta, and/or the pedal arteries. The paired epipodial arteries, supplying the epipodium and lateral margin of the foot muscle, are not shown.

Table 6.1. Haemocyanin concentrations of *H. rubra* haemolymph (mg mL^{-1} protein).

Animal	N	Haemocyanin (mg mL^{-1} protein)
Captive	9	9.63 ± 0.77
Wild	12	9.53 ± 0.69
Total	21	9.57 ± 0.50
Extra captive	5	10.65 ± 0.82

Values given as Mean ± S.E.M of duplicate recordings for N animals.

Table 6.2. Bound O_2 (mmol L^{-1}), O_2-carrying capacity (vols%), protein concentration (mg mL^{-1}), absorbance (Abs units) and copper content (mmol L^{-1}) of *H. rubra* Hc.

Sample	Bound O_2	Vols %	Protein	Abs 346 nm	Diff Abs 346 nm	Copper
1	0.21	0.47	13.64	5.6 ± 0.09	5.05	0.41 ± 0.01
2	0.16	0.35	12.41	3.9 ± 0.09	3.56	0.33 ± 0.01
3	0.19	0.69	14.46	7.73 ± 0.16	7.26	0.59 ± 0.01
4	0.11	0.25	10.26	3.1 ± 0.1	2.52	0.37 ± 0.01
5	0.06	0.12	5.88	1.72 ± 0.07	1.17	0.13 ± 0.01
6	0.03	0.08	5.36	1.64 ± 0.12	1.06	0.11 ± 0.01
Mean ± S.E.M	0.15 ± 0.04	0.33 ± 0.09	10.33 ± 1.40	4.00 ± 0.95	3.44 ± 0.96	0.33 ± 0.08

Bound O_2 = total O_2 - O_2 in solution (mean of duplicates)

Vols % = mL O_2 100 mL^{-1} haemolymph

Protein from biuret (mean of duplicates)

Absorbance at 346 nm and 1 cm light path for 1 mL undiluted haemolymph (mean of triplicates)

Diff Abs = absorbance corrected for scattering

Copper from µg L^{-1} (ppm) (mean of 4 trials)

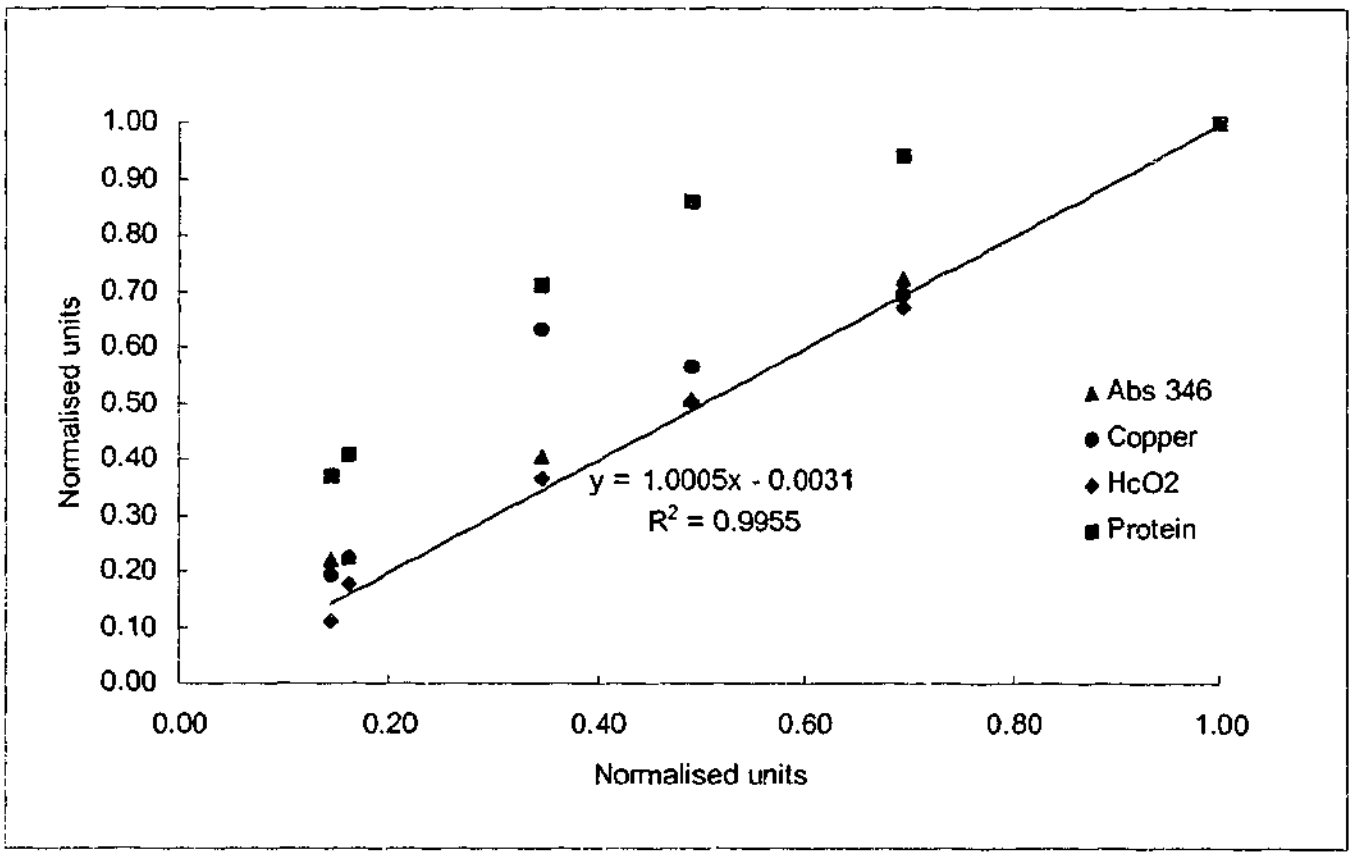


Figure 6.2. Normalised linear plot of *H. rubra* haemolymph absorbance (at 346 nm), copper, bound oxygen (HcO_2) and protein content against differential absorbance (at 346 nm, corrected for scattering). The regression shown is for HcO_2 against differential absorbance.

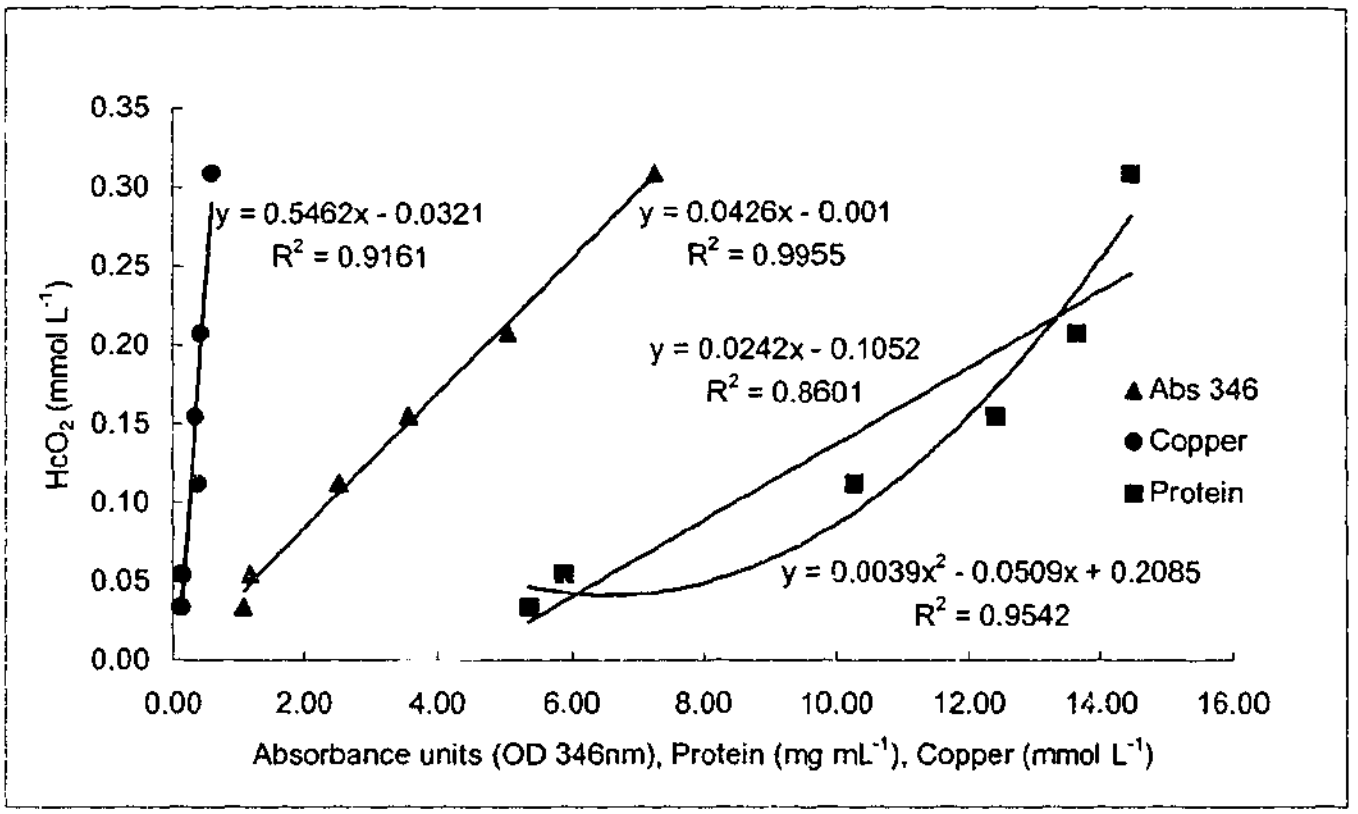


Figure 6.3. Linear regression plots examining relationship of *H. rubra* haemolymph absorbance (at 346 nm), copper (mmol L^{-1}) and protein (mg mL^{-1}) to bound oxygen (HcO_2, mmol L^{-1}). Regression equations are given for each curve. Relationship between protein and bound oxygen alternatively examined by polynomial regression.

Table 6.3. *Haliotis rubra* Hc extinction coefficients (E_{346} mM^{-1} cm^{-1}) based on Oxyhaemocyanin (HcO_2) and Copper (units Abs/(mmol L^{-1})) for data uncorrected and corrected for scattering, and ratio of extinction coefficients for corrected data.

Sample	HcO_2	Copper	HcO_2	Copper	Ratio
	Uncorrected		Corrected		
1	27.14	13.78	24.31	12.34	1.97
2	25.49	11.85	22.98	10.68	2.15
3	25.29	13.26	23.50	12.32	1.91
4	28.06	8.45	22.45	6.76	3.32
5	32.25	13.36	21.43	8.88	2.41
6	50.59	15.03	31.50	9.36	3.37
Mean ± S.E.M	31.46 ± 3.96	12.62 ± 0.93	24.36 ± 1.48	10.06 ± 0.88	2.52 ± 0.27

Table 6.4. Haemocyanin concentration of *H. rubra* haemolymph (mmol L^{-1} functional unit) calculated from haemolymph protein, extinction coefficients derived from bound O_2 (HcO_2) and copper analyses, and bound O_2 (HcO_2).

Sample	Protein[1]	HcO_2 coefficient[2]	Copper coefficient[3]	HcO_2[4]
1	0.26	0.21	0.25	0.21
2	0.23	0.15	0.18	0.16
3	0.27	0.30	0.36	0.31
4	0.19	0.10	0.13	0.11
5	0.11	0.05	0.06	0.06
6	0.10	0.04	0.05	0.03
Mean ± S.E.M	0.19 ± .03	0.14 ± 0.04	0.17 ± 0.05	0.15 ± 0.04

[1] = Protein values (mg mL^{-1}) converted to mmol L^{-1} by dividing by Mwt Hc functional unit (53,000 Da, Ellerton *et al.* 1983), multiplied by 1000.

[2] = Absorbance values converted to mmol L^{-1} using extinction coefficient based on bound O_2 (HcO_2).

[3] = Absorbance values converted to mmol L^{-1} using extinction coefficient based on copper.

[4] = Bound O_2 (HcO_2 mmol L^{-1})

Table 6.5. Oxygen affinity (P_{50}), binding cooperativity (n_{50}) and Bohr factor (φ) of *H. rubra* Hc at varying pH and 5 mmol L^{-1} D-lactate. Summary data refers to single curve for all data of the same treatment, whereas average data refers to mean ± S.E.M of all curves for each treatment.

Sample		Summary			Average		
	pH	P_{50} mmHg	n_{50}	φ	P_{50} mmHg	n_{50}	φ
pH 6.3	6.46	4.18	0.85		4.75 ± 1.26	0.71 ± 0.12	
pH 6.9	7.12	6.51	0.92	0.291	7.89 ± 2.1	0.72 ± 0.07	0.334
pH 7.5	7.74	13.77	1.81	0.525	15.22 ± 2.42	1.12 ± 0.46	0.460
pH 6.3 control	6.44	7.34	0.65		6.16 ± 1.99	0.74 ± 0.21	
pH 6.3 D-lactate	6.38	10.36	0.89	-2.50	9.71 ± 0.79	0.76 ± 0.16	-3.29
pH 6.9 control	7.10	9.02	0.95		10.33 ± 3.26	0.73 ± 0.13	
pH 6.9 D-lactate	7.04	10.48	1.21	-1.08	12.06 ± 4.06	0.69 ± 0.07	-1.12

N = 7 for pH 6.3 and pH 6.9 trials. N = 4 for pH 7.5, and all control and lactate trials

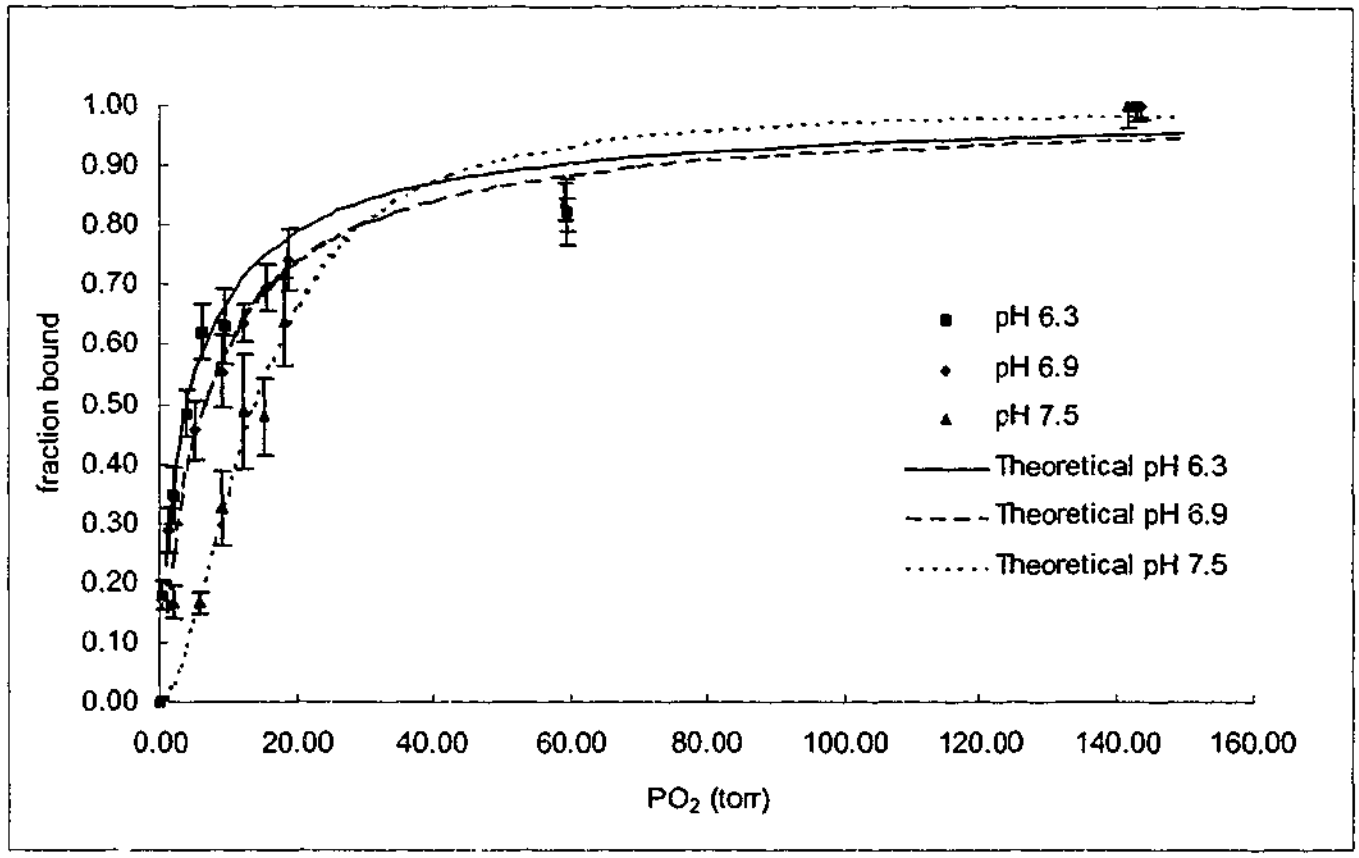
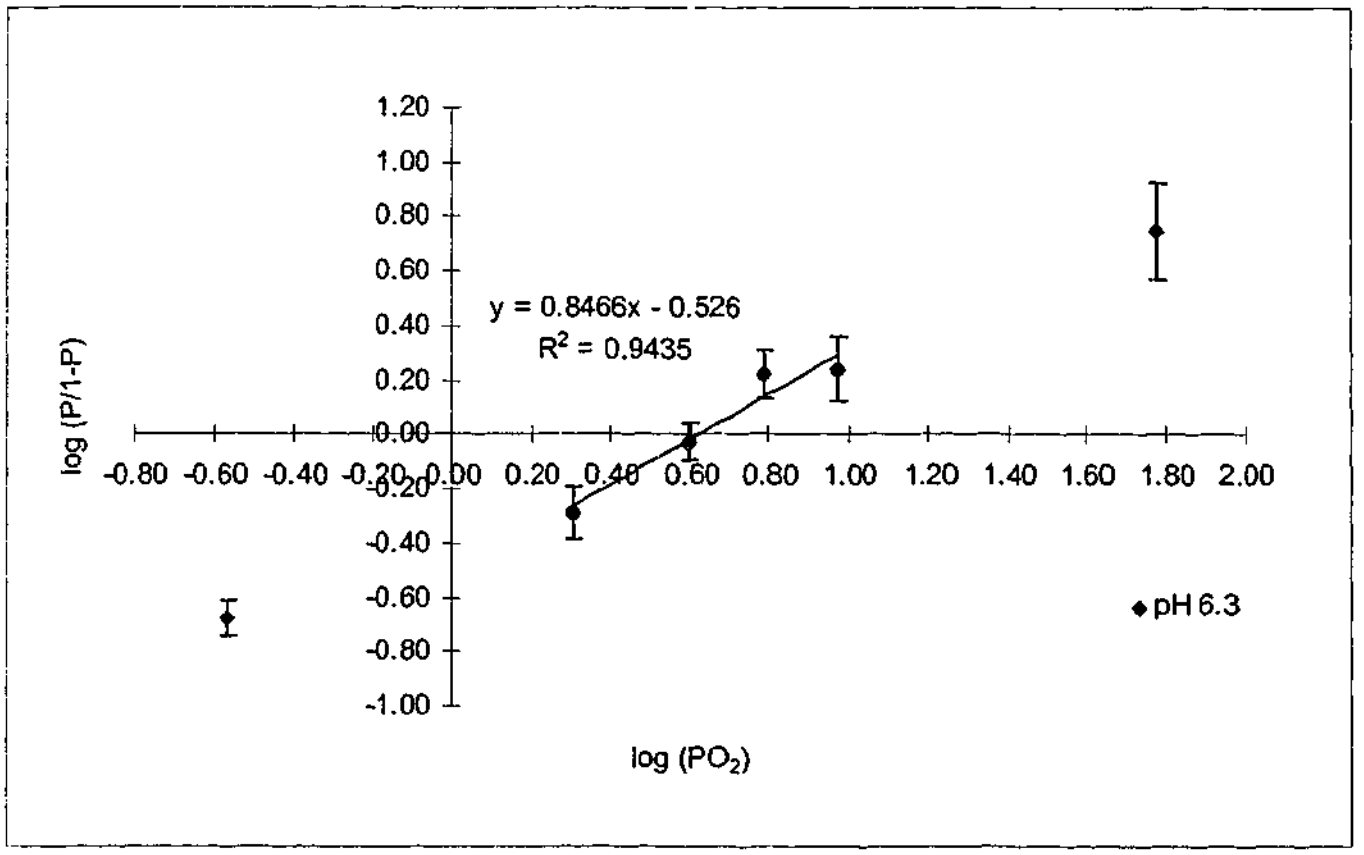


Figure 6.4. Oxygen-binding curves of *H. rubra* haemocyanin measured at 15°C in 100 mmol L^{-1} Bis-Tris or Tris-HCl buffer pH 6.3, 6.9 and 7.5. Theoretical curves calculated from Hill plot regression equations plotted over actual data (Mean ± S.E.M. fraction bound at each PO_2). Note left (reverse Bohr) shift of oxygen binding curve as pH decreases.

(A)



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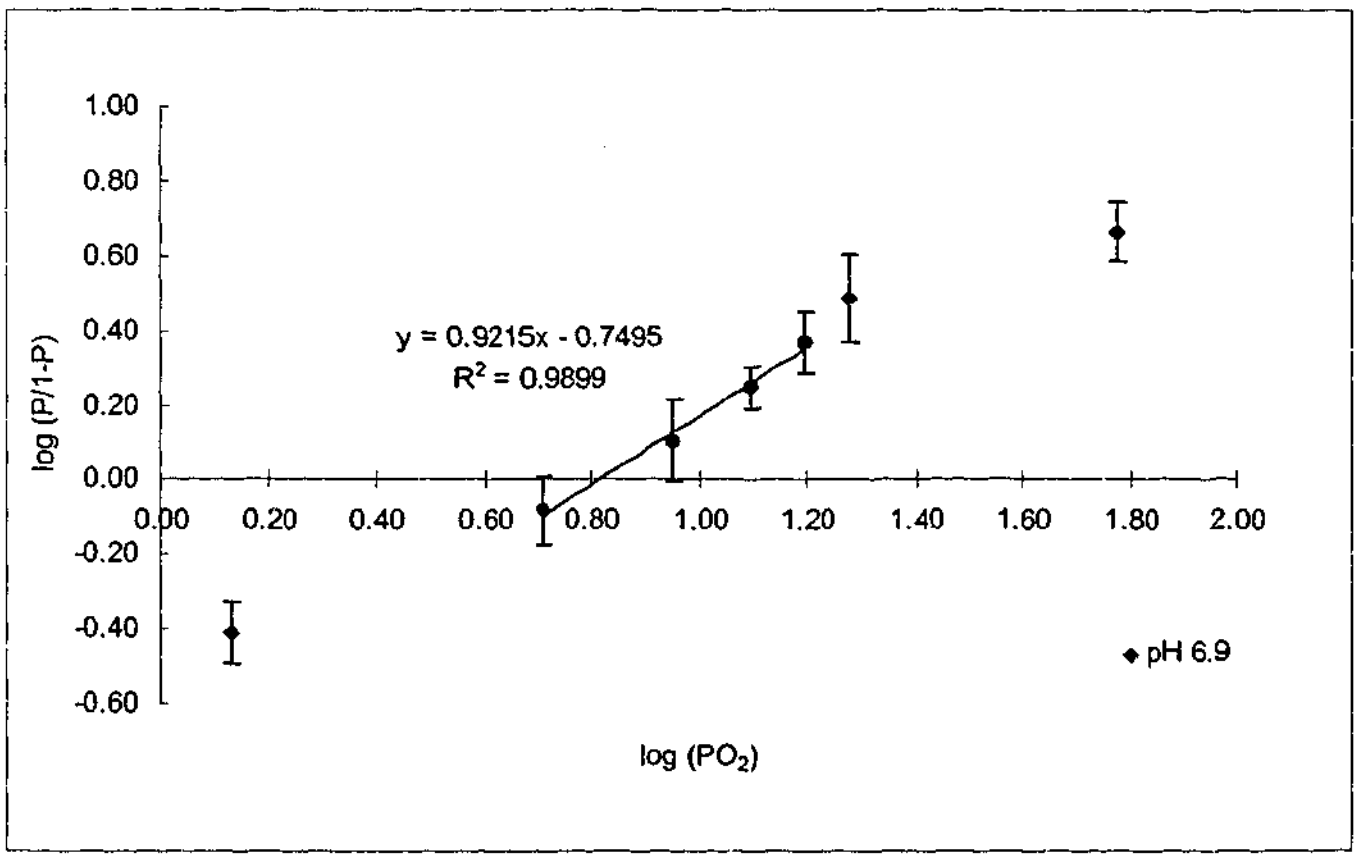


Figure 6.5. Hill plots of *H. rubra* haemocyanin oxygen binding measured at 15°C in 100 mmol L^{-1} Bis-Tris or Tris-HCl buffer at (A) pH 6.3 and (B) pH 6.9. Data are Mean ± S.E.M. log [P/(1-P)] (P, fractional oxygen saturation). Linear regression shown for data points used to calculate P_{50} and n_{50}.

(C)

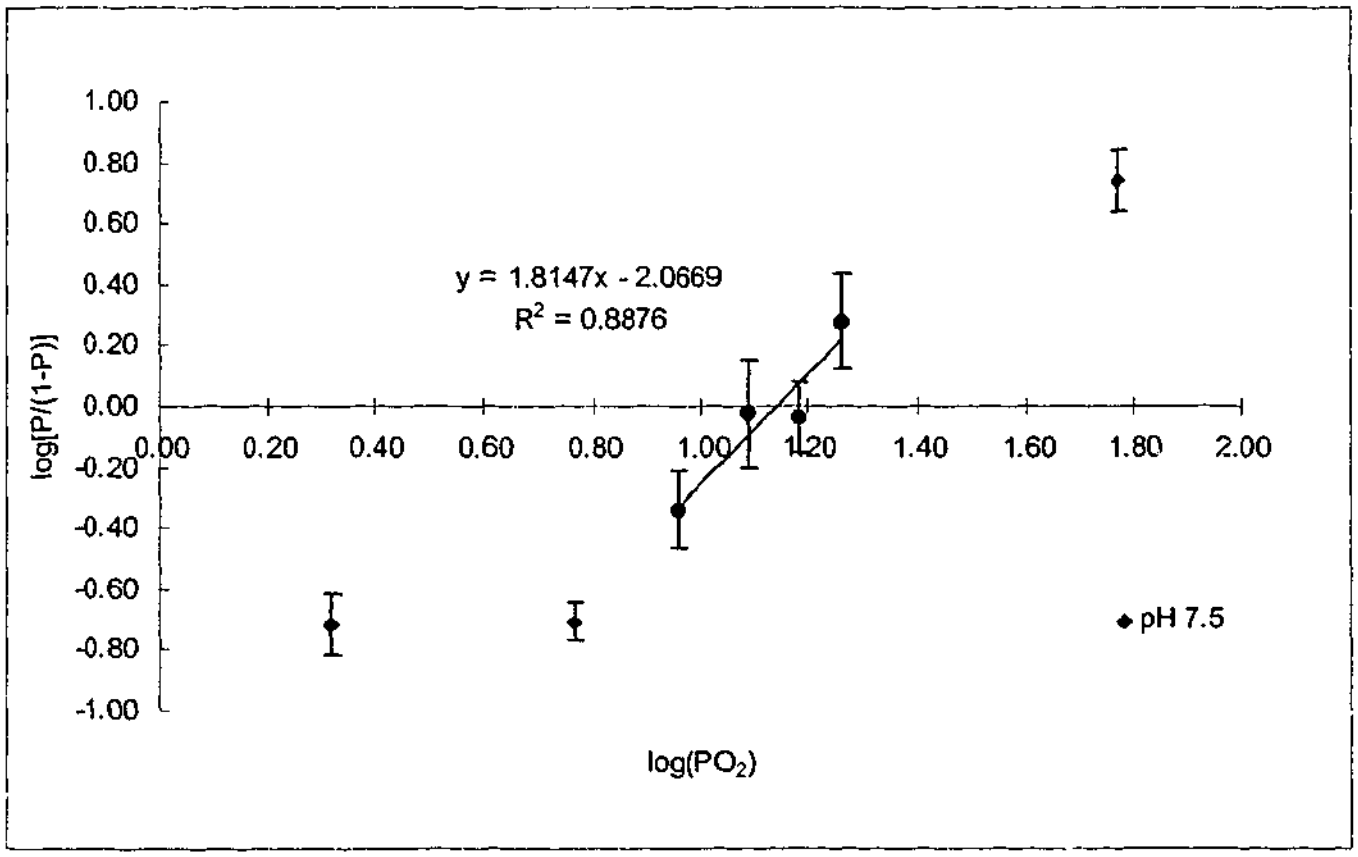
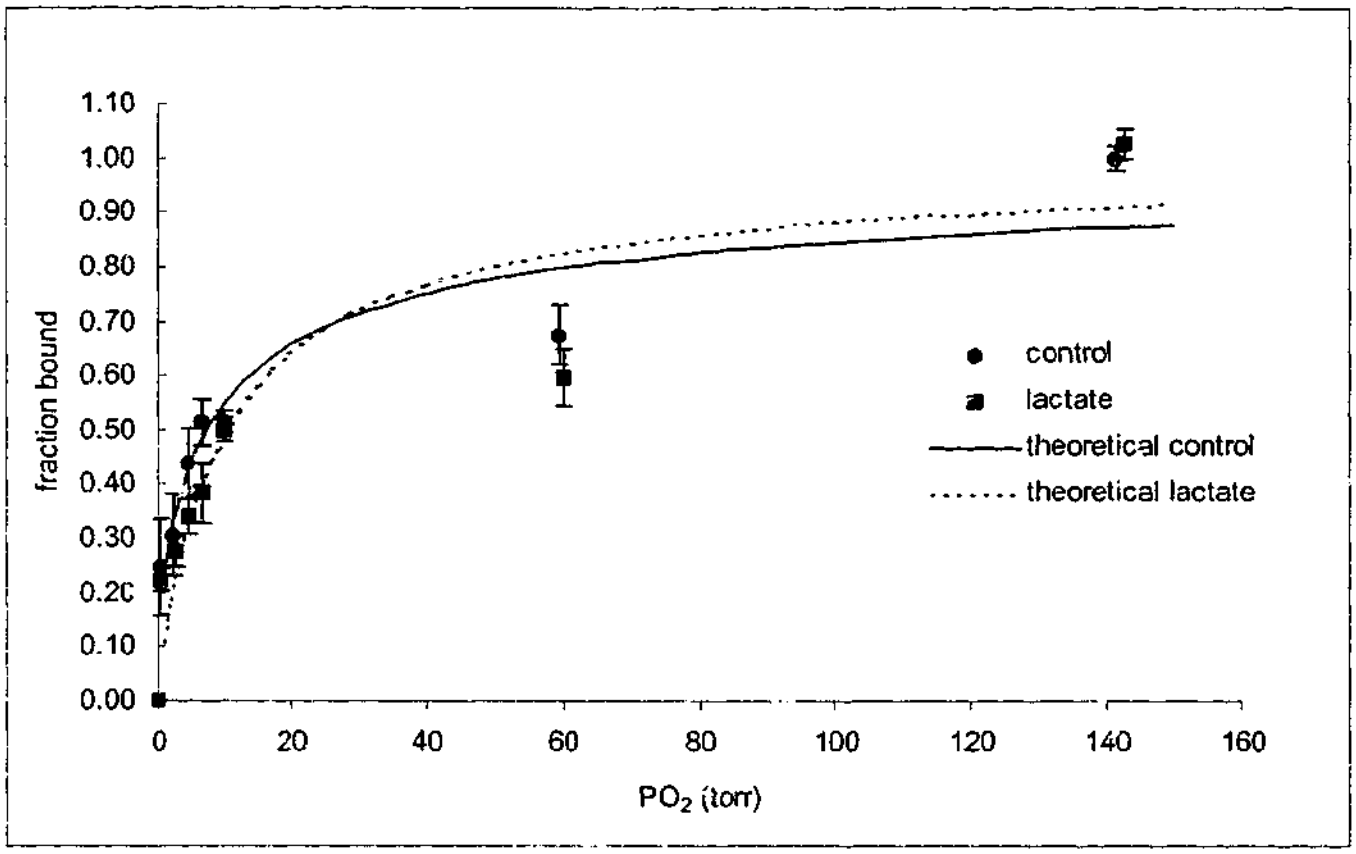


Figure 6.5 continued Hill plots of *H. rubra* haemocyanin oxygen binding measured at 15°C in 100 mmol L^{-1} Bis-Tris or Tris-HCl buffer at (C) pH 7.5. Data are Mean ± S.E.M. log [P/(1-P)] (P, fractional oxygen saturation). Linear regression shown for data points used to calculate P_{50} and n_{50}.

(A)



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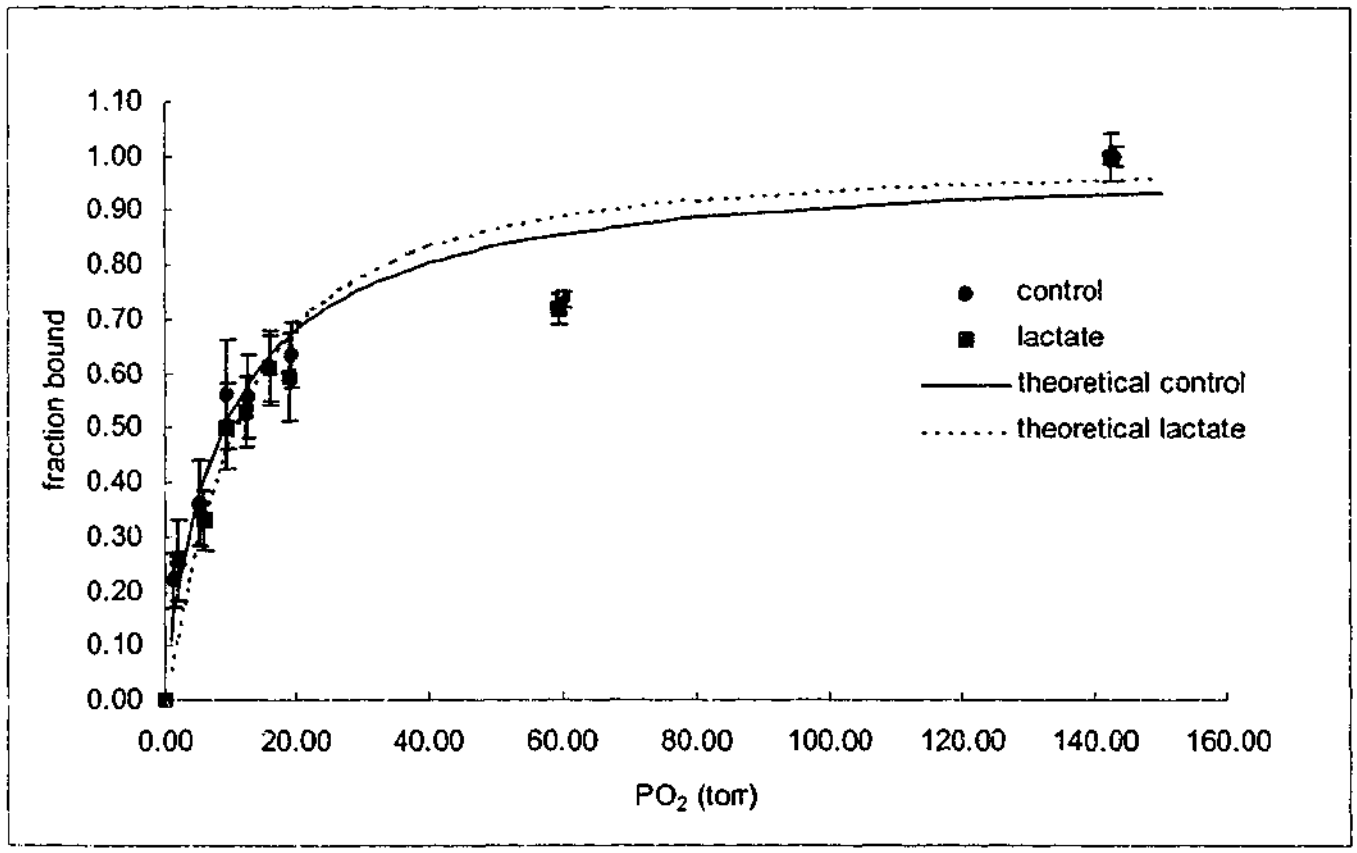
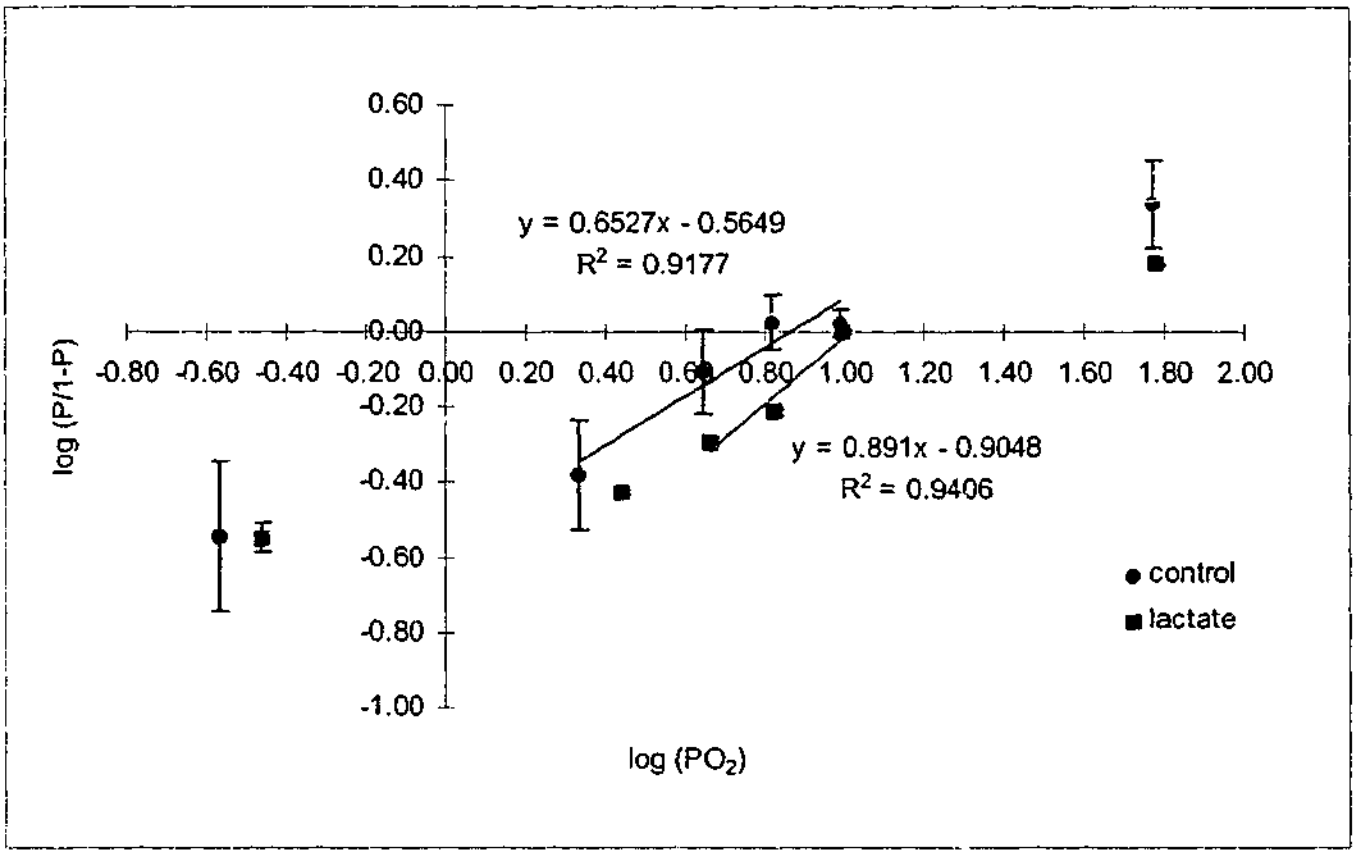


Figure 6.6. Oxygen binding curves of *H. rubra* haemocyanin measured in the absence (control) and presence (lactate) of 5 mmol L^{-1} D-lactate at 15°C in 100 mmol L^{-1} Bis-Tris of Tris-HCl buffer at (A) pH 6.3 and (B) pH 6.9. Theoretical curves calculated from Hill plot regressions plotted over actual data (mean ± S.E.M. fraction bound at each PO_2).

(A)



(B)

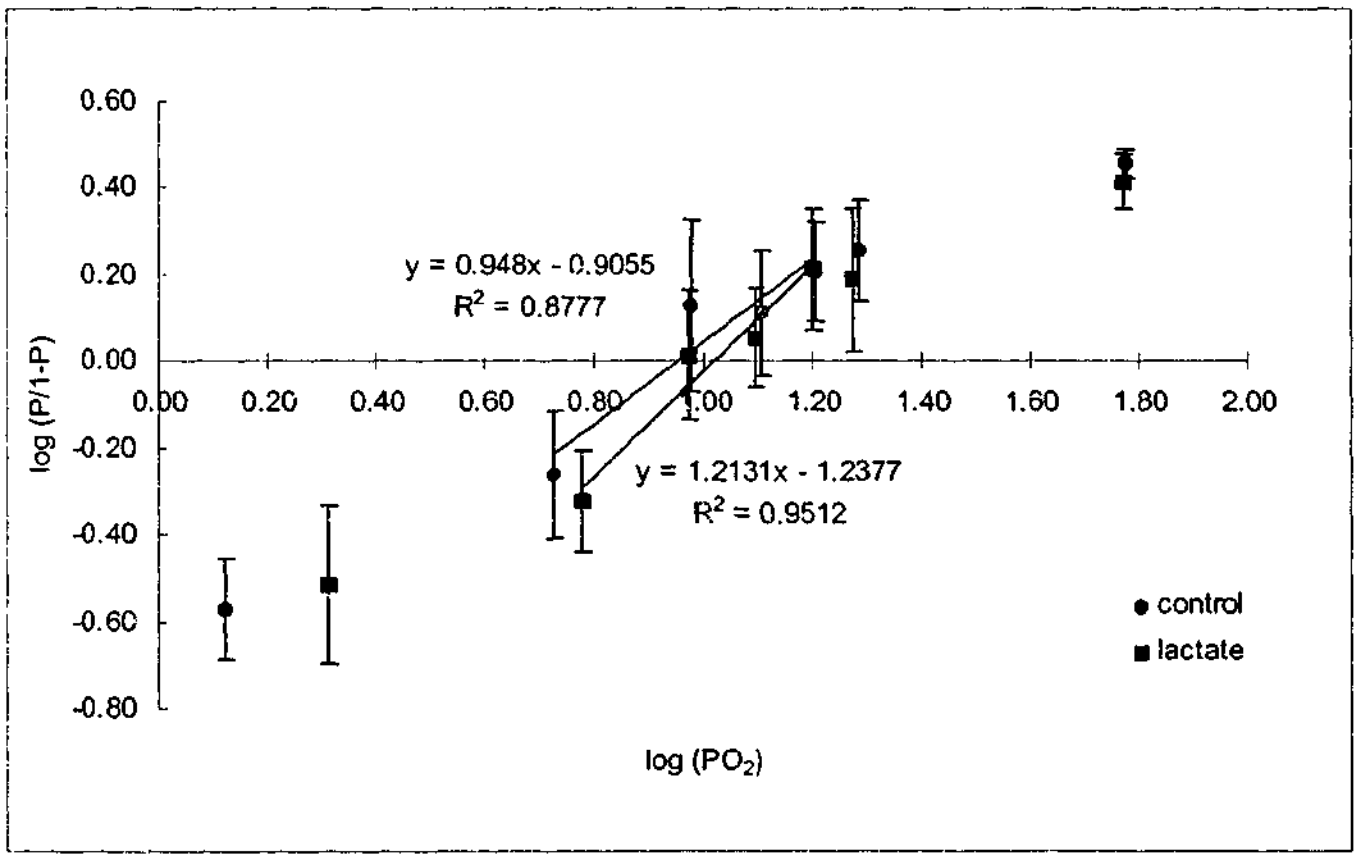


Figure 6.7. Hill plots of *H. rubra* haemocyanin oxygen binding measured in the absence (control) and presence (D-lactate) of 5 mmol L^{-1} D-lactate at 15C in 100 mmol L^{-1} Bis-Tris or Tris-HCl buffer at (A) pH 6.3 and (B) pH 6.9. Data are mean ± S.E.M. log [P/(1-P)] (P, fractional oxygen saturation). Linear regression shown for data points used to calculate P_{50} and n_{50}.

Table 6.6. Haemocyanin concentrations of *Haliotis* haemolymph (mg mL^{-1}). Values are mean ± S.E.M unless otherwise stated (range in parentheses).

Species	Haemocyanin (mg mL^{-1})	Reference
H. rubra	9.57 ± 0.50	This study
H. rubra	0.49 ± 0.04	Harris and Burke (2001)
H. laevigata	0.65 ± 0.04	
H. roei	8.6 (3.6-15.1)	Ainslie (1980a)
H. laevigata	5.9 (2.4-14.2)	
H. rubra	4.1 (1.0-9.9)	
H. roei	7.9 ± 2.2*	Ainslie (1980b)
H. laevigata	4.3 ± 1.2*	
H. rubra	4.6 ± 2.1*	
H. assinina	6.64	Baldwin *et al.* (in prep)
H. australis	3.43 ± 0.87	Wells *et al.* (1998a)
H. iris	4.22 ± 1.34	
H. iris (sheltered)	1.32 ± 0.35*	Wells *et al.* (1998b)
H. iris (exposed)	1.06 ± 0.22*	
H. kamtschatkana	6.5	Boyd and Bourne (1995)
H. cracherodii	3.8 (2.1-20.3)	Pilson (1965)
H. corrugata	1.5 (0.02-15.3)	
H. fulgens	5.4 (0.3-18.9)	

* = Mean ± SD

Table 6.7. Oxygen carrying capacity of *Haliotis* haemocyanin.

Species	Temp (°C)	mmol L^{-1}	Vols%	Reference
H. rubra	15	0.145 (0.196)*	0.325 (0.439)*	This study
H. laevigata	20	—	1.44	Ainslie (1977, 1980b)
H. roei	20	—	1.04	
H. rubra	20	—	1.01	
H. iris	15	—	0.522	Taylor 1993
H. kamtschatkana	—	—	2.77	Boyd and Bourne (1995)
H. cracherodii	—	0.16	—	Holste (1972)#
H. corrugata	—	0.12	—	
H. fulgens	—	0.15	—	

* = calculated for 4 best results

(—)=data not available

= Data cited in Mangum (1983).

Oxygen-content in vols % for which mmol L^{-1} O_2 is provided may be calculated by multiplying mmol L^{-1} by 2.24 (and vice versa for obtaining mmol L^{-1} from vols%).

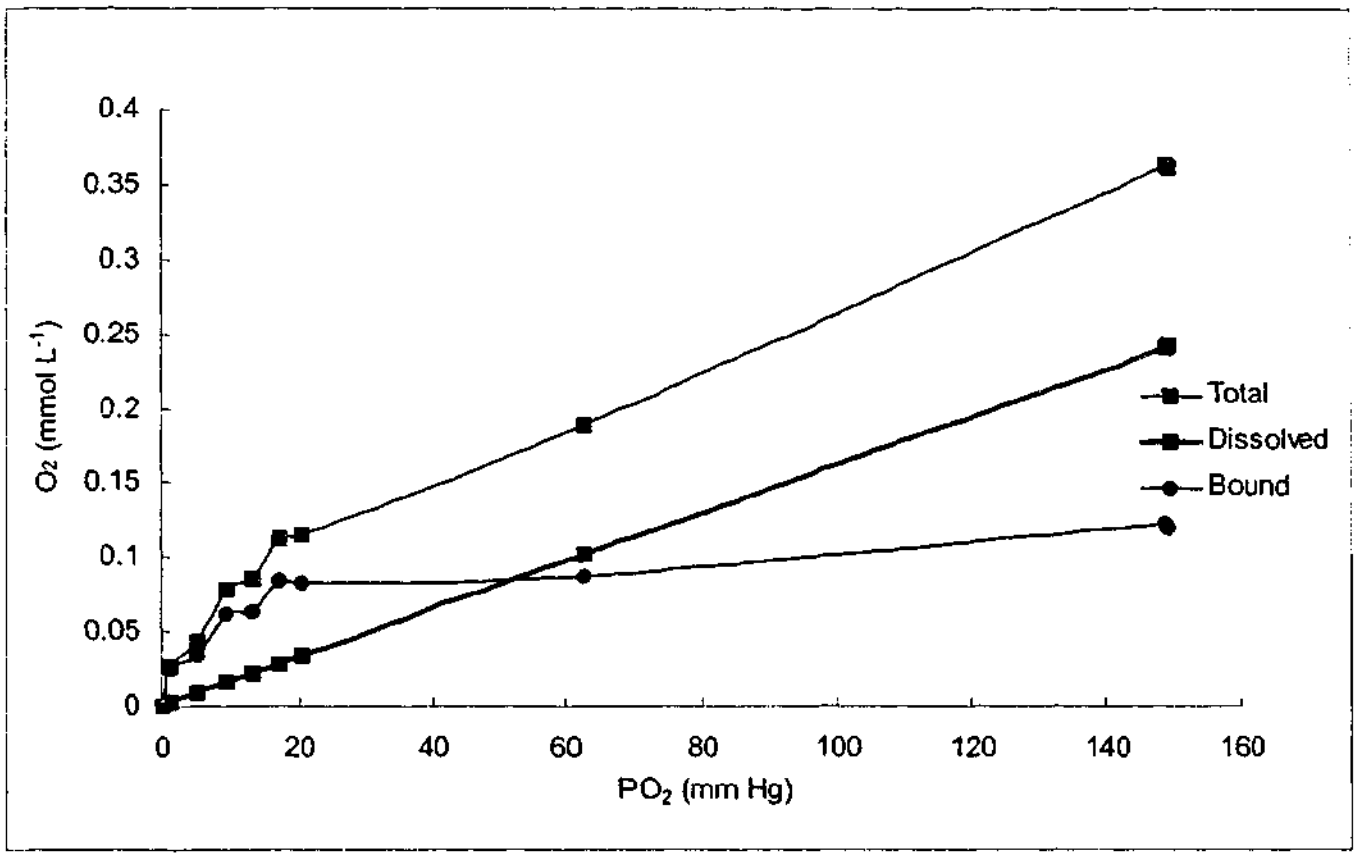


Figure 6.8. Typical raw oxygen-binding curve of *H. rubra* haemocyanin at 15°C in 100 mmol L^{-1} Tris-HCl buffer pH 6.9, showing total O_2, bound O_2 (HcO_2) and dissolved O_2 (O_2 in haemolymph).

CHAPTER SEVEN

GENERAL OVERVIEW AND SIGNIFICANCE OF THE STUDY

The Blacklip abalone *Haliotis rubra* of southern Australia forms the basis of the world's most important wild abalone fishery. Compared to other abalone species, very little information is available on its metabolic biochemistry. The primary aim of this study was to remedy this situation and provide new information important to an understanding of the ecophysiology of this relatively common gastropod of southern Australia, and also applicable to the harvesting, handling and transport of this valuable commercial species.

Previous studies on the metabolism of other abalone have concentrated on the functioning of the massive pedal musculature that accounts for the animal's commercial value, but little information is available on other important muscle systems.

The specific aims of this study therefore were as follows:

1. to perform a comparable study on the pedal musculature of *H. rubra* in order for comparison with data from other abalone species;
2. to extend this approach to an examination of a rarely studied muscle system of major importance to abalone, the radula muscles;
3. and to investigate and correlate characteristics of the oxygen-delivery system in *H. rubra* to muscle physiology and biochemistry.

Studies of this nature have not been conducted previously for any Australian abalone species, and as such increase our knowledge of the biology of an important component of temperate marine ecosystems in southern Australia.

Energy metabolism of the pedal musculature

This study has shown that the pedal musculature of *H. rubra* is metabolically similar to that of other abalone species. The adductor and foot muscles have a low aerobic scope and display considerable capacity for facultative anaerobiosis during both exercise and environmental induced hypoxia. These conclusions are supported by the low activities of key aerobic enzymes and low concentrations of myoglobin (Mb), high activities of pyruvate reductase enzymes, high pH buffering capacities and large concentrations of arginine phosphate and glycogen. Furthermore, the capacity for anaerobiosis is demonstrated by the accumulation of the pyruvate reductase end products D-lactate and tauropine in these muscles.

The recovery of *H. rubra* following intense muscle work is characterised by a rapid reduction in the muscle concentration of pyruvate reductase end products, a decrease in concentration involving little haemolymph-mediated removal.

The predominantly anaerobic metabolic poise of the pedal musculature is also reflected in the arrangement of the cardiovascular system. The pedal and adductor muscles of *Haliotis*, which may account for 50% of the weight of the animal, are supplied with haemolymph only via a pair of pedal arteries and a single small adductor artery. The haemolymph in abalone is not distributed in a tissue weight-specific manner.

From this study of the metabolic biochemistry of the pedal musculature of *H. rubra*, the new and most interesting aspects to emerge include the detection of pyruvate reductase end products in the haemolymph of *H. rubra*. D-lactate and, interestingly, very little tauropine is released into the haemolymph of *H. rubra* during whole animal exercise and emersion. As the duration of hypoxia increases, the concentration of these metabolites in the haemolymph increases and the haemolymph pH decreases. In addition, hypoxia results in an increase in the blood glucose titre in *H. rubra*. The presence of D-lactate in the haemolymph of an archaeogastropod mollusc was first recorded here for *H. rubra*. These findings have been repeated by subsequent work performed by the author on *H. iris* (Behrens *et al.* 2002), and suggest that facultative anaerobiois in the pedal musculature influences more than just the metabolism of these muscles. The changes in blood biochemistry following anaerobiosis have consequences for the aquaculture and commercial processing of these animals, and are discussed below in detail.

Energy metabolism of the radula muscles

This work is the first to examine the metabolism and physiology of the radula muscles of abalone and their *in vivo* function during actual feeding. The results clearly demonstrate that the radula muscles of *H. rubra* display a physiological and biochemical profile totally different to that of the pedal musculature, a profile in accordance with the appearance and function of the radula muscles. Relatively high activities of key glycolytic and obligate aerobic enzymes, low activities of pyruvate reductase enzymes, a low pH buffering capacity as well as high concentrations of Mb indicate that aerobic metabolism is of major importance to these muscles.

Radula muscles display no evidence of facultative anaerobiosis unlike the pedal musculature of *H. rubra*. The lack of significant concentrations of D-lactate, tauropine or hydrolysis of arginine phosphate implies that the continuous contractions of the radula and buccal muscles during feeding in *H. rubra* are supported aerobically, a feature that allows the

ingestion of large quantities of algae over extended periods. Feeding on drift algae and grazing on micro-algae do not stimulate different metabolic responses of the radula muscles. As with feeding, exercise and environmental hypoxia of the whole animal does not lead to the accumulation of pyruvate reductase end products in the radula muscles.

The metabolic poise of the radula muscles is reflected in both the role of, and haemolymph supply to, these muscles. The radula muscles are positioned favourably with respect to O_2-delivery at the expansion of the aorta into the cephalic arterial sinus. This arrangement provides these muscles with both a 'first-bite' at the oxygenated haemolymph, and a constant haemolymph, and therefore O_2 and fuel, supply. This is in contrast to the pedal musculature, which may experience a compromised supply of haemolymph during periods of muscular contraction.

The presence of D-lactate in the haemolymph of *H. rubra*, presumably released from the hypoxic pedal musculature, and the presence of D-LDH in the radula muscles, raised the possibility that D-LDH in more aerobic organs such as the radula muscles may be present as an isozyme form different from that in the pedal musculature. This isozyme may be poised for the aerobic utilisation of this metabolite, rather than its production. Kinetic and electrophoretic data however indicate that pedal and radula musculature contain a single D-LDH isozyme. It is proposed that the same enzyme which has been shown to function as a pyruvate reductase in the adductor and foot muscles, may also function as a lactate oxidase in the presumably aerobic radula muscle.

From this study of the metabolic biochemistry of the radula muscles of *H. rubra*, a number of new and interesting findings have emerged. As far as the author is aware, the current study appears to be the most complete study conducted so far on the metabolism of abalone, if not gastropod, radula function. This is of some importance given that these muscles, which account for only 1 % of animal weight, ultimately support the commercial pedal musculature that comprise 40-50% of the animal's wet weight.

While other studies have provided information on the enzyme profile and effects of *in vitro* stimulation few, if any, studies have examined the metabolism of gastropod radula muscles during *in vivo* normal feeding behaviour. The *in vitro* studies on whelk radula retractor muscles, which reveal a large anaerobic component to muscular contraction, suggest that the muscles of these carnivorous gastropods may function differently during feeding.

Other work has determined the concentration of Mb in the radula muscles of various gastropods and the current study provides the first equivalent results for abalone. The presence of a tissue haemoglobin has been long assumed for abalone, and although numerous studies have identified Mb in abalone radula muscles, there was no estimate of the

concentration. This study, in conjunction with that of Shepherd (2002) is the first to document the amount of Mb in abalone muscles. In addition, the results indicate that *H. rubra* probably possesses the unusual IDO-type Mb, first identified in the abalone *Sulculus diversicolor* and *Nordotis madaka*.

Haemolymph oxygen-delivery

Whereas the biochemical profile of *H. rubra* pedal and radula muscles may be used to estimate their metabolic potential, the O_2-delivery capacity of haemolymph supplied to these is more difficult to quantify. This study has showed that estimates of the concentration of haemocyanin (Hc) present in the haemolymph of abalone depends not only on the inherent variability of the protein within animal populations, but also to some extent on the methods employed to arrive at that value. While levels of Hc and the O_2-carrying capacity may differ across species, the possession of a reverse Bohr shift that lies within the physiological pH range appears to be a common feature of abalone, and was observed for *H. rubra*.

The present study suggests that *H. rubra* haemolymph has a higher concentration of Hc than previously reported for Australasian abalone species. Furthermore, *H. rubra* Hc has a higher affinity for O_2 than previously reported, but a lower carrying capacity. The presence of D-lactate in haemolymph did not significantly alter the Hc O_2-binding characteristics. Any effects of hypoxia and haemolymph anaerobic metabolites on Hc O_2-binding of *H. rubra*, and other abalone, are most probably the sole results of pH perturbations.

It appears from the haemolymph delivery system and metabolite studies that the haemolymph, and therefore O_2, is delivered preferentially to the radula muscles of abalone, relative to the pedal musculature. The aerobic function of the radula muscles may also be maintained by a haemolymph shunt, where cephalic sinus and pedal vessel valves may isolate the pedal circulation and ensure sufficient haemolymph and O_2 for other, more O_2-dependent organs. The possession of Mb, with a high O_2-affinity, further ensures delivery of O_2 to the radula muscle mitochondria.

Application of results to the ecophysiology and commercial processing of *Haliotis rubra*

Physiologically and biochemically, *H. rubra* appears to function in a similar fashion to other temperate abalone species, such as *H. iris*. Although generally subtidal, when encountering low oxygen conditions during either tidal cycles or as a result of commercial handling and transport, studies indicate that *H. rubra* shows a characteristic series of physiological and metabolic responses: a stressed appearance, an apparently altered

respiratory activity, a reduction in tissue and blood pH, a change in Hc O_2-binding behaviour and a switch to facultative anaerobiosis leading to the accumulation of the pyruvate reductase end products D-lactate and tauropine in the pedal musculature and haemolymph. Similar patterns are observed during enhanced musculature activity associated with righting behaviour.

Whilst an exact experimental protocol simulating commercial practices or live transport was not undertaken in this study, the metabolic responses of *H. rubra* may be followed to monitor the animal's health and viability during commercial processes. Even though studies have concerned themselves with examining the response of abalone to hypoxia, only a few have addressed these physiological processes in a context directly r applicable to the commercial industry.

One of the major problems facing the abalone industry is a possible reduction in animal health during transport, the management of which may be aided by monitoring metabolic responses to stress (James and Olley 1970, 1974; Baldwin *et al.* 1992; Ryder *et al.* 1994; Wells and Baldwin 1995; Fleming 2000). Anaerobic metabolism negatively affects pedal muscle quality, directly influencing the taste and texture of both live exported and processed abalone. This negative effect is most likely mediated by the accumulation of acidic end products and the decrease in pH. James and Olley (1970, 1971b) and Olley and Thrower (1977) demonstrated that pedal muscle toughness increased and texture deteriorated as pH fell, affecting the palatability. Air emersion also causes significant loss of fluid from abalone, and accounts for observed weight losses in live transported or stored individuals (Gorfine 2001). These losses directly affect weight estimates and therefore catch quotas. This has direct consequences on both the price and volume of allowable wild animal harvests.

Conditions under which abalone are exported live appear to take advantage of the biochemistry of these gastropods, although the packaging and transport of Australian species is most probably based on metabolic data accumulated on other species, such as paua (*H. iris*). Generally, abalone are transported emersed in sealed, insulated containers (dry consignment), containing ice packs to regulate temperature and slow metabolism, and either seawater or moist algae to maintain humidity (Fallu 1994; Ryder *et al.* 1994; Fleming 2000; Freeman 2000; Gorfine 2001). The practice of injecting O_2 into sealed bags containing abalone may ensure that some respiratory stress is avoided.

Muscle biopsies may be taken from a proportion of animals following transport or handling to gauge effects of shipment or conditions during shipment. Even though tauropine and D-lactate are very stable in frozen muscle (Baldwin *et al.* 1992), it may be considered advantageous to sample haemolymph instead. Rather than sacrificing a percentage of the

abalone for muscle samples, haemolymph can be withdrawn carefully via a fine syringe needle from the pedal sinus or CAS, with little detriment to animal health or meat quality. The levels of D-lactate, glucose and pH in *H. rubra* haemolymph could be a useful indicator of conditions abalone may experience during transport. No anaerobic metabolites are detected in the haemolymph of resting *H. rubra*, and hence appear as a consequence of hypoxia. Likewise, blood glucose titres rise with hypoxia. As demonstrated by *H. iris*, the levels of anaerobic metabolites are likely to rise as the duration of air exposure increases (Behrens *et al.* 2002). Unfortunately, confident quantifiable predictions as to the exact amount of metabolite in the tissues can not yet be made from haemolymph samples.

The use of haemolymph metabolites as indicators of stress is widely employed, amongst qualitative tests, as an indicator of animal health during the transport and handling of lobsters (Paterson and Spanoghe 1997; Paterson *et al.* 1997; Morris and Oliver 1999a, 1999b; Speed *et al.* 2001). Such a protocol is often teamed with a period of aerobic recovery to ensure animal health and quality prior to processing (Taylor and Waldron 1997; Taylor *et al.* 1997). The use of haemolymph samples as a rapid and accurate check for anaerobic metabolism has been identified previously by Wijsman *et al.* (1985) for freshwater gastropods.

Caution must be exercised however if blood samples are to be used for monitoring abalone as not only is tauropine accumulated in the pedal musculature at rest and not present in haemolymph samples, but infection around the sample site may result in necrosis of foot muscle (Taylor 1993; Chen 1996). This situation is obviously less desirable for the consumer.

To overcome any detrimental effects of metabolite accumulation on taste or quality, abalone may benefit from being kept under oxygenated conditions to full recovery, post transport (Fleming 2000; C. Spurrier Pacific Shoji Pty. Ltd., pers. comm). The short investigation carried out during this study on recovery from exercise should be augmented by longer monitoring studies on recovery of animals following actual transport and emersion.

Further studies

This study has highlighted the adage that science often raises more questions than it answers. Further interesting avenues of research arising from the present study include the examination of normal behaviour such as locomotion, foraging and clamping on *H. rubra* haemolymph physiology and muscle metabolism, and examination of the pattern of recovery from extended hypoxic periods. The characteristics of radula muscle Mb probably deserve as much attention, in regards to O_2-binding, as received by Hc. In particular, any effects of metabolites on Mb function would be interesting to examine. Further studies may also

attempt to integrate muscle metabolic and respiratory or cardiovascular responses to physiological challenge.

The question of whether D-lactate in the haemolymph is sequestered by other tissues such as the radula muscle and used as a fuel would benefit from tracer studies, where the distribution and concentrations of labelled D-lactate would be followed both during hypoxic events and recovery in oxygenated conditions.

The examination of biochemical indices and responses to hypoxia in *H. rubra* is not complete, a fact readily acknowledged by the author. Further studies might also investigate the importance of muscle phosphagens to metabolism in *H. rubra* pedal muscles. In addition to tauropine and D-lactate, low levels of succinate and alanine are also known to accumulate in the muscles of abalone following severe hypoxia (Gäde 1988; Watanabe *et al.* 1994b). It appears that prolonged or severe hypoxia, or total anoxia, involves supplementation of glycolysis with the co-fermentation of glycogen and aspartate, as has been observed for many molluscs (see Chapter 1).

Commercial applications of some of the current results would benefit from further experiments to achieve stronger correlations between muscle and haemolymph metabolite concentrations. If a strong correlation can be ascertained, simple non-destructive haemolymph samples may prove invaluable in assessing and monitoring animal health in the future. In addition, the data presented in this thesis could be augmented by further studies examining the fate of anaerobic metabolites in both muscle and haemolymph over a recovery regime following periods of hypoxia simulating transport. This could incorporate following animal O_2-consumption during the possible repayment of any O_2 debts.

Finally, any inter- and intraspecific patterns should be resolved. The biochemistry and physiology investigated here for *H. rubra* should be determined for other Australian temperate species, especially *H. laevigata* and *H. roei*, which are presently the focus of aquacultural studies and are of considerable economic and ecological importance. Additionally, research suggests oceanic *H. rubra* show anatomical, physiological and genetic differences to Port Phillip Bay individuals (Huang *et al.* 2000; Drew *et al.* 2001). However, nothing is known about any biochemical or metabolic differences. A distinct demarcation of sheltered and oceanic exposed populations is available among abalone from Victoria, and the author believes that these issues should be resolved.

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