LACTATE SEQUESTRATION IN THE CARAPACE OF THE CRAYFISH *AUSTROPOTAMOBIUS PALLIPES* DURING EXPOSURE IN AIR

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Summary

When held in air for up to 24 h, crayfish accumulated Ca^{2+} and Mg^{2+} in their haemolymph in direct proportion to raised levels of lactate. K^+ levels were highly variable, with elevated levels associated with morbidity. Lactate accumulation in the haemolymph was reflected in proportional increases in lactate levels in the carapace and muscle. Pieces of carapace incubated in saline containing elevated levels of lactate accumulated lactate to up to half the dissolved concentration. Measured levels in the carapace, relative to its water content, implied that lactate accumulated in the carapace in a combined form, possibly complexed to calcium. The exoskeleton seems to provide a reserve of buffering capacity and a sink for lactate during anaerobic metabolism. A similar mechanism has been identified in pond turtles.

Key words: Crustacea, crayfish, *Austropotamobius pallipes*, lactate, air-exposure.

Introduction

The British freshwater crayfish *Austropotamobius pallipes* lives in streams or pools that are liable to dry up or become stagnant, and these animals are known to leave water voluntarily to forage or to invade neighbouring habitats (Huxley, 1896; Taylor and Wheatly, 1980). When placed in air, the gills collapse and the crayfish suffer continuously from systemic hypoxia and hypercapnia. Associated with an initial virtual anoxia and a switch to anaerobic metabolism, lactate levels in the haemolymph increase to 13 times their submerged level within 1 h of aerial exposure, and the animals experience an uncompensated metabolic and respiratory acidosis (Taylor and Wheatly, 1981).

Despite the apparent impairment of gas exchange, the animals survived prolonged periods of aerial exposure, at least in part because of a mechanism first described for the shore crab *Carcinus maenas* (Truchot, 1975). This entails progressive mobilization of HCO_3^-, compensating for the initial acidosis on entry into air. This recovery in acid–base status compensates a rightward Bohr shift, restoring oxygen delivery to the tissues (Taylor and Wheatly, 1981). Although this description of the respiratory adaptations enabling crayfish to survive in air seemed sufficient as that paper went to press, we were encouraged to return to the problem by another novel contribution from Truchot (1980) that described how haemocyanin oxygen-affinity was increased by the accumulation of lactate. We were able to identify this effect in the crayfish, where it combined with an equally potent effect of Ca^{2+} on oxygen affinity. Levels of Ca^{2+} in the haemolymph increased progressively when crayfish were placed in air, an effect we interpreted as arising from mobilisation of HCO_3^- from internal stores of calcium carbonate (Taylor et al., 1987; Truchot, 1987; Whiteley, 1999). Together, these compensatory mechanisms increase the oxygen affinity to a point where submerged rates of oxygen uptake are restored (Taylor and Wheatly, 1981; Taylor and Innes, 1988; Taylor et al., 1991) so that survival in air is ultimately limited by the inability of crayfish to maintain water balance (Tyler-Jones and Taylor, 1986).

On recovery of oxygen transport capacity, it was noted that lactate levels in the haemolymph, after having been elevated for the first 5 h in air, fell progressively to submerged levels, while the crayfish remained in air (Taylor and Wheatly, 1981; Morris et al., 1986). This could be interpreted as biochemical recycling of lactate, possibly to reform glycogen stores, *via* gluconeogenesis from pyruvate. However, on replacement in water after 24 h in air, crayfish experienced an apparent lactate washout, with haemolymph lactate levels rising to those measured on initial exposure to air. This implies that lactate was sequestered somewhere in a body compartment while the animals were in air (Taylor and Wheatly, 1981). The site of this sequestration has not been determined. Lactate levels in abdominal muscle were elevated after 3 h in air but fell in line with changes in the haemolymph during prolonged aerial exposure (Tyler-Jones

and Taylor, 1988), so that muscle seems not to be involved. In addition, the internal source supplying both HCO_3^- and Ca^{2+} to the haemolymph is unknown, although mobilisation of calcium carbonate from the carapace, by a reversal of the mechanisms employed to calcify the exoskeleton following ecdysis (Cameron and Wood, 1985), was discussed (Taylor et al., 1987; Whiteley, 1999). The possible involvement of the carapace in lactate sequestration has not previously been considered.

This collaborative investigation of the exoskeleton as a possible site of lactate sequestration was stimulated by discussion following a communication to the Society of Experimental Biology (Jackson, 1999). Recently, it had been demonstrated that the shell of the freshwater turtle (*Chrysemys picta bellii*) plays a dramatic and ecologically relevant role in neutralising the lactic acid produced during prolonged anoxic submergence (Jackson et al., 1996; Jackson, 1997). The turtle's shell acts in two ways: first, it releases buffers (calcium and magnesium carbonates) to supplement extracellular buffering of lactic acid; and second, it accumulates lactate and buffers the associated protons and, by so doing, relieves the extracellular fluid of this additional acid load.

Crayfish in air encounter many of the same problems faced by turtles under ice (i.e. hypoxia, accumulation of acid metabolites), and the present investigation set out to determine whether the calcified exoskeleton had a combined role in acid–base regulation and lactate sequestration enabling prolonged survival of crayfish in air.

Materials and methods
Experimental animals

Crayfish (*Austropotamobius pallipes*, Lereboullet, N=30) of either sex, but predominantly males, were captured from Stowe pool (Litchfield, Staffordshire, UK). They were transported to The University of Birmingham, where they were maintained for 10 days in plastic aquaria receiving a continuous supply of dechlorinated Birmingham tap water at 15 °C. The animals were not fed during this period.

Experimental protocol and tissue sampling

The primary experimental group of crayfish was exposed in air by careful siphoning of water from the tanks. They were then left undisturbed for between 2 h and 28 h, prior to sampling. A second group was held in air for up to 24 h and sampled at intervals of 2–5 h. These animals were clearly stressed by repeated handling and sampling, and showed very high levels of lactate in the haemolymph and tissues. A control group was sampled without prior disturbance or removal from water.

Haemolymph was sampled into a syringe within 45 s of capture using a hypodermic needle (22 gauge) inserted dorsally in the midline from the posterior edge of the cephalothorax forward into the pericardial sinus. The animal was then killed by destruction of the central nervous system for immediate sampling of tissue. In each animal, both the dorsolateral branchiostegites and the right cheliped were removed. Samples of the exoskeleton from one branchiostegite and the dorsal face of the propodite of the cheliped were dissected free from underlying tissue and thoroughly cleaned by scraping away tissue with a scalpel then blotting with absorbent paper, before being frozen in liquid nitrogen. A sample of muscle from the propodite of the cheliped was removed, blotted and frozen. Muscle and carapace samples were then manually ground to powder at the temperature of liquid nitrogen, using a mortar and pestle. The powder was diluted tenfold in 8 % perchloric acid and left for extraction for 24 h before analysis of lactate. The other branchiostegite was used to measure the water content of the carapace.

In vitro *incubation of the carapace in lactate-rich Ringer*

To study the uptake of lactate into the carapace *in vitro*, small pieces of carapace (0.1–0.2 g) taken from the branchiostegites of 10 crayfish were placed in a Ringer's solution containing high levels of lactate (30 mmol l^{-1}) at two pH values (7.4 and 7.8). The intact pieces of carapace were placed in 10 ml of Ringer for up to 24 h at 20 °C in glass vials that were shaken continuously during the incubation. The Ringer consisted of 126 mmol l^{-1} NaCl, 35 mmol l^{-1} CaCl$_2$, 2 mmol l^{-1} MgCl$_2$, 5 mmol l^{-1} KHCO$_3$, 10 mmol l^{-1} NaHCO$_3$, 5 mmol l^{-1} Hepes and 30 mmol l^{-1} sodium lactate. The relatively high concentration of Ca^{2+} is based on values measured in the haemolymph of crayfish after 24 h in air (Morris et al., 1986). The pH of the solution was adjusted with 10 mmol l^{-1} NaOH and did not change by more than 0.05 units during the 24 h; the lactate concentration of the Ringer also remained constant.

Analytical techniques

Lactate concentrations of the haemolymph and of perchloric acid extracts of muscle and carapace were determined using an enzymatic technique (Sigma Diagnostics; Procedure No. 735). In this method, lactate is converted to pyruvate and H_2O_2 which, in the presence of peroxidase, leads to oxidative condensation of a chromogen producing a coloured dye with an absorption at 540 nm that is directly proportional to lactate concentration. The water content of the carapace was determined after drying to constant mass at 60 °C for a minimum of 36 h. Haemolymph cation (Na^+, K^+, Ca^{2+} and Mg^{2+}) concentrations were measured by atomic absorption spectrophotometry (Pye-Unicam SP9). Chloride concentrations were measured using an amperometric titrator (Aminco Cotlove).

Data are expressed as mean values ± S.E.M. The data were subjected to analysis of variance (ANOVA) for repeated measures, and the significance of any apparent differences between mean values was identified by *post hoc* Student–Newman–Keuls tests, with significance attributed at the 95 % confidence level (P<0.05). The relationship between measured variables was determined by linear regression least-squares analysis, including calculation of the coefficient of correlation (r^2).

Table 1. *Haemolymph ion levels in crayfish* (Austropotamobius pallipes) *submerged in aerated water and following 2, 5 and 24 h of exposure in air at 20 °C*

	Submerged	2 h in air	5 h in air	24 h in air
Ca^{2+} (mmol l^{-1})	6.2±0.4	7.9±1.5	25.0±1.5*	24.3±1.4*
Mg^{2+} (mmol l^{-1})	1.1±0.1	1.4±0.1	3.2±0.8*	2.8±0.5
K^+ (mmol l^{-1})	6.6±0.2	10.1±1.9	8.6±0.4	9.9±0.5
Lactate (mmol l^{-1})	0.2±0.1	2.7±1.1*	10.6±2.3*	6.2±1.2*

Values are mean ± 1 s.e.m. (*N*=5).

Mean values that are significantly different (*P*<0.05) from submerged levels are indicated by an asterisk.

Results

Sampling in vivo

The ionic composition of haemolymph obtained from crayfish held in water or following varying periods of exposure in air is shown in Table 1. In submerged animals, mean haemolymph lactate concentration was 0.2±0.1 mmol l^{-1} (mean ± s.e.m., *N*=5). This rose tenfold after 2 h in air, with further increases to 50 times the submerged level after 5 h. After 24 h in air, there was a huge variation in haemolymph lactate concentration between animals, from 0.04 to 70.33 mmol l^{-1}, apparently dependent on their physiological state. The extremely high levels of haemolymph lactate were found to have been measured in animals that appeared moribund and had elevated K^+ levels. When these animals were excluded, mean lactate level in the surviving group was found to have reduced slightly from the level reached after 5 h in air. However, this reduction was not significant, and lactate remained at 30 times the submerged level. The data from the surviving group of animals are illustrated in Fig. 1.

Both Ca^{2+} and Mg^{2+} levels rose during aerial exposure so that, after 24 h, Ca^{2+} levels had increased fourfold and Mg^{2+} levels had more than doubled from their levels in submerged animals. These changes appeared to be directly proportional to changes in haemolyph lactate levels (Fig. 2). K^+ levels were virtually unchanged from their submerged levels of approximately 7 mmol l^{-1} in a subpopulation of crayfish that survived and remained mobile during aerial exposure (Table 1). In contrast, other animals showed large increases in haemolymph K^+ levels during aerial exposure, up to a maximum at about 30 mmol l^{-1} (see Fig. 2). This maximum level was observed in six moribund animals following 24 h of exposure in air. These large variations in K^+ level did not appear to correlate in any systematic way with the changes observed in the other variables, including lactate levels (Fig. 2), and probably reflect differences between animals in their capacity to survive aerial exposure. Leakage of K^+ from tissues is recognised as an early indicator of incipient mortality in crayfish (Bowler et al., 1973). Consequently, animals having
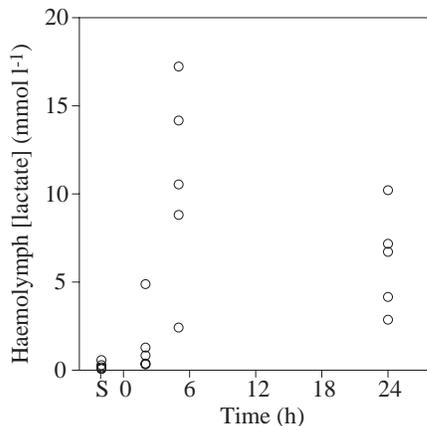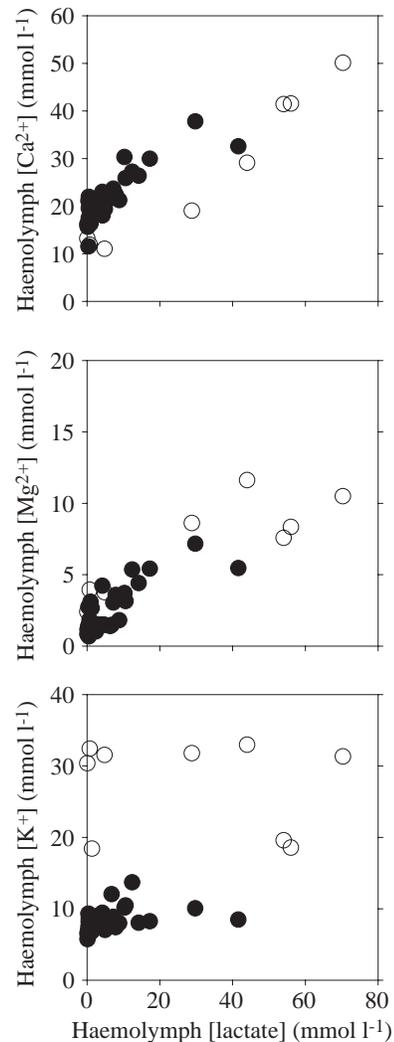
Fig. 1. Measured levels of haemolymph lactate in 20 crayfish either submerged in aerated water (S) or exposed to air for 2, 5 or 24 h at 20 °C.

Fig. 2. The relationship between levels of Ca^{2+}, Mg^{2+} and K^+ and the level of lactate in the haemolymph of crayfish submerged in aerated water or exposed in air for 2, 5 or 24 h at 20 °C. Open symbols identify animals having elevated levels of K^+. The regression equations for all animals are: for Ca^{2+}, *y*=17.8+0.42*x* (*r*^{2}=0.77); for Mg^{2+}, *y*=1.8+0.14*x* (*r*^{2}=0.78).
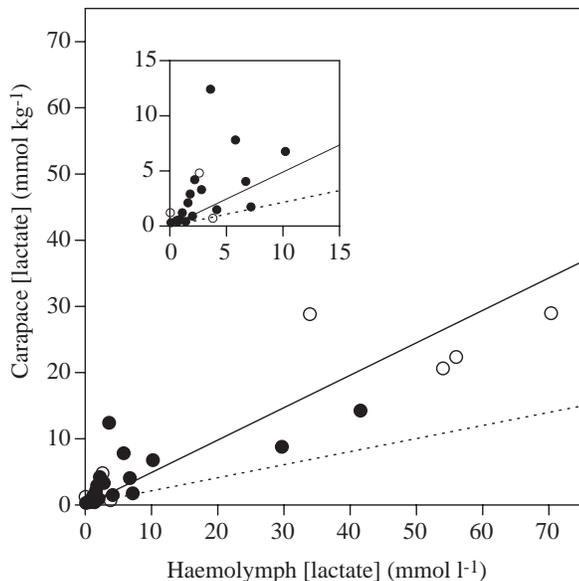
Fig. 3. The relationship between levels of lactate measured in the carapace and haemolymph of crayfish submerged in aerated water or exposed in air for 2, 5 or 24 h at 20 °C. Open symbols identify animals having elevated levels of K$^+$ in their haemolymph. The inset is an expanded portion of the graph to illustrate the distribution of values below 15 mmol l^{-1} and 15 mmol kg^{-1}. The solid line is a linear regression for all animals through the points (y=1.4+0.48x; r^2=0.85); the broken line indicates the notional relationship based on the partitioning of lactate into the water compartment of the carapace (see Discussion).

elevated K$^+$ levels are identified in Figs 2 and 3 and excluded from Fig. 1.

Levels of lactate measured in the carapace (Fig. 3) increased during aerial exposure in proportion to the levels in the haemolymph of individual animals. The levels of lactate that accumulated in the carapace were approximately half those measured in the haemolymph (linear regression y=1.4+0.48x; r^2=0.85). Lactate levels in cheliped muscle also varied with haemolymph level, showing a similar proportional change to that observed for the carapace samples (linear regression of all data for muscle lactate levels against haemolymph lactate level: y=2.24+0.44x, r^2=0.63).

Incubation of carapace *in vitro*

Pieces of carapace incubated at 20 °C in saline containing 30 mmol l^{-1} sodium lactate, buffered either to pH 7.4 or to 7.8, showed a significant increase (P<0.001) in lactate levels, to a concentration approximately half that in the saline (i.e. 15 mmol kg^{-1}). Equilibration was independent of saline pH and was complete in under 3 h, so that there were no further changes in lactate levels over the succeeding 21 h (Fig. 4).

Discussion

This study has confirmed that the crayfish *Austropotamobius pallipes*, like the turtle (Jackson, 1997), accumulates lactate in
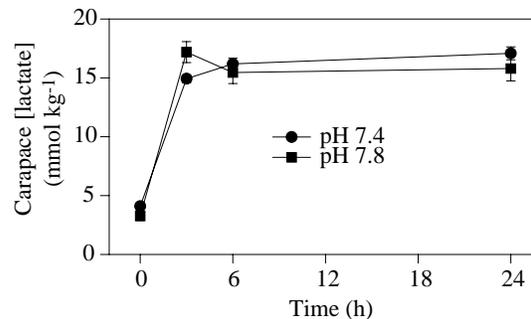

Fig. 4. Accumulation of lactate over time into pieces of crayfish carapace incubated with saline containing 30 mmol l^{-1} lactate at 20 °C. Equilibration was rapid up to a concentration half that in the saline. Values are means ± S.E.M. (*N*=6).

its shell (exoskeleton) during lactic acidosis. In both species, prolonged hypoxia leads to a reliance on anaerobic glycolysis and a rise in circulating lactate levels. Lactate entered the exoskeleton of the crayfish in proportion to the haemolymph concentration, and the regression equation relating exoskeletal to haemolymph concentrations indicated that the exoskeletal concentration (in mmol kg^{-1}) averaged 48 % of the haemolymph concentration (in mmol l^{-1}). In the anoxic submerged turtle, using the same units, shell lactate averaged 50 % of plasma lactate at 20 °C (Jackson et al., 1996), 89 % at 10 °C and 92 % at 3 °C. The durations of submergence, however, were 6 h, 9 days and 3 months, respectively, so that full equilibration between compartments was more likely to have been achieved at the colder temperatures.

The *in vitro* incubation of crayfish exoskeleton suggests that initial equilibration of lactate with haemolymph occurs relatively rapidly in this tissue. Steady-state exoskeletal lactate concentration was reached within 3 h (the shortest time tested) at a concentration that was approximately 50 % of the lactate level in the incubation medium. This is similar to the *in vivo* value of 48 % noted above, indicating that the *in vivo* samples are likely to have been close to equilibrium. We did not examine lactate efflux from the crayfish carapace in this study, but we assume that it occurs in response to low haemolymph lactate levels, just as lactate has been observed to leave the turtle shell both *in vivo* (Jackson, 1997) and *in vitro* (Jackson et al., 1999).

Accumulation of lactate by the crayfish exoskeleton *in vivo* was to levels similar to those measured in cheliped muscle. However, while the the water content of muscle is approximately 80 % (Taylor et al., 1987), the water content of the exoskeleton is only 21 %. Because of the relatively low water content of turtle shell (approximately 30 %), it was argued (Jackson, 1997) that much of the shell lactate must exist in combined or precipitated form. This argument also holds for the crayfish exoskeleton. If we assume that the exoskeletal lactate in the *in vitro* incubation existed in simple solution in the water phase of the exoskeleton, then the lactate concentration of 15 mmol kg^{-1} exoskeleton would be equivalent to 71.4 mmol kg^{-1} exoskeletal water. This is more

than twice the bathing solution concentration. In several of the *in vivo* samples, exoskeletal lactate levels were 30 mmol kg^{-1} exoskeleton or higher so, by the same reasoning, lactate concentration within the water phase would have to have been 140–170 mmol kg^{-1} exoskeletal water. These estimates are indicated by the broken lines in Fig. 3. In extreme cases in the turtle, shell lactate concentrations in the tissue water of over 500 mmol l^{-1} would have been necessary. We suggest that this is unlikely and that a more reasonable explanation is that the bulk of the exoskeletal lactate exists in combined form, perhaps complexed with calcium, as has been postulated for turtle shell (Jackson, 1997). It is possible that chemical binding of lactate within the exoskeleton facilitates its further uptake by keeping free lactate concentration low.

The exact nature of lactate binding within the shells of these animals is unknown. The suggestion that lactate is complexed to calcium is based on the known importance of this reaction in turtle extracellular fluid during prolonged anoxic submergence (Jackson and Heisler, 1982). Ca^{2+} and lactate combine to form a cationic complex (CaLactate^{+}) with an association constant (approximately 20 l mol^{-1}) that is too low to be of physiological significance under control extracellular conditions. However, in the extracellular fluid of an anoxic turtle, when circulating levels of both Ca^{2+} and lactate are very high, the formation of this complex can be quite significant and can account for as much as two-thirds of the total Ca^{2+} in the extracellular fluid (Jackson and Heisler, 1982). Under anoxic conditions in both the turtle and the crayfish, suitable conditions for this reaction may also exist within calcified tissues for lactate that gains entry. Assuming that such a complex does exist within the shell, a further uncertainty is its physical state, whether it is in solution, present as a precipitate or is possibly bound electrostatically to the mineral surface. Osmotic considerations favour one of the latter possibilities, but no experimental evidence is available to support this. Finally, because of uncertainty about the state of lactate within the shell, the nature of the equilibrium state is equally in doubt. However, on the basis of *in vitro* data from turtle shell (Jackson et al., 1999), we suggest that the lactate enters, in effect, accompanied by a proton, so that the buffering power of the shell is exploited by this process. This could occur, for example, either by the diffusion of undissociated lactic acid into the shell or by the entry of two lactate ions in exchange for one carbonate ion.

We also observed increases in the haemolymph concentrations of both Ca^{2+} and Mg^{2+} that correlated closely with haemolymph lactate concentration (Fig. 2). Although we have no direct evidence from this study, we suggest, as have others (e.g. de Fur et al., 1980), that the source of these divalent cations was the exoskeleton and that they were released, accompanied by carbonate, in response to haemolymph acidosis. Regression analysis of our data reveals that haemolymph Ca^{2+} and Mg^{2+} levels increased by 0.42 mmol l^{-1} and 0.14 mmol l^{-1}, respectively, for each mmol l^{-1} increase in haemolymph lactate (Fig. 2). Because these are divalent ions, the equivalent buffering that this represents (assuming

stoichiometric release of carbonate) would approximately match the increase in lactic acid concentration. Mobilisation of divalent carbonate ions could lead to the formation of univalent HCO$_3^-$ by a reversal of the mechanism proposed for calcification of the exoskeleton (Cameron and Wood, 1985), resulting in removal of protons from the haemolyph and the provision of an appropriate buffer against further acidosis (Taylor and Wheatly, 1981).

In summary, these results reveal a close similarity between the response of the crayfish to hypoxia-induced lactic acidosis and the response of the freshwater turtle to the same stress. Both species appear to utilize their shell in two ways: first, as a temporary storage site for lactate and, second, as a source for supplemental buffering of the extracellular fluid. These functions may be crucial in enabling these animals to survive periods of prolonged oxygen deprivation and the associated high circulating levels of lactate.

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