

EXERCISE IN THE TERRESTRIAL CHRISTMAS ISLAND RED CRAB *GECARCOIDEA NATALIS*

I. BLOOD GAS TRANSPORT

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Summary

The respiratory and circulatory physiology of the terrestrial Christmas Island red crab *Gecarcoidea natalis* was investigated with respect to exercise in the context of its annual breeding migration. Red crabs were allowed to walk for predetermined periods of up to 45 min. During this exercise period, blood gas measurements were made on venous, pulmonary and arterial samples to assess the function of the lungs in gas exchange and the performance of the circulatory system in gas transport and to determine the role and importance of the haemocyanin.

The lungs of *G. natalis* were very efficient at O₂ uptake, pulmonary blood being 80–90 % saturated throughout the 45 min exercise period. The maximum O₂-carrying capacity was 1.1 mmol l⁻¹, and haemocyanin (Hc) delivered 86 % of oxygen in resting crabs and 97 % during exercise. Oxygen delivery to the tissues was diffusion-limited during exercise. Indirect evidence, from the changes in haemolymph pH during transit through the lungs, suggested that the lung is the site of CO₂ excretion. The Bohr shift was high at high pH (pH 7.8–7.5, $\varphi = -1.23$) but decreased at low pH (pH 7.1–6.8, $\varphi = -0.48$). The decreased Hc affinity for O₂ during the exercise period facilitated O₂ delivery to the tissues without impairing O₂ loading at the lungs. The decrease in pH was sufficient to explain the change of affinity of Hc for O₂ during the exercise period. The marked acidosis (0.8 pH unit decrease) was largely metabolic in origin, especially during sustained locomotion, but less than could be predicted from concomitant lactate production.

Introduction

The Christmas Island red crab *Gecarcoidea natalis* (Pocock) is renowned for its annual breeding migration. During the migration, *G. natalis* travel up to several kilometres in a few days to reach the ocean (Hicks *et al.* 1990), but there is little information on the mechanisms and limitations of sustained exercise in migratory crabs.

Exercise physiology has been extensively studied in mammals, and their aerobic and anaerobic capacities and their limitations are well established (Alexander, 1991;

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Hoppeler and Billeter, 1991; Turner, 1991). Exercise has been examined in several terrestrial (Smatresk and Cameron, 1981; Wood and Randall, 1981*b*; Full and Herreid, 1983; Greenaway *et al.* 1988; Herreid and Full, 1988) and aquatic (McMahon and Wilkens, 1975; McMahon *et al.* 1979; Herreid, 1981; Booth *et al.* 1982) crustaceans. These studies largely focused, however, on selected aspects of respiratory physiology. There are good data for the cost of locomotion with respect to O₂ consumption and lactate production (Herreid *et al.* 1979; Wood and Randall, 1981*a*; Herreid *et al.* 1983; Full and Herreid, 1983; Full *et al.* 1985), the effect of exercise on heart and ventilation rate (Cameron and Mecklenburg, 1973; Wilkens, 1981; Booth *et al.* 1982) and the effect of activity on blood gas and acid–base status after exercise (McMahon *et al.* 1979; Wood and Randall, 1981*b*; Booth *et al.* 1984; Greenaway *et al.* 1988; Forster *et al.* 1989). Although data exist for O₂ and CO₂ exchange during exercise (Full and Herreid, 1984; Full *et al.* 1985; Hamilton and Houlihan, 1992), few studies have examined the changes in blood gas variables during the course of exercise.

Most of these studies have used rigorous conditions to assess endurance, capacity and speed, but few have related the results to the usual activities of the animals (e.g. Wheatly *et al.* 1985; Booth *et al.* 1982). However, since animals seldom move around in such an organised regimen, the resulting data can be difficult to relate to their ethology.

The common effect of exercise is a mixed respiratory and metabolic acidosis (e.g. Wood and Randall, 1981*b*; Booth *et al.* 1982; Wheatly *et al.* 1986; Greenaway *et al.* 1988; Milligan *et al.* 1988; Forster *et al.* 1989). The limitations of aerobic metabolism in exercising land crabs, however, have not been addressed directly. In terrestrial crustaceans, gas exchange can occur across gills and/or lungs (Taylor and Greenaway, 1979; Greenaway *et al.* 1983; Farrelly and Greenaway, 1987). The circulatory system of terrestrial crabs is relatively sophisticated (McMahon and Burnett, 1989) and highly organised with distinct afferent and efferent systems in the lungs (Greenaway and Farrelly, 1989). The pericardial sinus receives both post-branchial and pulmonary efferent (pulmonary vein) blood (Greenaway and Farrelly, 1989; Farrelly and Greenaway, 1993).

Oxygen delivery to the tissues in decapod crustaceans is facilitated by haemocyanin (Hc). In most water-breathing crabs, the oxygen affinity of Hc is modulated by metabolites such as L-lactate (Truchot, 1980) and urate (Morris *et al.* 1985) and by inorganic ions (Morris *et al.* 1987), whereas the haemocyanins of terrestrial crustaceans are apparently less sensitive to modulation (Morris *et al.* 1988; Morris, 1991). The role and importance of Hc in O₂ delivery tends to increase in decapod crustaceans during both hypoxia and exercise (Taylor, 1976; Booth *et al.* 1982; Bradford and Taylor, 1982; Wheatly *et al.* 1986; Greenaway *et al.* 1988; Lallier and Truchot, 1989).

Despite the wealth of data, there has been no attempt at an integrated study of the separate parts of respiratory/exercise physiology, i.e. gas exchange and transport, ventilation, perfusion, acid–base status and metabolic condition. Therefore, as part of an integrated investigation, the present study examined blood gas transport in exercising *G. natalis* to determine the ability of these crustaceans to sustain locomotion and to describe the limitations of the respiratory, gas exchange and circulatory systems.

Materials and methods

Husbandry

Gecarcoidea natalis (80–200 g) were collected from Christmas Island (under permit from the Australian Nature Conservation Agency) in November 1991, 1 week prior to the annual breeding migration. They were housed individually or in pairs in plastic fish boxes (30 cm×40 cm×15 cm) at 25±1 °C at over 80 % humidity. The boxes were cleaned and lettuce, carrots, apple, dry dog food and dry leaves were provided on a weekly basis. The red crabs used in this study were almost all males.

Preparation of animals for experiments and sampling

In each experiment, at least six individuals were sampled at each time period and different animals were sampled at each time period of exercise. To obtain arterial and pulmonary blood samples, it was necessary to pre-drill holes through the carapace using a dental drill (Dremel, model 396) without puncturing the underlying hypodermis. The hole was then sealed with vacuum grease and the crabs were allowed to recover until at least the following day (Greenaway *et al.* 1988). For a description of the anatomy of the circulatory system of various species of land brachyurans, including *G. natalis*, see Greenaway and Farrelly (1989).

Prior to experiments, animals were isolated overnight in darkened respiratory chambers aerated with water-saturated air, with no access to food or water. Rested animals were sampled immediately on removal from the respirometry chambers. To measure the rate of O₂ uptake of rested animals, the chambers with resting red crabs were connected to the respirometry equipment. Exercised animals were allowed to walk at a speed of their own choosing for 5, 10, 20 or 45 min in an exercise arena 2 m². The floor of the arena was kept wet at all times to maintain a high humidity. The crabs were allowed to walk from one end of the arena to the other and encouraged by gentle tactile stimulation to walk back across the arena. As soon as a red crab stopped walking, simple tactile stimulation was sufficient to encourage it to continue walking during prolonged exercise periods. Walking rates were recorded and are reported in Adamczewska and Morris (1994). All experiments were carried out at 25±1 °C unless stated otherwise.

Haemolymph samples were withdrawn from the pulmonary vein (pulmonary blood), the pericardium of the heart (arterial blood) and from the venous sinus (venous blood) *via* the arthrodial membrane at the base of the last walking leg. The holes in the carapace were immediately resealed with vacuum grease to prevent blood loss. Approximately 500 µl samples from the arterial and venous blood sites were obtained using 1 ml tuberculin glass syringes, whereas 100 µl of pulmonary blood was obtained using a 100 µl gas-tight Hamilton syringe. Syringes were prechilled on ice and blood samples were stored on ice to retard clotting.

Arterial and venous samples were immediately analysed at 25 °C for partial pressure and content of O₂ and CO₂ as well as blood pH. The smaller pulmonary blood sample was analysed for pH and for O₂ and CO₂ content. Samples of the blood were transferred using a short length (5 mm) of catheter tubing to a series of chilled Hamilton gas-tight syringes

to be used in the C_{CO_2} and C_{O_2} measurements (see below). All the measurements using the BMS 3 used blood injected directly from the sample syringe.

Blood gases and pH

The partial pressures of CO_2 and O_2 (P_{O_2} and P_{CO_2}) in the haemolymph were measured with an O_2 electrode (model E5047) and a CO_2 electrode (model E5037/SI) and recorded as mmHg (Torr, 1 mmHg = 0.1333 kPa). Both electrodes were housed in the BMS3 Mk II blood micro system, thermostatted at $25 \pm 0.2^\circ C$ and connected to a PHM73 pH/blood gas monitor (Radiometer, Copenhagen, Denmark).

The electrodes were calibrated with humidified gases each day before use. The O_2 electrode was calibrated using O_2 -free gas and with water-saturated air. The CO_2 electrode was calibrated using two humidified gases, one with 0.5 % and the other 2.5 % CO_2 (CIG certified analysis). P_{CO_2} of pulmonary blood was calculated according to the Henderson–Hasselbalch equation using $pK=6.1$, $\alpha_{CO_2}=0.0405$ and pH measured *in vivo*. P_{O_2} of pulmonary blood was calculated using pH and C_{O_2} measured *in vivo*, maximal haemocyanin oxygen-affinity ($Hc-O_2max$) determined for venous blood (assuming that the Hc content was the same in venous and pulmonary blood) and Hc O_2 -affinity and cooperativity were determined from the O_2 equilibrium curves constructed for *G. natalis* (see below). The calculations were standardised by accounting for different Hc concentrations in each animal.

Blood oxygen contents were measured using the modified Tucker chamber method (Tucker, 1967) according to Bridges *et al.* (1979) using 10 μl samples. The O_2 electrode was connected to an oxygen meter (Strathkelvin model 781) and changes in P_{O_2} were recorded on a pen recorder (Kipp and Zonen, model BD111). Calibration of the oxygen electrode was performed using an oxygen-free solution (Na_2SO_3) and air-saturated water.

The haemolymph CO_2 content was measured using a Corning 965 CO_2 analyser (calibrated with HCO_3^- standard, 15 mmol l^{-1}) using a 25 μl haemolymph sample.

Blood pH was measured using a G299a capillary electrode thermostatted at $25^\circ C$ in the BMS3 Mk II system. The pH electrode was calibrated regularly with Radiometer precision buffers of pH 7.410 (S1510) and 6.865 (S1500), accurate to ± 0.005 at $25^\circ C$.

Field measurements of P_{VO_2} were made on venous blood samples taken using chilled 1 ml syringes, from pre-migratory animals resting in their burrows. The P_{O_2} was determined at the burrow site using a Ni/Cd battery, Strathkelvin O_2 meter and Radiometer electrode housed in a D616 cell. Air temperature was constant at $25 \pm 1^\circ C$ (calibration temperature). Burrow P_{O_2} values at depths ranging from 15 to 50 cm were measured using a PE50 catheter to draw air from the burrow into a D616 cell.

Haemocyanin content and blood saturation

Blood haemocyanin content was measured by spectrophotometric scanning of a 10 μl blood sample in 1 ml of 50 mmol l^{-1} EDTA solution. The peak absorbance near 335 nm ($Hc \text{ Cu}^{2+}$) was used to calculate the Hc concentrations using the extinction coefficient $2.69 E_{1\%}^{1\text{cm}}$ (Nickerson and Van Holde, 1971).

Construction of O₂ equilibrium curves

Oxygen equilibrium curves were constructed using pooled blood samples collected from six animals exercised at each time period, 0, 10 or 45 min. Blood was equilibrated to various gas mixtures of N₂, O₂ and CO₂ at 25±0.1 °C in the BMS2 Mk II blood micro system using 80 µl tonometer equilibration tubes. Oxygen content of the blood samples was determined using the Tucker chamber. The required equilibration gas mixtures were prepared using gas-mixing pumps (Wösthoff, Bochum, Germany) and medical-grade gases. The pH of the samples was changed by altering the CO₂ concentration in the gas mixture (0.2–3 %) and measured near the *P*₅₀ using a microcapillary electrode (type G299a, Radiometer) thermostatted at the experimental temperature in the BMS3. Four equilibrium curves spanning the normal physiological pH range were produced for blood at each exercise time. The curves were analysed for O₂ affinity by calculating the *P*₅₀ and cooperativity of O₂ affinity as the *n*₅₀ according to the Hill equation, using values between 25 and 75 % saturation.

Non-bicarbonate buffering capacity of the blood

The buffering capacity of the blood was investigated by equilibrating blood with gas mixtures of different *P*_{CO₂}. Gas-mixing pumps and the BMS2 were used in the same way as for the construction of O₂ equilibrium curves. The equilibration gas mixture consisted of O₂, CO₂ and N₂, saturated with water before coming into contact with the blood. After equilibration, the pH of the blood (G299a pH electrode) and the CO₂ content (Corning CO₂ analyser) were measured in duplicate. The proportion of CO₂ in the equilibration gas ranged from 0.1 % to 5 %, producing pH values which spanned *in vivo* pH measurements (pH 6.739–7.528).

Osmolality of the blood was determined using an 8 µl sample in a Wescor 5100C vapour pressure osmometer. The osmometer was calibrated with two standards of known osmolalities (290 and 1000 mosmol kg⁻¹) covering the normal *in vivo* range.

Calcium in the blood

Blood calcium was determined for rested animals and for animals exercised for 5, 10, 20 and 45 min. Venous blood (200 µl) was mixed with an equal volume of 0.1 % nitric acid to deproteinise the sample, centrifuged, and 50 µl of the supernatant was diluted with 1.95 ml of 2.66 % LaCl₃·7H₂O. Three standards with [Ca²⁺] of 9, 15 and 20 mmol l⁻¹ were made up to span the expected concentration range of the samples to be analyzed. The Ca²⁺ concentration was calculated from the regression of the standard absorbance and concentration. The measurements were made using an atomic absorption spectrophotometer (Varian AA-175 series). The spectrophotometer was zeroed using Ca²⁺-free water and then checked using a blank solution of 1.95 ml of LaCl₃·7H₂O and 50 µl of Ca²⁺-free water.

Analysis of results

Variances were tested for homogeneity using Bartlett's (χ²) test (Winer *et al.* 1991) and/or Cochran's *C*. Time series data were compared by analysis of variance (ANOVA).

Post-hoc testing employed the Tukey HSD multiple means comparison test. All analysis was performed using the Systat statistical package.

Blood O₂ equilibrium data were subjected to analysis of covariance after testing for heterogeneity of slopes and covariance using software developed by Morris *et al.* (1985).

Significance of all data was taken at the probability level $P=0.05$ and all data are presented as mean \pm S.E.M., unless otherwise stated.

Definitions

CpCO ₂	Pulmonary blood carbon dioxide content (mmol l ⁻¹)
CaCO ₂	Arterial blood carbon dioxide content (mmol l ⁻¹)
CvCO ₂	Venous blood carbon dioxide content (mmol l ⁻¹)
CpO ₂	Pulmonary blood oxygen content (mmol l ⁻¹)
CaO ₂	Arterial blood oxygen content (mmol l ⁻¹)
CvO ₂	Venous blood oxygen content (mmol l ⁻¹)
PpCO ₂	Pulmonary blood partial pressure of carbon dioxide (mmHg=Torr)
PaCO ₂	Arterial blood partial pressure of carbon dioxide (mmHg)
PvCO ₂	Venous blood partial pressure of carbon dioxide (mmHg)
PpO ₂	Pulmonary blood partial pressure of oxygen (mmHg)
PaO ₂	Arterial blood partial pressure of oxygen (mmHg)
PvO ₂	Venous blood partial pressure of oxygen (mmHg)
Hc	Haemocyanin – Cu ²⁺ based respiratory pigment
Hc-O ₂	Haemocyanin-bound oxygen (mmol l ⁻¹)
Hc-O ₂ max	Maximum Hc oxygen-carrying capacity (mmol l ⁻¹)
S	Hc saturation coefficient (where 1.00=100 % saturation)
φ	Bohr factor ($\Delta \log P_{50}/\Delta \text{pH}$)
Arterial	Blood sampled from pericardium (mixed pulmonary and branchial return).

Note: 1 mmHg=0.133 kPa.

Results

Blood gases and pH

The O₂ content of pulmonary blood from quiescent animals was 0.98 ± 0.05 mmol l⁻¹ and remained high throughout the 45 min exercise period (0.98 – 0.85 mmol l⁻¹) (Fig. 1A). Arterial and venous blood both had significantly lower O₂ contents than pulmonary blood (ANOVA, $F_{2,85}=52.3$), with arterial blood carrying approximately 0.1 mmol l⁻¹ less O₂ than pulmonary blood in both rested and exercised animals. The oxygen contents of the pulmonary and arterial supplies were not significantly reduced even after severe exercise (more than 20 min). However, passage through the tissues resulted in significant oxygen removal, which was increased after 20 min of walking (ANOVA, $F_{4,25}=3.319$). For example, CvO₂ of resting *G. natalis* was 0.68 ± 0.14 mmol l⁻¹, but it was only 0.29 ± 0.07 mmol l⁻¹ after 20 min of exercise (Fig. 1A).

The measured partial pressure of O₂ in arterial and venous bloods and derived

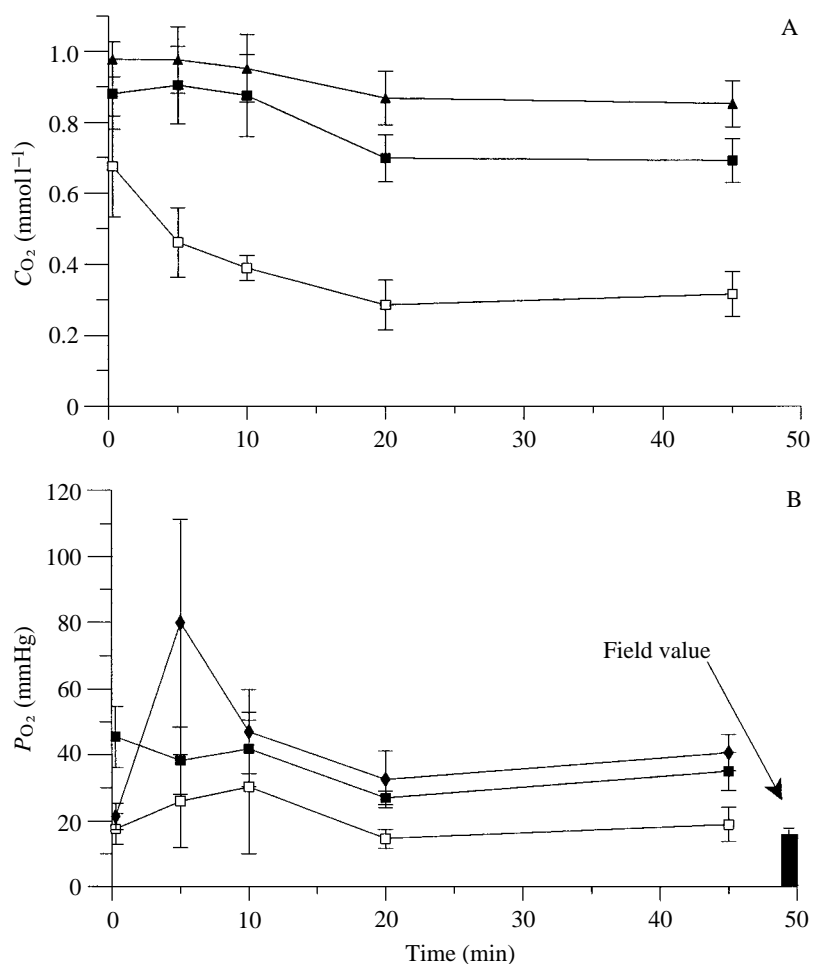


Fig. 1. (A) Oxygen content (CO_2 , mmol l^{-1}) in the haemolymph of *Gecarcoidea natalis* during exercise in pulmonary (▲), arterial (■) and venous (□) blood ($N=6$). (B) Oxygen partial pressure (P_{O_2} , mmHg) in the haemolymph of *Gecarcoidea natalis* during exercise. Calculated values for pulmonary samples (◆) and measured values for arterial (■) and venous (□) blood. The pulmonary values were calculated using haemocyanin (Hc) properties determined from the O_2 equilibrium curves determined for each individual animal at the pH measured *in vivo* and corrected for variation in Hc content ($N=6$ at each sampling time unless stated otherwise). The P_{vO_2} of animals sampled in their burrows on Christmas Island was 14.1 ± 1.8 mmHg and the P_{O_2} of the dry season burrows was 144 ± 1.2 mmHg. Values are mean \pm S.E.M.

pulmonary P_{O_2} are presented in Fig. 1B. Two-way ANOVA suggested that any difference between the three blood samples (pulmonary, arterial and venous) was dependent on the duration of exercise. Therefore, one-way ANOVA was used to test differences between P_{aO_2} , P_{vO_2} and P_{pO_2} over the 45 min.

Oxygen uptake and delivery were examined further by calculating the differences

between the O_2 contents of blood samples from each of the three sites; that is arterial–venous (a-v), pulmonary–venous (p-v) and pulmonary–arterial (p-a) differences. Despite the significant interaction in the previous ANOVA, one-way ANOVA on the a-v, p-v and p-a differences revealed no significant changes with time. Trends in this data set suggest that the P_{O_2} of venous blood is lower than that of arterial or pulmonary blood. Pa_{O_2} was 45.5 ± 9.3 mmHg in rested crabs and reached a minimum of 27.0 ± 2.0 mmHg in crabs exercised for 20 min, whereas the Pv_{O_2} was consistently lower,

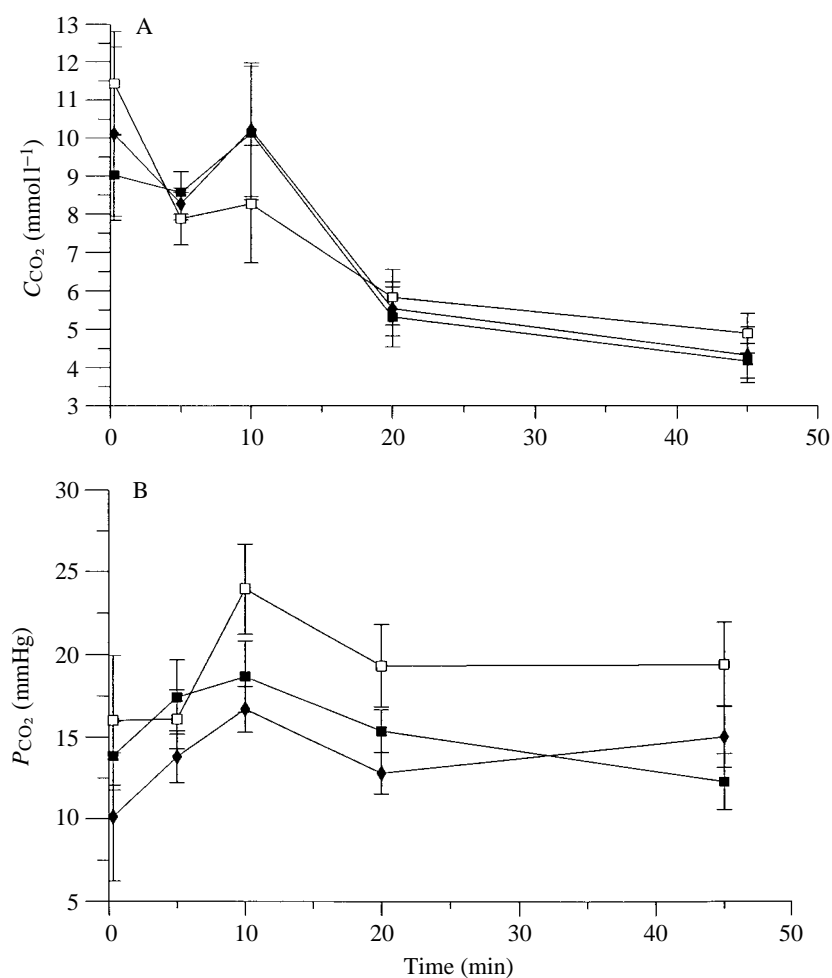


Fig. 2. (A) Carbon dioxide content (C_{CO_2} , mmol l^{-1}) in the haemolymph of *Gecarcoidea natalis* during exercise in pulmonary (◆), arterial (■) and venous (□) blood ($N=6$). (B) Carbon dioxide partial pressure (P_{CO_2} , mmHg) in the haemolymph of *Gecarcoidea natalis* during exercise. Calculated values for pulmonary (◆) blood and values measured *in vivo* for arterial (■) and venous (□) blood. P_{CO_2} for pulmonary blood was calculated according to the Henderson–Hasselbalch equation using $pK=6.1$, $\alpha_{CO_2}=0.0405$ and pH measured *in vivo* ($N=6$).

ranging from 14.6 ± 2.9 to 26.0 ± 14.0 mmHg (Fig. 1B). Field P_{vO_2} averaged 14.1 ± 1.8 mmHg in animals resting in their burrows and was thus indistinguishable from the laboratory values. The pulmonary P_{O_2} was low (21.4 ± 4.0 mmHg) in quiescent red crabs and increased at the onset of exercise, remaining above the arterial P_{O_2} throughout the exercise period.

In contrast, the CO_2 contents of the three categories of blood (pulmonary, arterial and venous) were not significantly different from each other. However, the average haemolymph CCO_2 decreased from $10.06 \text{ mmol l}^{-1}$ in rested animals to 4.46 mmol l^{-1} in animals exercised for 45 min (ANOVA, $F_{4,87}=32.17$) (Fig. 2A). It must be noted that there was a marked heterogeneity of variances in this data set, even after logarithmic and square-root transformations. Examination of Fig. 2A does show, however, a clear trend of a decreased CCO_2 over the course of the exercise period.

Venous P_{CO_2} was significantly higher than that of the pulmonary blood (ANOVA, $F_{2,73}=5.557$) (Fig. 2B), but did not increase significantly during the 45 min of exercise. P_{vCO_2} ranged from 16.0 ± 3.9 to 24.0 ± 2.7 mmHg and P_{pCO_2} from 10.1 ± 3.4 to 16.7 ± 1.4 mmHg, but the arterial P_{CO_2} was more variable, although generally remaining between that of the pulmonary and venous blood.

Prior to exercise, the blood had an overall mean pH 7.47 ± 0.04 , but during exercise a steady significant decrease to pH 6.89 or lower by 45 min was observed (ANOVA $F_{4,85}=45.85$) (Fig. 3). Individual red crabs showed a progressive decrease in pH from pulmonary to arterial to venous blood, such that the pH values of pulmonary blood, mean 7.188, and venous blood, mean 7.094, were statistically different (ANOVA $F_{2,85}=3.64$).

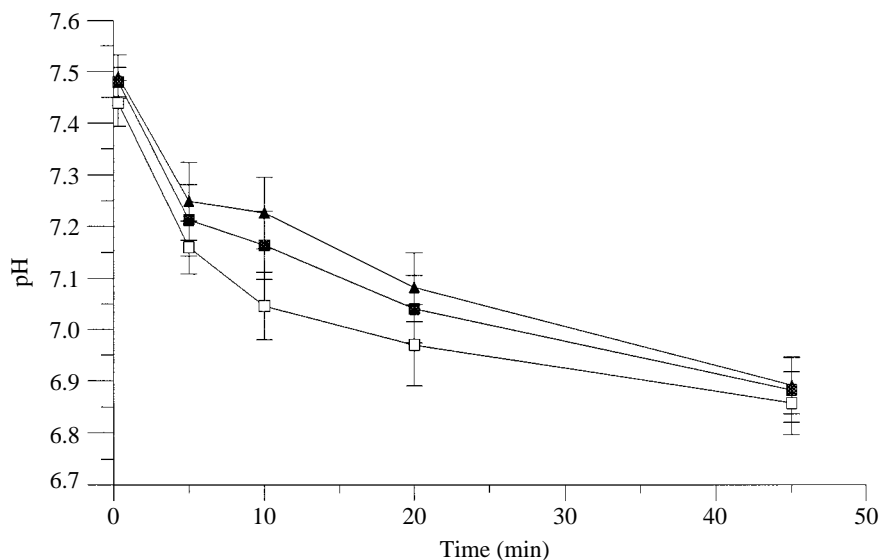


Fig. 3. The haemolymph pH of pulmonary (▲), arterial (■) and venous (□) haemolymph in *Gecarcoidea natalis* during exercise ($N=6$).

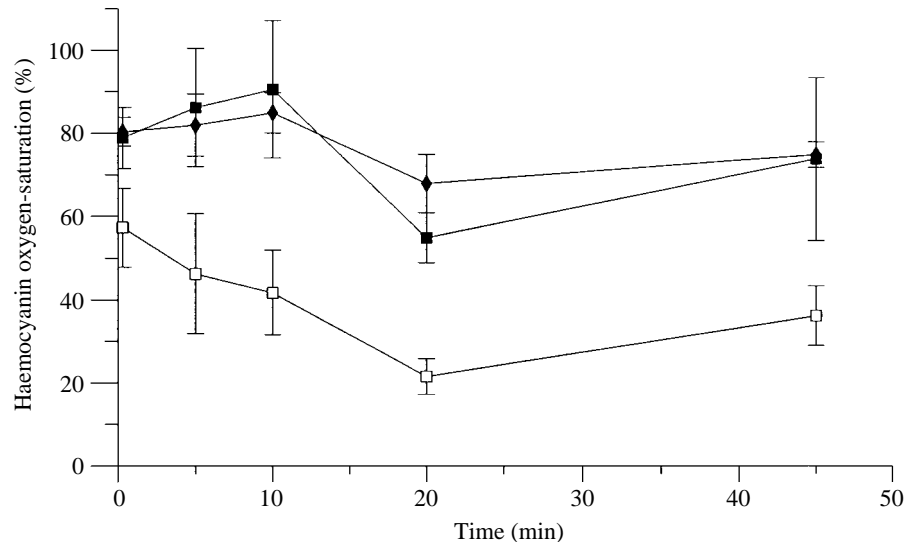


Fig. 4. The percentage of haemocyanin bound to O_2 in pulmonary (◆), arterial (■) and venous (□) blood during a 45 min period of exercise ($N=6$).

Haemocyanin O_2 -saturation

The haemocyanin in arterial and pulmonary blood samples was significantly more saturated with O_2 than that of the venous blood (ANOVA, $F_{2,72}=25.778$) (Fig. 4). Both arterial and pulmonary blood were highly saturated during the 45 min of exercise (80 % or more) and saturation tended to increase during early exercise (less than 10 min). There was a significant decrease in Hc O_2 -saturation at 20 min of exercise (ANOVA, $F_{4,72}=3.522$); this lowered saturation was seen in all three types of blood sample and corresponds to an increased Hc content of the blood in this group of red crabs (Table 1).

Oxygen equilibrium curves

The O_2 equilibrium curves constructed for the blood of resting crabs and crabs exercised for 10 and 45 min are presented in Fig. 5A–C. The curves for the blood of quiescent crabs indicated the greatest oxygen affinity (Fig. 5A). Curves constructed from the blood of exercised animals were shifted to the right (Fig. 5B,C), displaying a decrease in Hc oxygen-affinity. Analysis of covariance showed no significant change in the O_2 affinity of Hc simply as a result of time. Changes in pH during exercise were sufficient to

Fig. 5. (A) Oxygen equilibrium curves constructed with pooled blood from quiescent *Gecarcoidea natalis* at four different pH values (pH 7.822, 7.757, 7.566, 7.420). Individual data points have been omitted for clarity. (B) Oxygen equilibrium curves constructed with pooled blood from *Gecarcoidea natalis* exercised for 10 min (pH 7.629, 7.451, 7.369, 7.193). (C) Oxygen equilibrium curves constructed with pooled blood from *Gecarcoidea natalis* exercised for 45 min (pH 6.923, 6.818, 6.801, 6.767). The insets summarise the properties of each curve. The slope of each line describes the cooperativity of the haemocyanin and the x -intercept is the affinity of the curve ($\log P_{50}$) at each pH value.

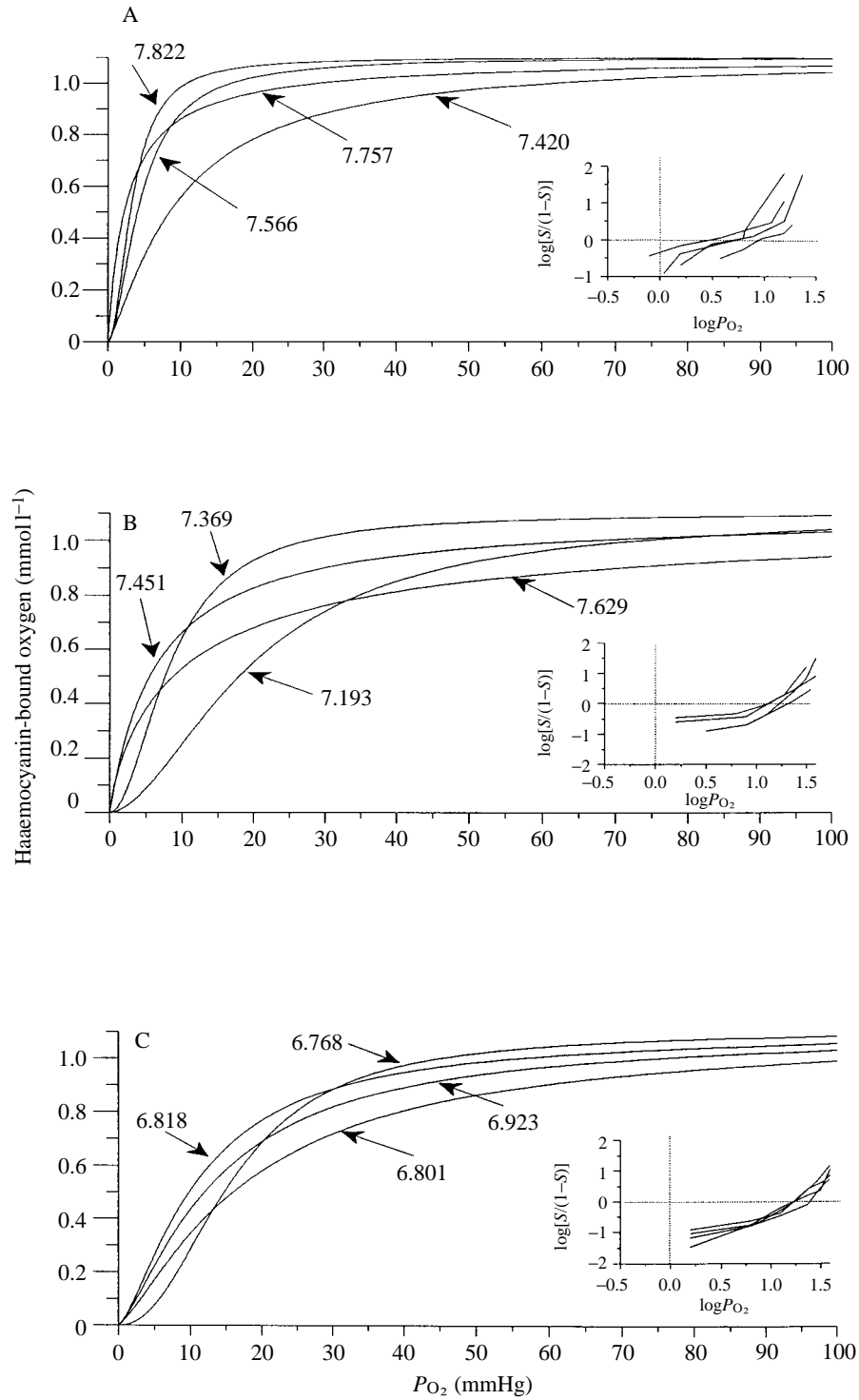


Fig. 5

Table 1. *Mean haemocyanin content (mmol l⁻¹) in Gecarcoidea natalis at rest and after exercise for different periods*

Time	Rest	Exercise			
		5 min	10 min	20 min	45 min
Mean \pm S.E.M.	1.08 \pm 0.08	0.91 \pm 0.12	0.99 \pm 0.16	1.22 \pm 0.09	0.93 \pm 0.10
Number of replicates	9	7	8	8	8

account for the observed changes in Hc O₂-affinity and currently there is no requirement to invoke other modulators. The relationships between O₂ binding cooperativity and the Hc O₂-affinity for each curve are summarised for the four pH values at each time period in the Hill plots (Fig. 5A–C, insets).

Further analysis of the data showed that the O₂ affinity of Hc (as log P_{50}) changed in a curvilinear fashion as a function of blood pH (Fig. 6A) and that O₂ binding cooperativity (n_{50}) of the pigment did not change during exercise (Fig. 6B) since the slope of the regression line was not significantly different from zero (t -test, d.f.=10, t =0.77). To determine the relationship between Hc O₂-affinity and pH, data for all three periods were plotted as log P_{50} against pH on one graph (Fig. 6A). Although data points for each period cluster around the associated *in vivo* pH range, over the entire *in vivo* physiological range of pH a general trend is apparent. At high pH, a change in pH of 0.1 unit (i.e. from 7.6 to 7.7) results in a large change in log P_{50} ; however, a change in pH from 6.7 to 6.8 has almost no effect since there is little dependency of O₂ affinity on pH (Fig. 6A).

Blood Ca²⁺ levels

Blood calcium levels were relatively constant during the first 10 min of exercise (22.8 mmol l⁻¹ in the resting crabs) and increased after 45 min of exercise to 25.24 mmol l⁻¹ (Table 2). Although this difference in [Ca²⁺] was not significant, a 2 mmol l⁻¹ increase in [Ca²⁺] would be of biological significance if it were to represent CaCO₃ mobilisation from the exoskeleton (see Discussion).

Non-bicarbonate buffer line

Buffering of the blood involving proteins and other poly-anions did not change during the course of the exercise, since there was no significant change in the value of $\Delta[\text{HCO}_3^-]/\Delta\text{pH}$. There was no significant change in the slope of the buffer lines during

Table 2. *Haemolymph [Ca²⁺] (mmol l⁻¹) at rest and after different periods of exercise in Gecarcoidea natalis*

Time	Rest	Exercise			
		5 min	10 min	20 min	45 min
Mean	22.81	22.90	22.76	24.26	25.24
S.E.M.	0.86	1.14	0.93	0.97	0.99

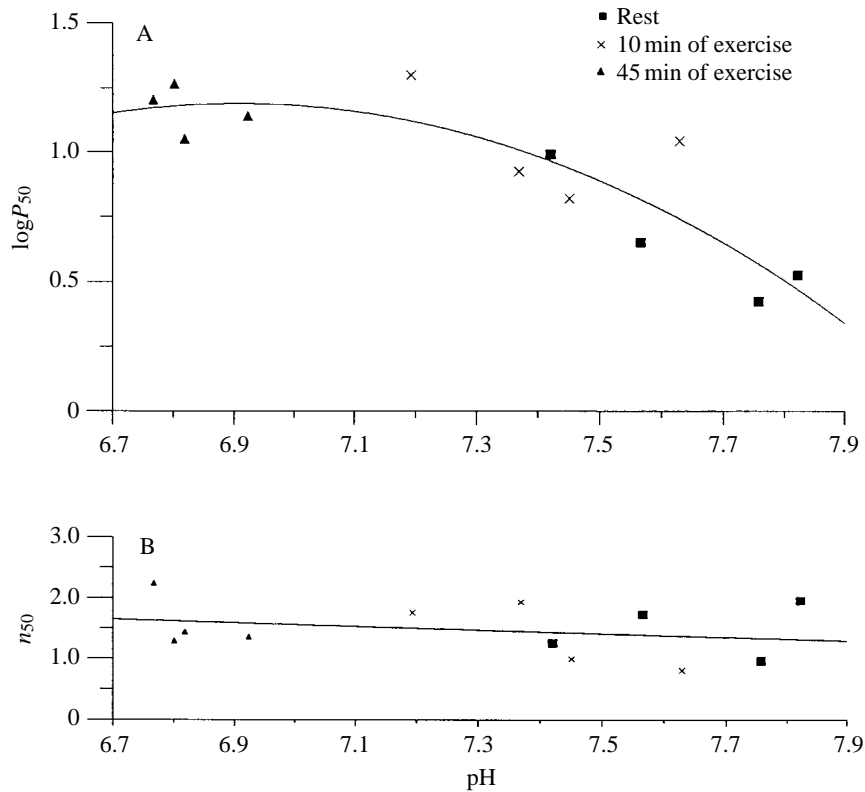


Fig. 6. (A) Comparison of the Bohr shift in whole blood of *Gecarcoidea natalis* from animals exercised for different periods. Owing to the curvilinear nature of $\Delta \log P_{50} / \Delta \text{pH}$ over the range of pH values measured *in vivo*, the Bohr factors were calculated from the regression equation over reduced pH ranges. pH 7.5–7.8: $\log P_{50} = 10.08 - 1.23 \text{pH}$ ($r^2 = 0.84$); pH 7.1–7.5: $\log P_{50} = 5.57 - 0.61 \text{pH}$ ($r^2 = 0.21$); pH 6.8–7.1: $\log P_{50} = 4.46 - 0.48 \text{pH}$ ($r^2 = 0.12$). (B) The cooperativity of haemocyanin from *Gecarcoidea natalis* exercised for 0, 10 and 45 min did not change over the measured *in vivo* pH range.

the 45 min exercise period, but the y-intercept decreased progressively with exercise (analysis of covariance, $F_{2,19} = 189$), indicating an overall decrease in bicarbonate buffering capacity at a similar pH with exercise (Table 3).

Discussion

Oxygen transport in blood

The pulmonary and arterial O_2 values for resting red crabs closely correspond to the values obtained by Greenaway and Farrelly (1989). Constant pulmonary O_2 levels throughout the 45 min exercise period demonstrate that extraction of O_2 from the air by *G. natalis* was not compromised. The consistent high level of saturation of both the pulmonary and arterial blood indicates that the air/blood diffusion interface at the lung is

Table 3. Regression equations for non-bicarbonate buffer lines for rested *Gecarcoidea natalis* and red crabs exercised for 10 and 45 min

Time exercised	Non-bicarbonate regression equation	r^2
Rest	$[\text{HCO}_3^- + \text{CO}_3^{2-}] = 96.48 - 11.86\text{pH}$	0.95
10 min	$[\text{HCO}_3^- + \text{CO}_3^{2-}] = 73.82 - 9.63\text{pH}$	0.88
45 min	$[\text{HCO}_3^- + \text{CO}_3^{2-}] = 56.66 - 7.65\text{pH}$	0.98

not a limiting factor in O_2 uptake. Thus, when the Hc is nearly saturated, large changes in P_{pO_2} (Fig. 1B) occur with very little change in O_2 content (Fig. 5A). The blood leaving the lungs was not 100 % saturated in *G. natalis*, but full saturation has been demonstrated in the terrestrial anomuran *Birgus latro* (Greenaway *et al.* 1988). Individual variations in [Hc] (determined by Cu^{2+} content), together with a constant blood O_2 content (Fig. 1A), can produce quite variable saturation levels (Fig. 4).

Assuming that efferent pulmonary and branchial supplies are the only returns to the heart, the lowered arterial CO_2 (cf. pulmonary) indicates that O_2 -rich pulmonary blood is mixing with relatively oxygen-poor postbranchial blood, showing that the gills do not play an important role in O_2 uptake. No direct measurements of postbranchial haemolymph O_2 content in brachyuran crabs are available, and thus the exact role of the gills is difficult to quantify, but Greenaway *et al.* (1988) have shown the gills are not involved in O_2 uptake in the anomuran crab *B. latro*. The lungs of *G. natalis*, like those of other land crabs, are well vascularised and provide a large surface area for gas exchange (Farrelly and Greenaway, 1993).

In aquatic species, Hc plays a relatively small role in O_2 delivery under normoxic resting conditions (>50 % of delivered O_2 in solution) but, in terrestrial species, Hc has been shown to deliver most of the O_2 to the tissues (Redmond, 1968; Greenaway *et al.* 1988). In *G. natalis*, the proportion of O_2 delivered by Hc increased from 86 % at rest to 97 % after exercise. In *B. latro*, this value remained unchanged at approximately 93 % (Greenaway *et al.* 1988). Additionally, the P_{aO_2} and Hc- O_2 max for *G. natalis* are comparable with values for *B. latro* (Greenaway *et al.* 1988), indicating that the role of Hc in O_2 delivery is very similar in both of these species.

Maintenance of a constant P_{vO_2} , and thereby the O_2 diffusion gradient into the tissues, concomitant with progressively declining CvO_2 requires that the O_2 affinity of Hc must decrease. Thus, changes in Hc O_2 -affinity must play an important role in optimising O_2 delivery to the tissues in *G. natalis*. The progressive decrease in CvO_2 during exercise was significant, but after 45 min of exercise the blood was still 36 ± 7 % saturated. A persistent large venous oxygen 'reserve' suggests that the limitation on O_2 delivery to the tissues is at the point of diffusion from the blood into and through the tissues.

Acid-base and CO_2 status

The excretion of CO_2 and the consequences for haemolymph pH are fundamental in determining circulating O_2 levels, the requirement for anaerobiosis and the *in vivo* functioning of the Hc. Clearly, *G. natalis* has little problem with CO_2 excretion since there was no persistent increase in CO_2 during the exercise period. It was not possible to

identify the site of CO_2 excretion conclusively, however, since it is difficult to demonstrate pre- and postpulmonary/branchial C_{CO_2} differences because of the large CO_2 pool in the blood (Fig. 2A).

Despite the absence of any difference in C_{CO_2} , venous blood had a significantly higher P_{CO_2} than pulmonary blood. The P_{CO_2} values (16.0–19.4 mmHg) in *G. natalis* are high compared with those of other terrestrial crustaceans (e.g. *Birgus*, 9.2 mmHg, Cameron and Mecklenburg, 1973; *Coenobita clypeatus*, 6.8 mmHg, McMahon and Burggren, 1979; *Gecarcinus lateralis*, 10 mmHg, Smatresk *et al.* 1979; *Cardisoma carnifex*, 15 mmHg, Wood and Randall, 1981b). This elevated P_{vCO_2} provides a high diffusion gradient for CO_2 excretion and, when plotted as a function of P_{O_2} according to Taylor and Innes (1988), it suggests that *G. natalis* is using the lung. During lung transit, haemolymph P_{CO_2} decreased while pH increased but, contrary to the Henderson–Hasselbalch principle, this was not accompanied by a decrease in C_{CO_2} (Fig. 7). Oxygenation of Hc at the lung liberates bound H^+ ; however, the blood pH

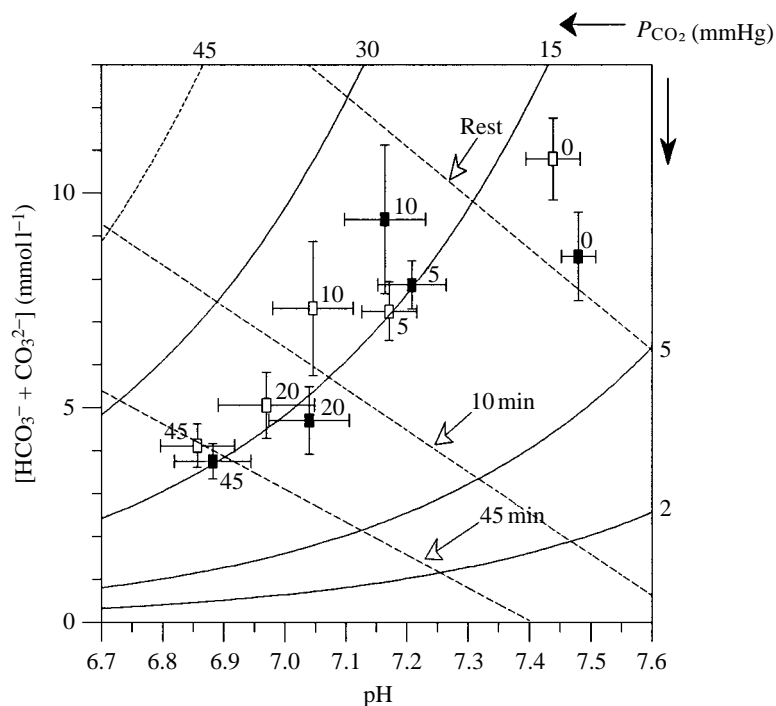


Fig. 7. The pH/[HCO_3^-] diagram for measured acid–base variables at 25 °C *in vitro*. Arterial (■) and venous (□) values are plotted, but since the acid–base perturbations in both blood samples were similar, the data were discussed for pooled blood (see text) ($N=6$). The broken lines represent the non-bicarbonate buffer lines determined *in vitro* for animals at rest and after 10 and 45 min of exercise. The blood for the *in vitro* determinations was removed from a separate group of animals. The equations for these lines are given in Table 3. Compensatory mechanisms of the acidosis not obvious from the pH/[HCO_3^-] diagram were detected using the method described by Wood *et al.* 1977 (see text).

increased after passage through the lungs (Fig. 3), indicating either that CO₂ is excreted or, less likely, that the lung is a site of oxidative metabolism (Morris and Greenaway, 1989).

Anaerobiosis was significant in *G. natalis* from the onset of exercise (Adamczewska and Morris, 1994), causing a marked blood acidosis that presumably reflects a severe tissue acidosis. The haemolymph acidosis in *G. natalis* (−0.79 pH units) was greater than that in the aquatic *Cancer magister* (−0.41 pH units, McMahon *et al.* 1979) and the terrestrial *Cardisoma carnifex* (−0.18 pH units, Wood and Randall, 1981b), *Coenobita compressus* (−0.4 pH units, Wheatly *et al.* 1986) and *Gecarcinus lateralis* (−0.45 pH units, Smatresk *et al.* 1979), but comparable to that of *B. latro* (−0.7 pH units, Greenaway *et al.* 1988). Differences in exercise regimen make it difficult to ascribe differences in acidotic tolerance to species or habitat, but *G. natalis* is clearly capable of tolerating unusually severe haemolymph acidosis.

Exercise-induced haemolymph acidosis in *G. natalis* has two discrete phases, as seen in the pH–bicarbonate diagram (Fig. 7). Phase 1 (0–10 min of exercise) was characterised by a mixed respiratory and metabolic acidosis (Fig. 7) as in previous findings (Smatresk *et al.* 1979; Wood and Randall, 1981b; Booth *et al.* 1984; Greenaway *et al.* 1988; Forster *et al.* 1989). During phase 2 (10–45 min of exercise), *P*CO₂ remained constant, indicating the further large blood acidosis is of purely metabolic origin.

Marked haemolymph acidosis in exercised *B. latro* was partially compensated, possibly by H⁺ excretion, *via* the gills during urine modification, as was initially suggested by Wolcott and Wolcott (1985, 1991) (Greenaway *et al.* 1988, 1990; Morris *et al.* 1991), or by hyperventilation (Smatresk and Cameron, 1981). An analysis of acid–base changes in exercising *G. natalis* as outlined by Wood *et al.* (1977) and Greenaway *et al.* (1988) shows that an equimolar influx of L-lactate and H⁺ into the blood would reduce haemolymph pH to near pH 5.5, considerably below the measured pH. This is equivalent to more than 15 mmol l^{−1} protons buffered in the blood during exercise. Mobilisation of skeletal CaCO₃ has been reported as a mechanism for compensating metabolic acidosis (e.g. Henry *et al.* 1981; Wood and Randall, 1981b; Cameron, 1985; Greenaway *et al.* 1988). In exercised *G. natalis*, blood Ca²⁺ levels increased from 22.8 mmol l^{−1} to 25.2 mmol l^{−1}, which could potentially account for the buffering of 5 mmol l^{−1} protons. A more likely explanation is that the remaining base equivalents are not required, since protons are buffered within the cells (Heisler, 1986). The relative contributions of these possible compensatory mechanisms require further investigation.

Blood function in O₂ transport

Evidence is accumulating to suggest that land crabs are relatively insensitive to organic modulators (Morris *et al.* 1985; Wheatly *et al.* 1986; Morris, 1990, 1991). The changes in Hc O₂-affinity of exercising *G. natalis* could be accounted for by the changes in pH measured *in vivo*. However, further detailed studies should determine whether there is any role for modulators (i.e. L-lactate and urate) in optimising Hc functioning in the context of natural activity levels.

The Hc of the red crabs showed significant pH sensitivity between pH 7.8 and pH 7.5 ($\varphi = -1.23$), but much less so between pH 6.8 and pH 7.1 ($\varphi = -0.48$). Lower pH

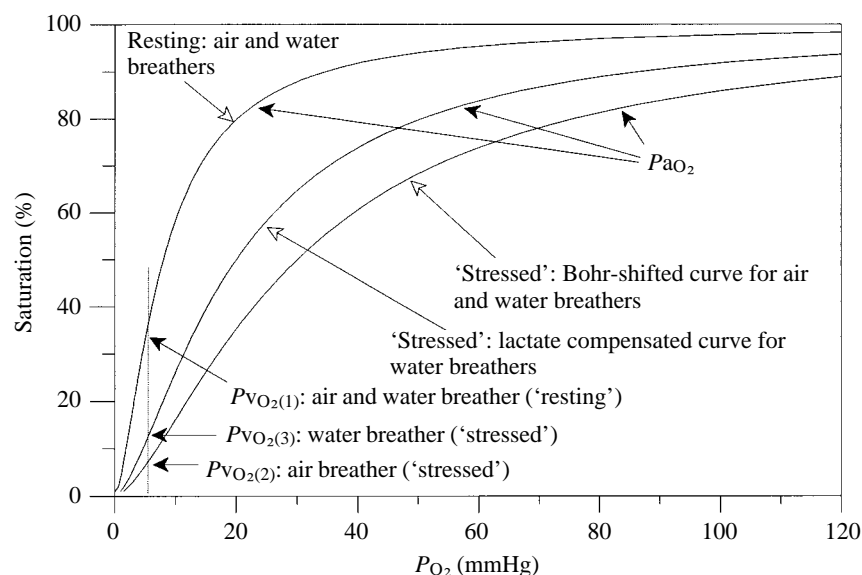


Fig. 8. Model equilibrium curves for a hypothetical crustacean. The changes in haemocyanin oxygen-affinity as a result of the Bohr shift are counteracted by L-lactate in aquatic crustaceans but not in terrestrial crabs. In aquatic crabs, L-lactate increases haemocyanin oxygen-affinity to maintain O_2 uptake at the gills. In terrestrial crustaceans, the haemocyanin remains fully saturated during stress and an increase in haemocyanin oxygen-affinity would result in decreased O_2 delivery to the tissues.

sensitivity at low pH is a property common to respiratory pigments (Morris, 1990) and of functional importance in *G. natalis*. At resting pH, the O_2 affinity and arterial saturation of Hc are high ($P_{50} \approx 5$ mmHg; $S = 80\text{--}90\%$). During mild exercise, delivery of O_2 to the tissues is facilitated by the markedly reduced Hc O_2 -affinity caused by a respiratory acidosis. However, further decreases in Hc O_2 -affinity induced by severe metabolic acidosis during sustained exercise are potentially maladaptive, compromising O_2 loading in the lung (cf. *Callinectes sapidus*; Booth *et al.* 1982), but further decreases are avoided by reducing the pH sensitivity of the Hc. The maximal oxygen-carrying capacities of *G. natalis* Hc (1.1 mmol l^{-1}) are within the range reported for other land crabs, e.g. 1.1 mmol l^{-1} in *B. latro* (Greenaway *et al.* 1988), 0.73 mmol l^{-1} in *Ocypode saratan* (Morris and Bridges, 1985) and 1.55 mmol l^{-1} in *Coenobita compressus* (Wheatly *et al.* 1986), while the affinity in quiescent animals ($P_{50} \approx 5$ mmHg) is relatively high compared with that of other species of land crab (Morris, 1991; Morris and Bridges, 1994).

Circulating L-lactate concentrations exceeded 30 mmol l^{-1} in exercised *G. natalis* (Adamczewska and Morris, 1994), but model O_2 equilibrium curves indicate that lactate is unlikely to be useful in optimising oxygen transport (Fig. 8). Prior to exercise, the Hc exhibits a high oxygen affinity (left-most curve, Fig. 8), which decreases during exercise owing to the hypercapnic lactacidosis of the blood inducing a Bohr shift of the curve (right-most curve, Fig. 8). Potential partial compensation of the modelled Bohr shift by a 'L-lactate effect' increases the affinity of the pigment (middle curve, Fig. 8). Analysis of

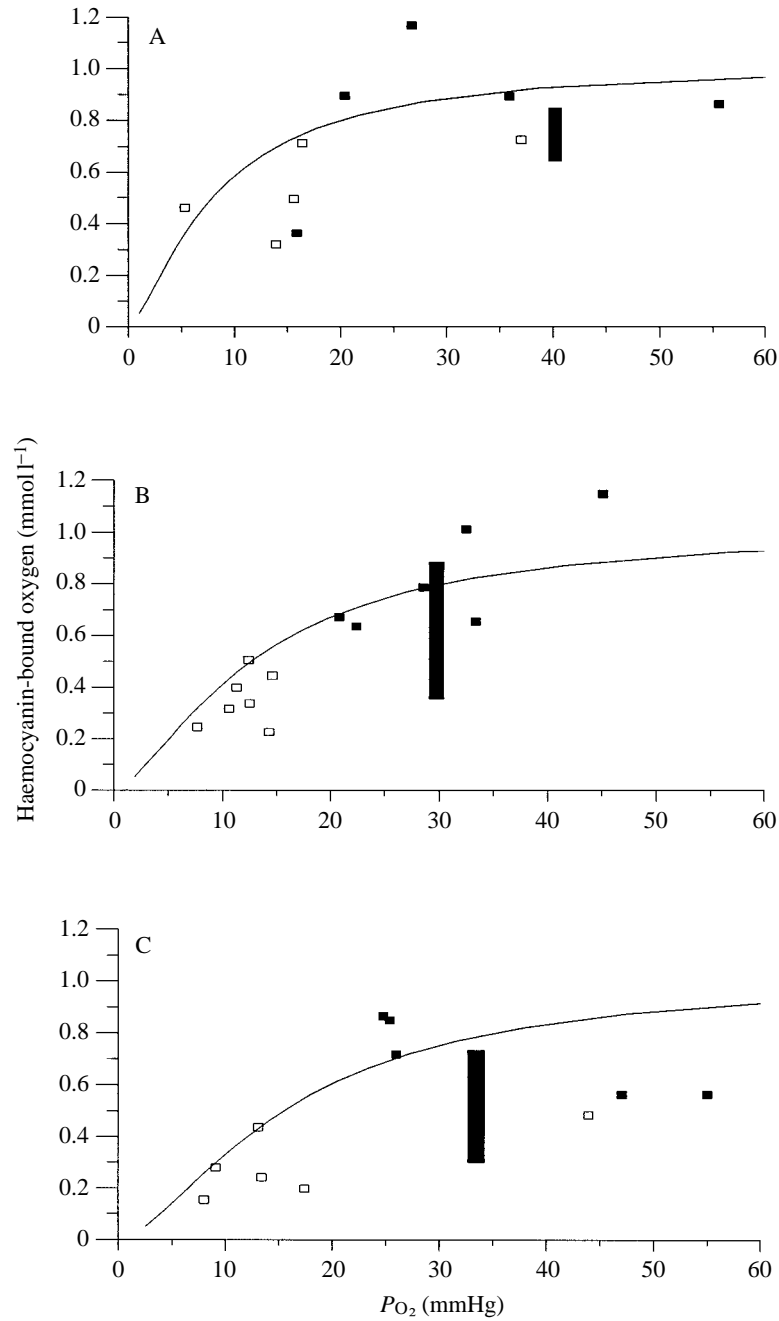


Fig. 9

O₂ equilibrium curves for resting *G. natalis* shows that Hc unloads a relatively small amount of the total bound O₂ to the tissues (Fig. 9A). During exercise, the saturation curves move, as predicted, to the right (Fig. 9B,C), lowering O₂ affinity and thus more

Fig. 9. Oxygen equilibrium curves constructed *in vitro* at 25 °C for *Gecarcoidea natalis* at rest (A) and after 10 min (B) and 45 min (C) of exercise. A shift of the equilibrium curve to the right with exercise indicates a decrease in haemocyanin oxygen-affinity. The decreased affinity of haemocyanin does not affect oxygenation at the lung but facilitates O₂ off-loading at the tissues. The black vertical bars represent the mean amount of O₂ delivered to the tissues by haemocyanin after different periods of exercise plotted against the mean P_{aO_2} . Individual data points for arterial (■) and venous (□) haemocyanin O₂-affinity measured *in vivo* show the relationship between P_{O_2} and haemocyanin oxygen-affinity in red crabs.

than doubling the Hc-bound O₂ delivered to the tissues. The change in affinity is such that P_{aO_2} and P_{vO_2} come to lie near either end of the steep part of the equilibrium curve, ensuring the maximal unloading of O₂ for a small change in P_{O_2} (i.e. a-v difference). The increased O₂ delivery is due largely to maintained O₂ loading at the lungs of *G. natalis* (and other land crabs). Compensatory modulation by L-lactate cannot increase O₂ loading at the lungs (since Hc remains nearly saturated with oxygen, Fig. 1A), but instead would decrease the O₂ delivered to the tissues at a constant P_{vO_2} (cf. a-v saturation for P_{aO_2} - $P_{vO_2(2)}$ and P_{aO_2} - $P_{vO_2(3)}$, Fig. 8). Thus, providing land crabs such as *G. natalis* can maintain O₂ loading, any L-lactate modulation of Hc would seem to be maladaptive.

Concomitant changes in levels of metabolites and energy metabolism are reported in Adamczewska and Morris (1994), but further work considering different walking strategies, including additional field observations/sampling, is required to elucidate fully the limiting factors of locomotion in these animals.

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