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THE FUNCTIONING OF THE HAEMOCYANIN OF THE TERRESTRIAL CHRISTMAS ISLAND RED CRAB *GECARCOIDEA NATALIS* AND ROLES FOR ORGANIC MODULATORS

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Summary

***Gecarcoidea natalis* is a land crab that migrates annually several kilometres to breed. The O_2-binding properties of haemocyanin in *G. natalis* were investigated *in vitro* to test the idea that the O_2-binding properties of the haemocyanin of land crabs are not dependent on circulating modulators and to provide a model of haemocyanin functioning during exercise. The affinity of the haemocyanin for O_2 decreased with increasing temperature (change in the heat of oxygenation; ΔH=–59 kJ mol^{-1}). The haemocyanin of *G. natalis* apparently differs from that of other terrestrial crabs in showing haemocyanin O_2 modulation by both organic and inorganic molecules. Haemocyanin O_2-affinity was not affected by Mg^{2+} but was sensitive to changes in Ca^{2+} concentration ($\Delta\log P_{50}/\Delta\log[Ca]$=–0.61, where P_{50} is the partial pressure of O_2 required for half-maximal O_2 binding). The Bohr factor was modest (ϕ=–0.26±0.03, N=4, in whole haemolymph at 25 °C) and there was no specific effect of CO_2 on the O_2-binding properties of the haemocyanin. An increase in urate concentration increased haemocyanin O_2-affinity, but the effect was linear ($\Delta\log P_{50}/\Delta$[urate]=–0.06) and not logarithmic as is the case in other species. The effect of L-lactate on the haemocyanin O_2-affinity in *G. natalis* was unique among the crustaceans, because an increase in L-lactate concentration decreased the haemocyanin O_2-affinity. The effect of L-lactate on haemocyanin O_2-affinity ($\Delta\log P_{50}/\Delta\log$[lactate]) was time-dependent and decreased from a maximum of 0.044 on day 1 to 0.001 after 4 days of storage at 4 °C. The presence of an unknown dialysable and unstable factor in the haemolymph is postulated to explain the time-dependent effect of L-lactate on haemocyanin O_2-binding properties. Model oxygen equilibrium curves constructed for *in vivo* conditions showed that the reverse effect of L-lactate was advantageous by decreasing the O_2-affinity of the haemocyanin beyond that predicted by the Bohr shift alone and assisted in O_2 off-loading at the tissues. This effect of lactate can only provide an advantage if the gas-exchange organs maintain arterial O_2 loading and thus is dependent on lung function in land crabs and must have occurred coincident with the evolution of these other features.**

Key words: *Gecarcoidea natalis*, Christmas Island red crab, haemocyanin, modulation, oxygen transport.

Introduction

The major respiratory pigment in the haemolymph of decapod crustaceans is the copper-based protein haemocyanin. Haemocyanin markedly increases the O_2-carrying capacity of the haemolymph and is now appreciated to be under the control of a complex suite of effectors and organic modulators that regulate O_2-binding by haemocyanin (for reviews, see Mangum, 1983; Morris, 1990, 1991; Burnett, 1992; Truchot, 1992; Morris and Bridges, 1994). Thus, haemocyanin functioning is apparently controlled in a manner analogous to vertebrate haemoglobin O_2-binding. A significant role for haemocyanin in O_2 transport in quiescent crabs has been questioned, and the involvement of haemocyanin in O_2 transport varies among species. The importance of O_2 delivery by haemocyanin increases in response to increased O_2 demand and during environmental hypoxia (e.g. McMahon *et al.* 1979; Greenaway *et al.* 1988; Lallier and Truchot, 1989; Morris *et al.* 1996*b*).

Recently, a dichotomy in the functioning of haemocyanin in terrestrial and aquatic decapod crustaceans has become apparent: terrestrial decapods appear to have haemocyanin with little or no sensitivity to most modulators. In some cases, this appears to be due to specific blocking of binding sites by dialysable compounds (e.g. Morris *et al.* 1988) and in others to an absence of binding (Morris and Bridges, 1994). The haemocyanins of terrestrial crustaceans tend to become readily saturated with O_2 at the gas-exchange surfaces, despite a haemolymph acidosis, partly as a result of the efficiency of the gas-exchange organs (e.g. Wood and Randall, 1981; Greenaway *et al.* 1988; Adamczewska and Morris, 1994*a*, 1998). In addition, the lower viscosity of air, compared with

water, coupled to the relatively greater abundance of O_2 over CO_2 in air compared with water allows, a relative hypoventilation in air-breathers and reduces the costs of O_2 extraction from air. Consequently, the need for modulators to increase haemocyanin O_2-affinity in terrestrial crabs appears to be largely redundant (Morris, 1990). Instead, terrestrial crustaceans appear to rely on altering ventilation and circulation rates to maintain O_2 delivery by haemocyanin (Morris, 1991; Morris and Bridges, 1994). Haemocyanin is also the main protein in the haemolymph of crustaceans (Chen and Cheng, 1993), and higher concentrations of haemocyanin in terrestrial compared with aquatic species are thought to provide increased buffering for the higher P_{CO_2} and consequent CO_2 content in air-breathers (for reviews, see Mangum, 1983; Morris and Bridges, 1994).

The ability to increase the rate and capacity of O_2 delivery is essential if the increased metabolic rate is to be supported aerobically and, thereby, to increase the scope for activity. While increased activity can also be supported by anaerobic metabolism, anaerobiosis is very inefficient compared with aerobiosis and results in acidosis and an O_2 debt that must be repaid later (Herreid and Full, 1988).

The affinity of haemocyanin for O_2 can be affected by temperature, pH and salinity (for a review, see Truchot, 1992) as well as by neurohormones (Morris and McMahon, 1989; McMahon and Morris, 1990), L-lactate, urate (Truchot, 1980; Morris *et al.* 1985*a*) and various inorganic molecules (Mangum, 1983; McMahon, 1986; Morris, 1990, 1991). Changes in the O_2-affinity of respiratory pigments in response to temperature and salinity represent perturbing effects rather than regulatory mechanisms (Jokumsen and Weber, 1982; Morris, 1991; Lallier and Truchot, 1997). In contrast, L-lactate, urate and Ca^{2+} are thought to modulate haemocyanin O_2-affinity to optimise O_2 delivery to the tissues during exercise or environmental hypoxia (for a review, see McMahon, 1985; Morris, 1990; Burnett, 1992; Truchot, 1992). For example, anaerobiosis in crustaceans generates L-lactate, and the concomitant acidosis results in decreased haemocyanin O_2-affinity. In many aquatic crustaceans, L-lactate can partially offset the Bohr shift, thereby assisting O_2 uptake at the gas-exchange surfaces by increasing the O_2-affinity of haemocyanin (Booth *et al.* 1982; Morris *et al.* 1986*a*,*b*; McMahon, 1985; Morris, 1990). It is these organic modulator effects that seem to be specifically absent in land crabs.

The condition of greatest potential demand on haemocyanin functioning is likely to be either during moderate environmental hypoxia or during exercise at close to the maximum aerobic speed or during repeated sprinting. Thus, functionally important modulations of O_2-binding by haemocyanin in terrestrial crabs might be most important during exercise since they rarely encounter environmental hypoxia.

The Christmas Island red crab *Gecarcoidea natalis* provides a model to assess haemocyanin function in land crabs since, with the exception of a 3 week larval phase, it is completely terrestrial. In addition, these red crabs undertake an annual breeding migration during which they may walk distances of 1 km per day for a number of consecutive days. Importantly, this species does not exhibit exceptional aerobic capacity (ability to increase exercise intensity) compared with that of other crustaceans, and O_2 shortage at the tissue level during exercise results in anaerobiosis (Adamczewska and Morris, 1998). A characterisation of O_2 binding by the haemocyanin of *G. natalis* was carried out to determine the response to any effectors of haemocyanin O_2-affinity. This characterisation of the haemocyanin O_2-affinity and the sensitivity of O_2-binding to haemocyanin to modulators *in vitro* was used, together with *in vivo* data (Adamczewska and Morris, 1998), to model the functioning of the pigment *in vivo* in exercising *G. natalis*. The models of haemocyanin O_2-function could then be used to assess whether anaerobiosis during exercise occurs as a result of insufficient O_2 delivery to the tissues or whether it is due to other limitations, such as the aerobic capacity of the tissues.

Materials and methods

Construction of oxygen equilibrium curves in vitro

Red crabs *Gecarcoidea natalis* (Pocock) with a body mass ranging from 114 to 232 g (190±6 g; mean ± S.E.M., N=50) were collected from Christmas Island and maintained in the laboratory as described previously (Adamczewska and Morris, 1994*a*). To determine the functioning of the haemocyanin of *G. natalis* in oxygen transport, *in vitro* oxygen equilibrium curves were constructed using a spectrophotometric diffusion chamber method (Sick and Gersonde, 1969; Morris *et al.* 1985*b*). Samples of venous haemolymph were obtained by sampling *via* the arthrodial membrane at the base of the last walking leg. In the diffusion chamber, a subsample of haemolymph (15 µl) was deoxygenated with CO_2/N_2 mixtures (Wösthoff gas-mixing pumps), and the change in absorbance was determined after stepwise addition of O_2. Changes in absorption at 335 nm were proportional to the O_2 saturation of the haemocyanin. The pH of the haemolymph was controlled by changing the proportion of CO_2 (0.1–3.6 %) to span the pH range measured *in vivo*. The pH of the haemolymph was measured near the P_{50} (the partial pressure of O_2 at which the haemocyanin is 50 % saturated) with a capillary electrode (G299a, Radiometer) housed in a BMS2 (Blood Micro System) and thermostatted at the experimental temperature of 25±0.1 °C, unless otherwise specified.

Haemolymph from at least eight red crabs was pooled to produce each 'batch' of haemolymph used for the construction of oxygen equilibrium curves (OECs). Because of the number and duration of treatments, it was necessary to use several different batches of haemolymph. The effects of all treatments on the O_2-binding properties of a batch of haemolymph were compared with the O_2-binding properties of that batch of whole haemolymph. All OECs were constructed using fresh haemolymph refrigerated at 4 °C or held on ice. The individual crabs used for haemolymph sampling were different for each batch of haemolymph. At the end of a series of determinations, and for each batch of haemolymph, the haemocyanin O_2-

affinity was checked to ensure that the haemolymph had not deteriorated.

Effect of temperature

The effects of temperature on haemocyanin O_2-affinity and the cooperativity of O_2-binding in whole haemolymph were determined at 5 °C intervals over the range 15–35 °C. The change in heat of oxygenation (ΔH; kJ mol^{-1}) was calculated as:

$$\Delta H = -2.303\boldsymbol{R}\,\frac{\Delta \log P_{50}}{(1/T_1) - (1/T_2)}\ ,$$

where $\boldsymbol{R}$ is the gas constant (in kJ) and T is temperature (in K).

Effect of metabolites

The concentrations of L-lactate and urate in the haemolymph were manipulated to determine their effect on the haemocyanin O_2-affinity and binding cooperativity at 25±0.1 °C. A 500 µl sample of haemolymph was enriched with uric acid by equilibration with 4 mg of uric acid (Sigma, no. U-2625) overnight. The haemolymph was then centrifuged for 10 min at 10 000 $\boldsymbol{g}$ to precipitate any excess solid urate. Haemolymph enriched with urate was then diluted with the original whole haemolymph to obtain different concentrations of uric acid. The use of uric acid resulted in slight acidification of the haemolymph samples (see Results), but sodium urate did not appear to bind to the haemocyanin since it was not possible to increase the urate concentration in the haemolymph using the sodium salt. Urate concentrations in the haemolymph were determined using a Sigma Diagnostics test kit (no. 685).

The effect of L-lactate was investigated using four separate batches of haemolymph, each taken from a different group of eight red crabs, which had been collected from Christmas Island on one of two different occasions. The L-lactate concentration in whole haemolymph withdrawn from the crabs was always less than 1 mmol l^{-1}; in haemolymph enriched with L-lactate, the concentration of L-lactate ranged from 12.3 to 26.5 mmol l^{-1} (see Table 1). To determine the effect of L-lactate, haemolymph was enriched with L-lactate by the ultracentrifugation and replacement method (Bridges and Morris, 1986). Briefly, in the morning of the day of construction of the OEC, a sample of whole haemolymph (300 µl) was centrifuged (Air-Fuge, Beckman, USA) for 20 min at 160 000 $\boldsymbol{g}$ to pellet the haemocyanin. A proportion (10 %) of the plasma solution was removed and replaced with an equal volume of concentrated L-lactate (Sigma, no. L-2250 lithium lactate) solution in *G. natalis* Ringer, consisting of (in mmol l^{-1}): NaCl, 335; KCl, 8.5; $MgSO_4$, 1; $MgCl_2$, 10.4; $CaCl_2$, 17.5; and $NaHCO_3$, 1. The salt concentrations of the Ringer's solution were based on the measured salt concentration in the pooled whole haemolymph sample (see below). The sample was remixed and used for OEC determination. The concentration of L-lactate in each sample was determined using a Boehringer Mannheim test kit (no. 138 084). Control OECs were constructed on a different pooled haemolymph sample containing 20 mmol l^{-1} LiCl.

The combined effect of L-lactate and urate on the haemocyanin O_2-affinity was also examined. A series of OECs was constructed using haemolymph enriched with three different concentrations of uric acid and containing 21 mmol l^{-1} L-lactate.

Effect of dialysis and divalent cations

Haemolymph samples (400 µl) were dialysed for 20 h at 4 °C in two washings, each consisting of 2 l of *G. natalis* Ringer's solution at pH 8.1. The effects of Mg^{2+} and Ca^{2+} on the haemocyanin O_2-binding properties were investigated by dialysing haemolymph in Ringer's solutions containing three different concentrations of Mg (5.2, 10.4 and 16 mmol l^{-1}) and Ca (10, 17.5 and 24 mmol l^{-1}). The concentrations in the dialysed haemolymph were determined as total elemental concentration by the Atomic Adsorption Spectrophotometer (AAS) (GBC 906). To suppress interference, determinations of Na and K were made in the presence of 5.9 mmol l^{-1} $CsCl_2$ and of Mg and Ca in the presence of 7.2 mmol l^{-1} $LaCl_3$.

Separate haemolymph samples were similarly dialysed but in *G. natalis* Ringer's solution containing sufficient L-lactate to raise the concentration in the sample to 21 mmol l^{-1}. Some of these samples were further manipulated to alter the urate concentration (see above) so as to produce dialysed haemolymph containing 21 mmol l^{-1} and a range of urate concentrations. The maximum concentrations of L-lactate used were well within the physiological range for *G. natalis* which, during intensive exercise, may have haemolymph levels in excess of 30 mmol l^{-1} (Adamczewska and Morris, 1994*b*). The upper limit for the urate concentration used was determined by the maximum carrying capacity of the haemolymph and thus the maximum effect achievable.

Specific effect of CO_2

The specific effect of CO_2 on haemocyanin O_2-binding was determined by manipulating haemolymph pH by the addition of either 0.01 mol l^{-1} of HCl-enriched or 0.01 mol l^{-1} NaOH-enriched Ringer's solution to induce a 'fixed acid' Bohr shift rather than a CO_2 Bohr shift. A subsample of haemolymph (150 µl) was ultracentrifuged for 20 min at 160 000 $\boldsymbol{g}$ to sediment the haemocyanin (Beckman, Air-Fuge) and 40 µl of the plasma was removed. The plasma was then mixed with different volumes (range 10–40 µl) of the acidic or basic red crab Ringer's solution, and 40 µl of this mixture was returned to the haemolymph, and the haemocyanin was resuspended (Bridges and Morris, 1986). The OECs were constructed at constant CO_2 levels of either 0.4 % or 4 % CO_2. Analysis of covariance (ANCOVA) was used to test for heterogeneity in the variances (slopes) between the data sets to determine the dependence of $\log P_{50}$ on pH. For data sets with homogeneous variances, the elevations of the regression lines were compared to determine whether changes in haemocyanin O_2-affinity had occurred. The significance level of P=0.05 was applied to all the resulting F values. Values are presented as means ± S.E.M.

Results

For each OEC, the P_{50} at the physiological pH of 7.6 was calculated from the regression equations describing the dependence of logP_{50} on pH and used for comparisons among treatments. The P_{50} and cooperativity of O_2-binding (n_{50}) were calculated using saturation values between 25 % and 75 %.

Effect of temperature on haemocyanin O_2-affinity and binding cooperativity

The affinity of red crab haemocyanin for oxygen was clearly dependent on temperature (Fig. 1A). The ANCOVA showed that the dependence of logP_{50} on pH (Bohr factor) was the same for haemocyanin at 20, 25 and 35 °C (i.e. homogeneous variances) and that the reduction in haemocyanin O_2-affinity induced by increased temperature was statistically significant (significant change in elevation of slopes). The affinity of haemocyanin for O_2 across the range 15–35 °C decreased from a P_{50} of 0.80 kPa to 3.31 kPa at pH 7.6 (Fig. 1A). The haemocyanin sensitivity to temperature was lowest in the range 30–35 °C (ΔH=–31.9 kJ mol^{-1}) and highest between 15 and 20 °C (ΔH=–62.2 kJ mol^{-1}), with an overall ΔH of –59.0 kJ mol^{-1} over the temperature range examined (15–35 °C). The magnitude of the Bohr shift (ϕ=ΔlogP_{50}/ΔpH) was lowest at the lowest temperature of 15 °C (ϕ=–0.13) but increased to a maximum at 30 °C (ϕ=–0.49) (Fig. 1A). The cooperativity of haemocyanin O_2-binding (n_{50}=3.16±0.07, N=27) of the red crab haemocyanin was not dependent on either pH or temperature (Fig. 1B).

The specific effect of CO_2: fixed-acid Bohr shift

There was clearly no difference between the ‘fixed acid’ and CO_2 Bohr shifts, and ANCOVA did not reveal any specific effect of CO_2 on haemocyanin O_2-affinity. The relationship between logP_{50} (measured in kPa) and pH could be described by a common equation for both 0.4 and 4.0 % CO_2: logP_{50}=2.03–0.235pH (r^2=0.943). Similarly, there was no obvious difference between haemocyanin O_2-binding cooperativity at 0.4 % or 4 % CO_2, with a mean n_{50} of 3.13±0.06 (N=19).

The effect of Mg^{2+} and Ca^{2+} on haemocyanin O_2-binding properties

Haemolymph dialysed against three concentrations of Mg showed no change in either affinity or cooperativity of haemocyanin O_2-binding. The relationship between logP_{50} (measured in kPa) and pH could be described by a common

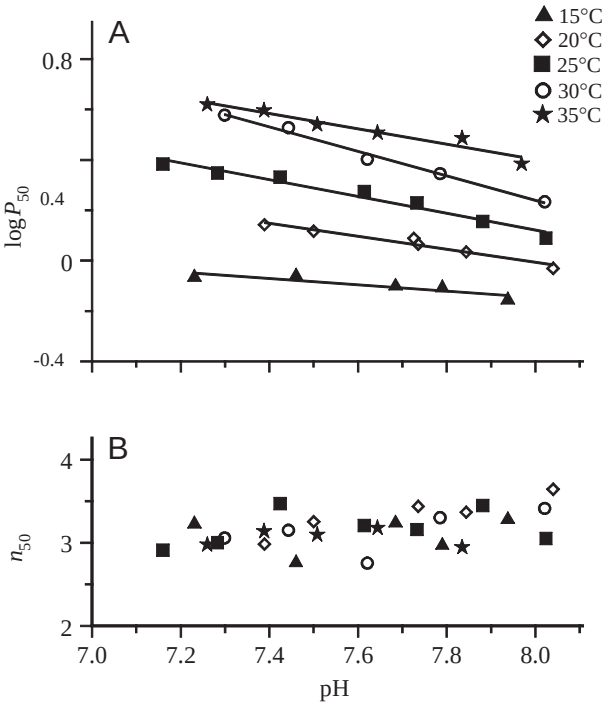

Fig. 1 (A) The haemocyanin O_2-affinity of *Gecarcoidea natalis* and the relationship between logP_{50} (measured as kPa) and pH at different temperatures: 15 °C, logP_{50}=0.853–0.125pH (r^2=0.819); 20 °C, logP_{50}=2.061–0.258pH (r^2=0.953); 25 °C, logP_{50}=2.794–0.334pH (r^2=0.967); 30 °C, logP_{50}=4.139–0.487pH (r^2=0.990); 35 °C, logP_{50}=2.841–0.305pH (r^2=0.939). (B) The cooperativity of O_2-binding (n_{50}) by haemocyanin in the temperature range 15–35 °C over a range of pH.

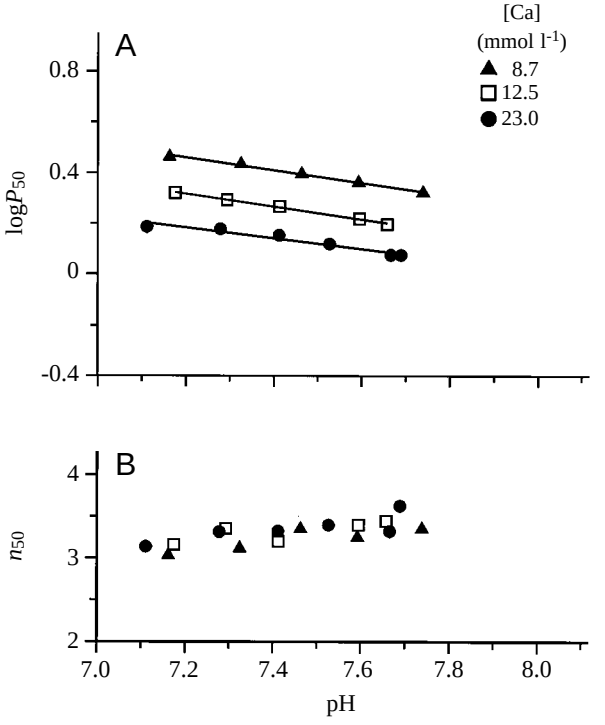

Fig. 2. (A) The effect of Ca concentration on the haemocyanin O_2-binding properties of *Gecarcoidea natalis*. The following equations describe the dependency of haemocyanin O_2-affinity (P_{50} measured as kPa) on pH at different concentrations of Ca: at 8.7 mmol l^{-1} Ca, logP_{50}=2.251–0.249pH (r^2=0.993); at 12.5 mmol l^{-1} Ca, logP_{50}= 2.140–0.253pH (r^2=0.996); at 23 mmol l^{-1} Ca, logP_{50}=1.71–0.212pH (r^2=0.942). (B) Oxygen-binding cooperativity (n_{50}) of the haemocyanin for the different Ca concentrations within the physiological pH range.

equation for Mg concentrations between 6.1 and 19.7 mmol l^{-1}: $\log P_{50}$=1.892–0.220pH (r^2=0.946) with a mean n_{50} of 3.32±0.04 (N=15). In contrast, dialysing haemolymph against increasing concentrations of Ca resulted in a progressive increase in the haemocyanin O_2-affinity (Fig. 2A). An increase in Ca concentration from 8.7 to 23 mmol l^{-1} resulted in a decrease in P_{50} at pH 7.6 from 2.29 to 1.26 kPa. The change in the affinity of haemocyanin for O_2 at different concentrations of Ca could be described by the following equation: $\log P_{50}$=–0.61log[Ca]+0.91 (r^2=0.958) (Fig. 2A). The Bohr factor remained similar at all concentrations of Ca used (mean ϕ=–0.24±0.02, N=3). Similarly, the n_{50} value of 3.30±0.04 (N=16) was not dependent on either Ca concentration or pH (Fig. 2B).

The effect of urate

Increased concentrations of urate did not alter the mean Bohr shift of whole haemolymph (ϕ=–0.26±0.03, N=4 at 25 °C) but did produce a progressive increase in haemocyanin O_2-affinity (Fig. 3A). At pH 7.6, P_{50} decreased from 1.82 kPa at 0.08 mmol l^{-1} uric acid to 1.00 kPa in the presence of 4.32 mmol l^{-1} uric acid (Fig. 3A). Interestingly, the haemocyanin O_2-affinity changed linearly with increasing [urate] rather than as log[urate] as described for other haemocyanins (Morris, 1991). Therefore, the effect of urate on the affinity of haemocyanin for O_2 at pH 7.6 could be quantified by the following relationship: $\log P_{50}$=0.267–0.060[urate] (r^2=0.997). The cooperativity of haemocyanin O_2-binding, mean n_{50}=3.08±0.05 (N=23), was not affected by different urate concentrations (Fig. 3B).

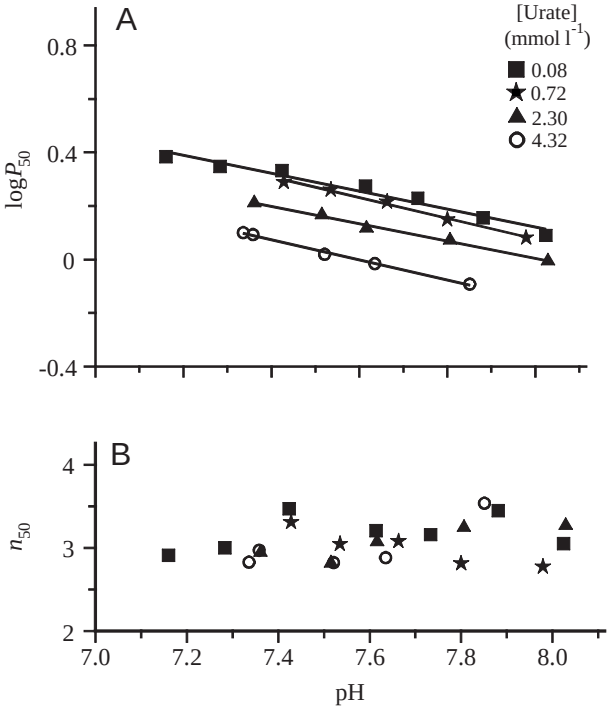

Fig. 3. (A) The effect of urate on haemocyanin O_2-affinity in whole haemolymph of *Gecarcoidea natalis*. The relationships between $\log P_{50}$ (measured as kPa) and pH at different urate concentrations are as follows: at 0.08 mmol l^{-1} urate, $\log P_{50}$=2.794–0.334pH (r^2=0.967); at 0.72 mmol l^{-1} urate, $\log P_{50}$=3.180–0.388pH (r^2=0.993); at 2.3 mmol l^{-1} urate, $\log P_{50}$=2.59–0.324pH (r^2=0.994); at 4.4 mmol l^{-1} urate, $\log P_{50}$=2.85–0.375pH (r^2=0.996). (B) The oxygen-binding cooperativity (n_{50}) of haemocyanin at different urate concentrations.

The combined effect of urate and L*-lactate enrichment on whole haemolymph*

In the presence of 21 mmol l^{-1} L-lactate, increasing the amount of urate in the haemolymph from 0.08 to 3.8 mmol l^{-1} influenced both the affinity of haemocyanin for O_2 and the Bohr effect (Fig. 4A). However, there was no clear relationship between the Bohr effect and the concentration of urate: ϕ varied between –0.47 and –0.27. In the presence of 21 mmol l^{-1} L-lactate, increasing the urate concentration increased the affinity of haemocyanin for O_2 (Fig. 4A) from a P_{50} (at pH 7.6) of 1.82 kPa at 0.08 mmol l^{-1} uric acid to a P_{50} of 1.23 kPa (at pH 7.6) at 3.8 mmol l^{-1} uric acid. The dependence of haemocyanin O_2-affinity on urate

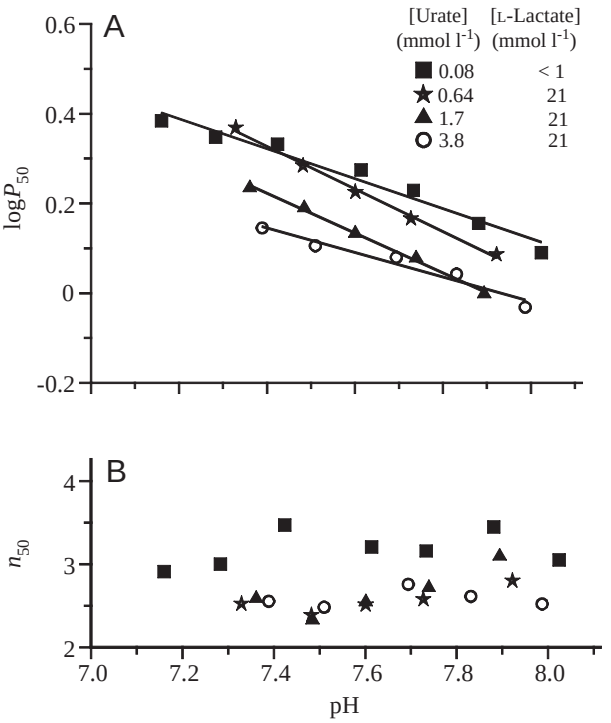

Fig. 4. (A) The haemocyanin affinity for O_2 in whole haemolymph enriched with both L-lactate (21 mmol l^{-1}) and different concentrations of urate and compared with whole haemolymph (filled squares, [urate] 0.08 mmol l^{-1}, [lactate] <1 mmol l^{-1}). The regression equations describing the relationship between $\log P_{50}$ (measured as kPa) and pH are: at 0.08 mmol l^{-1} urate, $\log P_{50}$=2.794–0.334pH (r^2=0.967); at 0.64 mmol l^{-1} urate, $\log P_{50}$=3.843–0.475pH (r^2=0.996); at 1.7 mmol l^{-1} urate, $\log P_{50}$=3.501–0.444pH (r^2=0.997); at 3.8 mmol l^{-1} urate, $\log P_{50}$=2.166–0.273pH (r^2=0.955). (B) The binding cooperativity (n_{50}) of haemocyanin in the presence of 21 mmol l^{-1} L-lactate and varying concentrations of urate compared with that of whole haemolymph (filled squares).

concentration in the presence of 21 mmol l^{-1} L-lactate could be described by the following equation: $\log P_{50}=0.248-0.045[\text{urate}]$ ($r^2=0.898$).

While high concentrations of either L-lactate (see below) or urate had no effect on the cooperativity of O_2-binding by haemocyanin, the combined effect of 21 mmol l^{-1} L-lactate and urate in whole haemolymph resulted in a significant decrease in the mean O_2-binding cooperativity of the haemocyanin from 3.18±0.09 (N=7) to 2.60±0.05 (N=5) (Fig. 4B).

The effect of L-lactate

The effect of L-lactate on the haemocyanin O_2-affinity of *G. natalis* was very unusual: increasing the concentration of L-lactate in the haemolymph resulted in a decrease in the haemocyanin O_2-affinity. Furthermore, the magnitude of the effect did not appear to be simply dependent on L-lactate concentration, but rather on the ageing of the haemolymph sample (Fig. 5; Table 1). The decrease in haemocyanin O_2-affinity in haemolymph enriched with L-lactate was greater in 'fresh haemolymph' (1 day after sampling) than in aged haemolymph (4–21 days old; Table 1). The O_2-affinity of haemocyanin in the presence of 20 mmol l^{-1} Li^+ (P_{50}=1.92 kPa at pH 7.6) was indistinguishable from that of the original whole haemolymph (P_{50}=1.93 kPa at pH 7.6).

Haemolymph enriched with L-lactate had a significantly lower haemocyanin O_2-affinity than the corresponding whole haemolymph in all cases examined (Table 1; Fig. 5). Haemocyanin O_2-affinity in the haemolymph enriched with 20 mmol l^{-1} l-lactate determined 1 day after collection was less than that of whole haemolymph (Fig. 5B–D; Table 1): at pH 7.6, the P_{50} increased by 0.31±0.14 kPa (N=3). In contrast, in haemolymph enriched with L-lactate several days after collection, the increase in P_{50} was only 0.14±0.02 kPa (N=4). Thus, the effect of L-lactate on haemocyanin O_2-affinity ($\Delta\log P_{50}/\Delta\log[\text{lactate}]$) was time-dependent and decreased from a maximum value of 0.044 on day 1 to 0.001 after 4 days of storage at 4 °C. The range of concentrations of L-lactate in the haemolymph (0.1–26.5 mmol l^{-1}) did not affect the binding cooperativity of the haemocyanin (mean n_{50}=3.16±0.02, N=67).

Effect of metabolites on haemocyanin O_2-binding properties in dialysed haemolymph

The Bohr shift of dialysed haemolymph (ϕ=–0.25) was greater than that of the corresponding whole haemolymph (ϕ=–0.18; Fig. 6A) but did not differ from the overall mean value (ϕ=–0.26). However, increasing the L-lactate concentration in the dialysed haemolymph (to 21 mmol l^{-1}) partially reversed this effect (ϕ=–0.22). Dialysed haemolymph

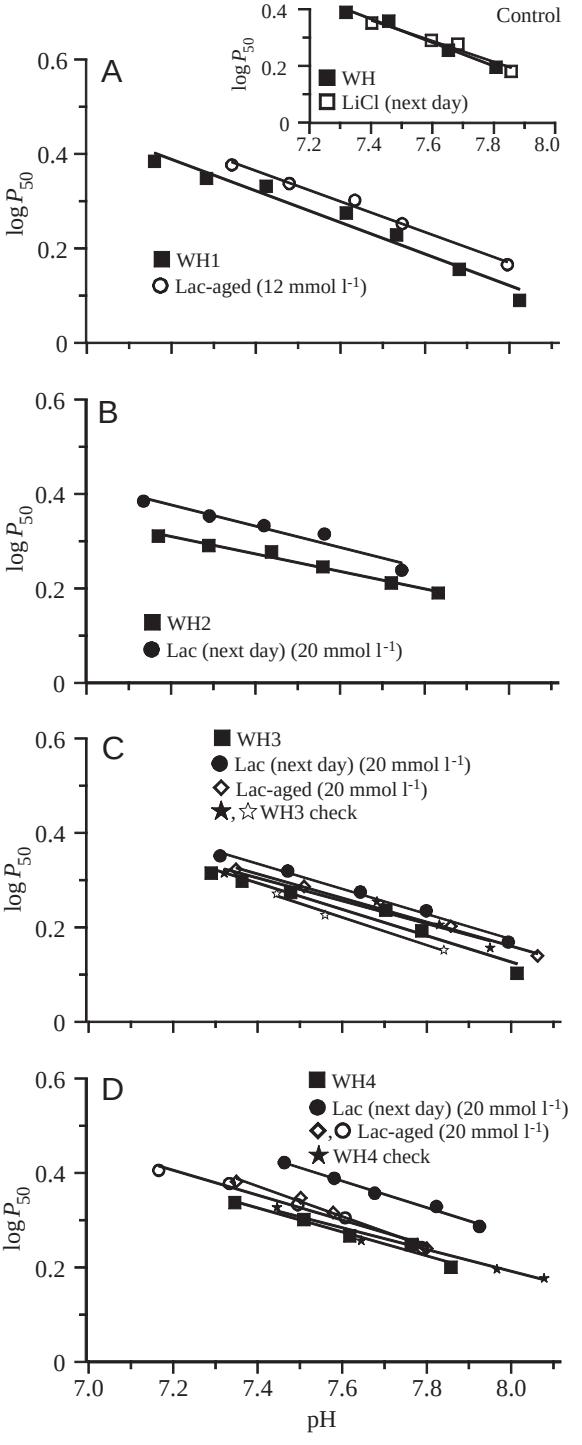

Fig. 5. The effect of L-lactate on haemocyanin O_2-affinity (P_{50}, measured as kPa) in whole haemolymph (WH) of *Gecarcoidea natalis*. (A–D) Four separate batches of haemolymph (WH1–WH4) originating from different crabs. The inset in A shows data for a separate pool of whole haemolymph with and without 20 mmol l^{-1} LiCl added to control for the Li^+ counterion of the lactate salt used. Samples of different age enriched with L-lactate were used to construct oxygen equilibrium curves (OECs). The lactate samples (WH enriched with L-lactate at the concentration shown) are identified by age, either 1 day post-sampling (next day) or a number of days later: for WH1, 21 days post-sampling (open circles); for WH3, 9 days post-sampling (open diamonds); and for WH4, 4 days (open diamonds) and 7 days (open circles) post-sampling. WH3 check and WH4 check represent values from OECs of whole haemolymph repeated several days later (see Table 1).

Table 1. *The effect of* L*-lactate on the haemocyanin O_2-affinity in* Gecarcoidea natalis

Sample	[L-Lactate] (mmol l^{-1})	Day of OEC construction	ϕ	*a*	r^2	Calculated P_{50} (kPa) at pH 7.6
WH 1	0.08	0	–0.33	2.794	0.967	1.799
Lac	12.3	21	–0.33	2.767	0.99	1.991*
WH 2	0.5	0	–0.18	1.636	0.985	1.722
Lac	19.8	1	–0.22	1.992	0.94	1.936*
WH 3	0.01	0	–0.28	2.357	0.958	1.730
Lac 1	20.1	1	–0.27	2.307	0.991	1.910*,A,C,D
Lac 2	20.3	9	–0.26	2.2	0.997	1.828*,A,E
WH 3 check	0.01	4	–0.24	2.095	0.953	1.803B,C
WH 3 check	0.01	11	–0.29	2.455	0.991	1.663B,D,E
WH 4	0.07	0	–0.25	2.203	0.969	1.888
Lac 1	20.1	1	–0.28	2.523	0.989	2.415*,A,B
Lac 2	17.4	4	–0.26	2.305	0.983	1.995*,A
Lac 3	26.5	7	–0.32	2.745	0.989	2.032
WH 4 check	0.07	3	–0.23	2.033	0.968	1.923

The measured concentrations of L-lactate are provided for each of the four batches of whole haemolymph.

The days on which the oxygen equilibrium curves (OECs) were constructed are also specified, where day 0 was the day that the haemolymph was sampled from the crabs and when the whole haemolymph OECs were constructed.

The slope (ϕ) and *y*-intercept (*a*) of the regression equations for each set of curves are provided where: $\log P_{50}=\phi pH + a$.

The affinity for O_2 (calculated as P_{50}) is supplied for each treatment at the physiological pH of 7.6.

WH, whole haemolymph; Lac, WH enriched with L-lactate.

An asterisk indicates that the haemocyanin O_2-affinity of the treatment group is different from the haemocyanin O_2-affinity of WH on the day of sampling (day 0).

A,B,C,D,E, like symbols represent a significant difference in haemocyanin O_2-affinity between two treatments.

Changes in the L-lactate sensitivity of O_2-binding by the haemocyanin with ageing of the sample were determined, as were inherent ageing effects in samples WH3 and WH4 several days after sampling (WH3 check, WH4 check).

enriched with L-lactate had a lower haemocyanin O_2-affinity (P_{50}=1.82 kPa) than either dialysed haemolymph (P_{50}=1.26 kPa) (ANCOVA, elevation: P<0.001) or whole haemolymph (P_{50}=1.74 kPa; ANCOVA, elevation: P<0.001; Fig. 6A).

Increasing the concentration of urate in dialysed haemolymph reduced the Bohr shift (ϕ=–0.12) in comparison with that of both dialysed and whole haemolymph (Fig. 6A). The haemocyanin O_2-affinity in dialysed haemolymph enriched with 4.32 mmol l^{-1} urate (P_{50}=0.87 kPa) was significantly higher than that in dialysed haemolymph (P_{50}=1.26 kPa) at the physiological pH 7.6 (Fig. 6A). The mean n_{50} of whole haemolymph (n_{50}=3.23±0.07, N=5) was not significantly different from that of dialysed haemolymph (Fig. 6B). However, the O_2-binding cooperativity of dialysed haemolymph enriched with urate (n_{50}=2.8±0.19, N=8) was significantly lower than that of dialysed haemolymph (n_{50}=3.31±0.06, N=5).

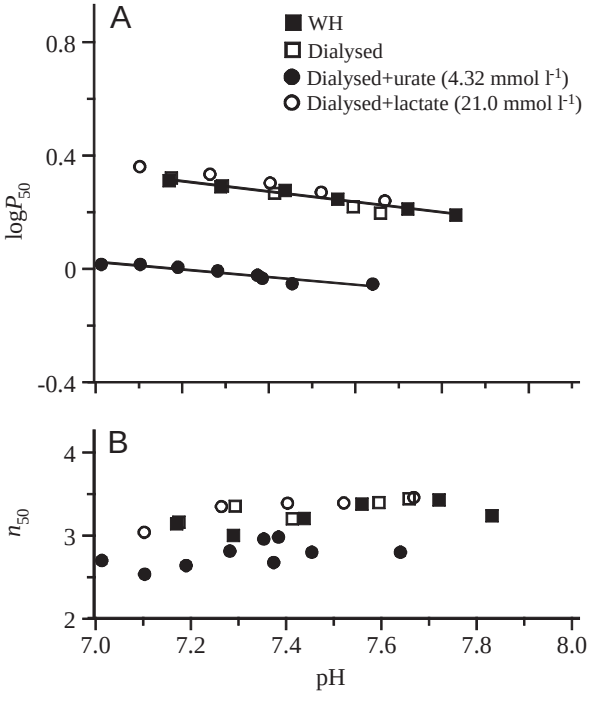

Fig. 6. (A) The effect of dialysis on haemocyanin O_2-affinity (P_{50}, measured as kPa) of *Gecarcoidea natalis* and with L-lactate (○) or urate (●) compared with whole haemolymph (WH, filled squares). The regressions describing the relationships between $\log P_{50}$ and pH are: WH, $\log P_{50}=1.636–0.184pH$ (regression line shown); dialysed haemolymph, $\log P_{50}=2.140–0.253pH$; dialysed haemolymph with 4.32 mmol l^{-1} urate, $\log P_{50}=1.917–0.219pH$; dialysed haemolymph with 21.0 mmol l^{-1} L-lactate, $\log P_{50}=0.885–0.124pH$ (regression line shown). (B) Binding cooperativity (n_{50}) of the haemocyanin in dialysed haemolymph and in the presence of urate or L-lactate.

Discussion

Functional properties of G. natalis *haemocyanin*

In *G. natalis* under resting conditions (pH 7.6 at 25 °C), the affinity of haemocyanin for O_2 (P_{50}=1.77 kPa) and the Bohr shift (ϕ=–0.26) were comparable with those of other terrestrial crustaceans (Morris, 1991). Attempts to correlate haemocyanin O_2-affinity with terrestriality have not been very successful. The mean haemocyanin affinities for O_2 at pH 7.6 are not substantially different when aquatic (P_{50}=2.21 kPa), amphibious (P_{50}=2.03 kPa) and terrestrial (P_{50}=2.10 kPa) species (data from Mangum, 1983; Morris, 1991; Truchot, 1992; Morris and Bridges, 1994) are compared, but such comparisons are phylogenetically confounded. Comparison among closely related species, for example within the Grapsidae, do show a progressive increase in haemocyanin O_2-affinity with increased reliance upon air breathing (Morris and Bridges, 1994). Within the Gecarcinidae, however, the haemocyanin O_2-affinity of red crabs is similar to that of the closely related *G. lallandi* and *Gecarcinus lateralis* (Morris and Bridges, 1994; Redmond, 1968) but lower than that of *Cardisoma* spp. (P_{50}<1 kPa; Redmond, 1962; Dela-Cruz and Morris, 1997) which, while obligate air-breathers, are still restricted in their distribution to areas with access to open water (e.g. Morris and Adamczewska, 1996).

The affinity of *G. natalis* haemocyanin for O_2 was quite sensitive to temperature changes (ΔH=–59 kJ mol^{-1}), but the tropical habitat of red crabs presents an environment of constant temperature with little selection pressure for the evolution of a haemocyanin of reduced temperature-sensitivity (Burnett *et al.* 1988; Morris, 1991; Eshky *et al.* 1996). The cooperativity of haemocyanin O_2-binding (n_{50}) of 3.2 in *G. natalis* was well within the general range of 2–4 reported for other decapods (Mangum, 1983; Truchot, 1992; Morris *et al.* 1996*a*) and was not affected by manipulating the concentrations of inorganic ions or by temperature changes. However, high concentrations of urate added to either whole haemolymph in the presence of 21 mmol l^{-1} L-lactate or to dialysed haemolymph reduced n_{50} to 2.6 and 2.8, respectively. Allosteric binding to haemocyanin of modulators of O_2-affinity can change the binding cooperativity of the respiratory pigment by preferentially affecting either the oxy- or the deoxy-conformation state of the haemocyanin (Zeis *et al.* 1992) or by disrupting the interactions between subunits (Decker *et al.* 1989). Since both urate and L-lactate alter the conformational structure of haemocyanin by binding to the subunits (Johnson *et al.* 1984; Nies *et al.* 1992), simultaneously high concentrations of both these modulating substances may change the cooperativity of O_2-binding.

A reduction in the O_2-binding cooperativity in dialysed haemolymph enriched with urate, but not in whole haemolymph enriched with urate, provides evidence for the presence of some dialysable factor in whole haemolymph which stabilised the quaternary structure of the haemocyanin. The presence of dialysable factors in the haemolymph which affect the response of haemocyanin to modulators or to O_2-binding have been postulated previously in the terrestrial anomuran *Birgus latro* (Morris *et al.* 1988) and more recently in other species of decapod (Bridges *et al.* 1997; Lallier and Truchot, 1997).

The concentrations of urate measured in the haemolymph of crustaceans range from 0.01 to 0.7 mmol l^{-1} (Henry and Cameron, 1981; Lallier *et al.* 1987; Lallier and Truchot, 1989; Dela-Cruz and Morris, 1997; Morris *et al.* 1996*b*; Morris and Callaghan, 1998). Since the effect of urate on haemocyanin O_2-affinity in red crabs was relatively small ($\Delta\log P_{50}/\Delta$[urate]=–0.06) and linearly dependent on the urate concentration, the increase in haemolymph [urate] in *G. natalis* after 20 min of intermittent exercise (0.04 mmol l^{-1}; Adamczewska and Morris, 1998) would result in an increase in haemocyanin O_2-affinity of only 0.05 kPa and have minimal significance in optimising the function of the haemocyanin in O_2 transport *in vivo*.

The sensitivity of decapod crustacean haemocyanins to Ca^{2+} ($\Delta\log P_{50}/\Delta\log$[Ca]) ranges from 0 to –0.82, with terrestrial crustaceans showing a marked reduction in sensitivity (for reviews, see Morris, 1990; Morris and Bridges, 1994). In comparison with other terrestrial crustaceans, the sensitivity of *G. natalis* haemocyanin to Ca^{2+} ($\Delta\log P_{50}/\Delta\log$[Ca]=–0.61) was quite large. During O_2 shortage, Ca^{2+} concentrations increase relatively slowly in the haemolymph over a period of hours, but the concentration of L-lactate may increase within minutes (Wood and Randall, 1981; Morris *et al.* 1986*b*, 1996*b*; Lallier *et al.* 1987; Lallier and Walsh, 1990). While L-lactate and urate may generally be of reduced utility in land crabs, retaining Ca^{2+} as a modulator of haemocyanin O_2-affinity may be advantageous for air-breathing crabs experiencing chronic acidosis (for a review, see Morris and Bridges, 1994).

The importance of L*-lactate in determining haemocyanin* O_2*-affinity*

The reverse effect of L-lactate, decreasing haemocyanin O_2-affinity in *G. natalis*, is unique among the Crustacea (Truchot, 1980; Morris *et al.* 1985*b*; McMahon, 1985; Morris, 1990, 1991; Morris and Bridges, 1994; Truchot, 1992). Additionally, the effect of L-lactate on the haemocyanin O_2-affinity was not related to the L-lactate concentration but instead to the time that the haemolymph had been stored *in vitro*. As a result of this time-dependent effect, the magnitude of the effect of L-lactate on haemocyanin O_2-affinity was difficult to determine precisely. However, on the day after the haemolymph had been withdrawn from the animals, $\Delta\log P_{50}/\Delta\log$[lactate] ranged from 0.013 to 0.044, with a mean value of 0.029±0.009 (N=3) (Fig. 5A–D; Table 1) but declined to 0.001 thereafter.

The fact that storage of haemolymph *in vitro* resulted in a change in the sensitivity of haemocyanin to L-lactate modulation within 4 days while the haemocyanin O_2-binding properties of whole haemolymph remained unchanged for up to 11 days (Table 1) suggests that the effect of L-lactate is independent of the fundamental haemocyanin O_2-binding properties of the haemocyanin in red crabs. Allosteric binding of L-lactate to haemocyanin is quite specific (Graham, 1985)

and results in conformational changes in the haemocyanin that affect the affinity of O_2-binding by this protein (Johnson *et al.* 1984; Nies *et al.* 1992). The presence of an unstable and dialysable unidentified cofactor in the haemolymph could account for the diminished effect of L-lactate on haemocyanin O_2-affinity with time. The current data provide further evidence supporting the presence of unknown factors in the haemolymph of crustaceans which affect the O_2-binding properties of haemocyanin (Morris *et al.* 1985*a*, 1988; Bridges *et al.* 1997; Lallier and Truchot, 1997).

The 'reverse' lactate effect represents a unique and novel potential adaptation to life on land among the Crustacea. Given that the availability of O_2 in air is more than sufficient to maintain haemocyanin oxygenation at the gas-exchange surfaces in red crabs (Farrelly and Greenaway, 1994; Adamczewska and Morris, 1994*a*, 1998), the decrease in haemocyanin O_2-affinity will assist in O_2 unloading at the tissues without compromising loading (for reviews, see McMahon, 1986; Morris, 1990). Fish and aquatic crustaceans possess feedback mechanisms to increase haemoglobin or haemocyanin O_2-affinity and thereby to maximise O_2 uptake from a relatively O_2-poor environment (Weber, 1980; Booth *et al.* 1982; Lallier and Truchot, 1989; Morris, 1990; Burnett, 1992). In contrast, the ease of O_2 extraction from air allows mammals to decrease the haemoglobin O_2-affinity during increased O_2 demand to maximise O_2 delivery to the tissues but still maintain O_2 uptake from air. Like mammals, red crabs appear to possess mechanisms to reduce the affinity of haemocyanin for O_2 during the initial stages of O_2 shortage during exercise to optimise O_2 unloading at the tissues at an unchanged P_{O_2}.

The binding sites for L-lactate in crustacean haemocyanin have been estimated at 0.2–0.5 per O_2-binding site, with a high affinity for L-lactate (Johnson *et al.* 1984; Nies *et al.* 1992). The rate of L-lactate production in red crabs can reach 1.8 mmol l^{-1} min^{-1} (Adamczewska and Morris, 1994*b*) and, assuming that the number and affinity of L-lactate binding sites on the haemocyanin of *G. natalis* are similar to those of other species, haemocyanin would become saturated with L-lactate within the first few minutes of exercise. Thus, the increase in L-lactate concentration during an initial period of exercise would assist O_2 off-loading at the tissues by decreasing the O_2-affinity of haemocyanin; subsequent increases in L-lactate concentration would not reduce haemocyanin O_2-affinity further and thus would not compromise O_2 loading.

Function of haemocyanin in O_2 transport in vivo

To describe the functional role of haemocyanin in *G. natalis*, model OECs were constructed, at the haemolymph pH measured *in vivo* and using the haemocyanin O_2-binding properties determined *in vitro*, for crabs at rest and for crabs after 20 min of intermittent exercise (Adamczewska and Morris, 1998). Superimposed on the model OECs were the P_{O_2} and the concentration of haemocyanin-bound O_2 measured *in vivo* for individual crabs (Adamczewska and Morris, 1998). After 20 min of intermittent exercise, the Bohr shift arising from the haemolymph acidosis predicts a decrease in the O_2-affinity of haemocyanin (0.43 kPa increase in P_{50}) compared with that of the crabs at rest (Fig. 7). This predicted Bohr shift would increase O_2 unloading at the tissues by 70 % (Fig. 7,

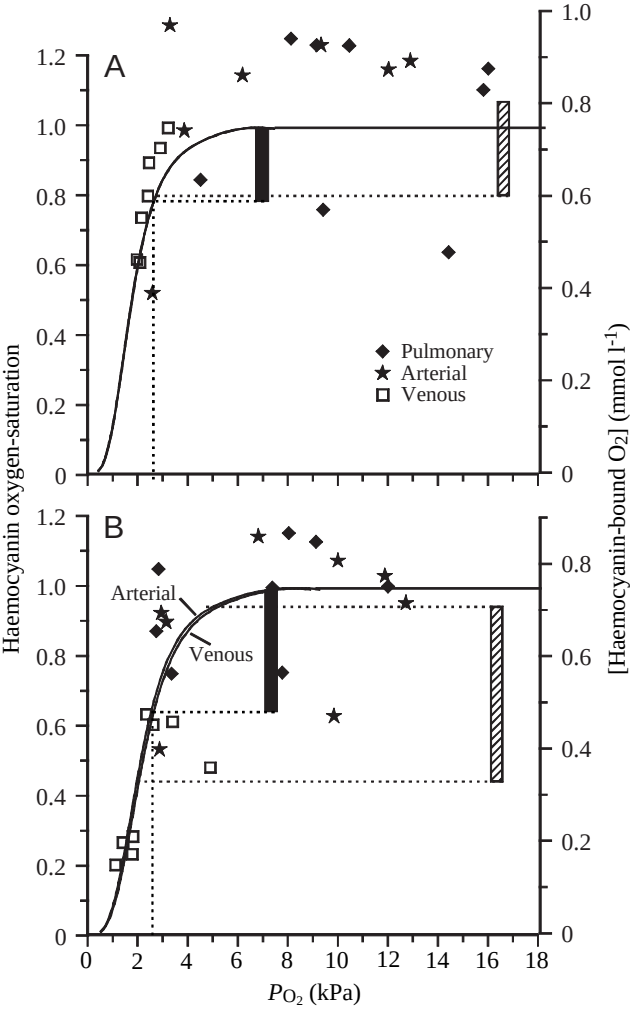

Fig. 7. Oxygen equilibrium curves (saturation given as relative haemocyanin-saturation) simulated for *Gecarcoidea natalis* at rest (A) (N=8) or after 20 min of intermittent exercise (B) (N=8) based on pH changes *in vivo*. The individual points show the concentration of haemocyanin-bound O_2 determined in pulmonary (◆), arterial (★) and venous (□) haemolymph for the eight crabs in each treatment (data from Adamczewska and Morris, 1998). For clarity, the haemocyanin-bound O_2 values were all normalised to a common haemocyanin content of 0.75 mmol l^{-1}. This was achieved by dividing the concentration of haemocyanin-bound O_2 by the maximum concentration of haemocyanin-bound O_2 for that sample (for method and unmodified data, see Adamczewska and Morris, 1998) to obtain relative saturation, which was the multiplied by the value of 0.75 mmol l^{-1} so that values could be compared. The hatched bars show the mean arterial–venous difference in normalised haemocyanin-bound O_2 concentration determined *in vivo*, i.e. the actual Δhaemocyanin-O_2. The filled bars show the amount of O_2 released from the haemocyanin as predicted by the *in vitro* data and the *in vivo* P_{O_2} changes.

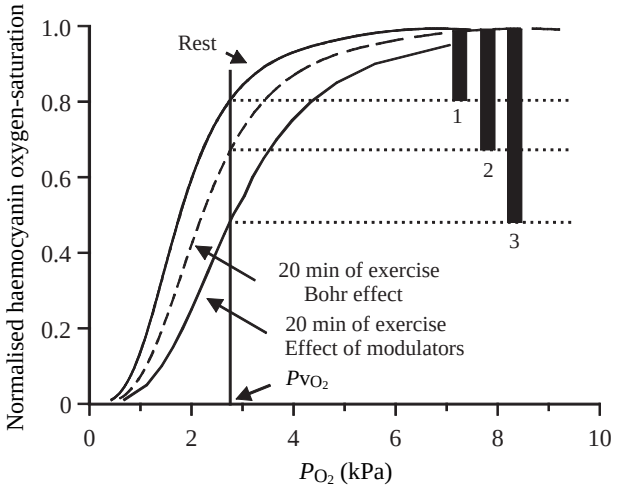

Fig. 8. Oxygen equilibrium curves constructed for the haemolymph of *Gecarcoidea natalis* at rest (1), after 20 min of intermittent exercise based on the Bohr shift only (2) and when the effects of affinity modulators (Ca^{2+}, urate and L-lactate) were taken into account (3). The effects of Ca^{2+}, urate and L-lactate were assumed to be additive in the model curves. The filled columns associated with each curve show the predicted amount of haemocyanin-bound O_2 released. P_{VO_2}, venous oxygen partial pressure. Normalised haemocyanin oxygen-saturation was calculated as for Fig. 7.

filled bars); however, the *in vivo* data show that the haemocyanin O_2 delivery to the tissues increased by more than 100 % (Fig. 7, hatched bars).

In an attempt to describe the haemocyanin O_2 functioning *in vivo* more precisely, the modulating effects of Ca^{2+}, urate and L-lactate were incorporated into the modelled OEC (Fig. 8). An increase in haemolymph Ca (of 2 mmol l^{-1}) and urate (of 0.04 mmol l^{-1}) concentration after 20 min of intermittent exercise (Adamczewska and Morris, 1998) could theoretically increase the haemocyanin O_2-affinity at the P_{50} by up to 0.14 kPa. However, the increase in L-lactate concentration after exercise would predict a decrease in affinity of approximately 0.3 kPa, thus partially opposing the effect of Ca^{2+} and urate and decreasing the haemocyanin O_2-affinity at the P_{50} by 0.17 kPa more than predicted by the Bohr effect alone (Fig. 8).

It is important to note that the effects of modulators on haemocyanin O_2-affinity are not necessarily additive (Morris *et al.* 1986*a*,*b*, 1987; Zeis *et al.* 1992), and model curves for *in vivo* functioning of haemocyanin in O_2 transport must be used with caution. However, the *in vitro* modelling of O_2 transport by haemocyanin in *G. natalis* appeared to approximate closely the data collected *in vivo* regarding O_2 transport (Adamczewska and Morris, 1998). The unique effect of L-lactate, to decrease the binding of O_2 by haemocyanin beyond that predicted by the Bohr shift, appeared to be adaptive in red crabs by maximising the delivery of O_2 to the tissues during exercise. This role for L-lactate is dependent on maintained O_2 loading and thus the exchange efficiency at the lungs, and these two features must have evolved in concert.

In red crabs, up to 80 % of the haemocyanin-bound O_2 was released to the tissues during exercise; thus, the O_2 partial pressure and O_2 reserves in the venous haemolymph were relatively low and did not provide a large O_2 diffusion gradient into the tissues (Adamczewska and Morris, 1998). During intermittent exercise, red crabs were operating at the limit of the O_2-transport system and any additional work would have to be supported by anaerobic metabolism. Whether the O_2-binding properties of haemocyanin limit exercise during the migration of red crabs awaits field evaluations of the *in vitro* models generated in the present study.

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