A comparative study of the oxygen transporting properties of the haemocyanin of five species of thalassinidean mud-shrimps

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Abstract

Comparative studies of the haemocyanin of five species of thalassinidean mud-shrimps showed that all five species exhibited a high oxygen affinity ($P_50$ range 1.0–9.3 Torr, at in vivo pH, 10°C). Each of the mud-shrimps exhibited a moderately large Bohr coefficient ($-1.06$ to $-1.48$) and values for the co-operativity of the haemocyanin did not differ greatly between the species ($n_{so}$ range 2.3–3.8). The highest oxygen affinities were recorded for the haemocyanin of Callianassa subterranea, Jaxea nocturna and Calocaris macandreae, whereas those for the two species of Upogebia were slightly lower but were still higher than in many other decapods. The higher oxygen affinity of the haemocyanin of the deposit feeding shrimps C. subterranea, J. nocturna and C. macandreae compared with that of the haemocyanin of the filter feeding upogebiids may be correlated with the fact that conditions within the burrows of the deposit feeders may be more severely hypoxic. The oxygen affinity of all five species showed a moderate temperate sensitivity ($D_kH_2O$ range 56.8 to $-82.1$ kJ mol $^{-1}$ over temperature range 5–10°C). Studies of the haemocyanin of one species (C. macandreae) showed that L-lactate did not affect the oxygen affinity of the haemocyanin as has been reported for a number of other decapod species. The oxygen carrying capacity of the haemocyanin (C$_{O_2}$) was similar in four of the species studied (0.22–0.42 mmol l $^{-1}$) but that of C. subterranea was significantly greater (0.83 mmol l $^{-1}$). The higher protein concentration of the haemolymph of this species also resulted in the haemolymph having a greater buffering capacity ($-7.67$ mmol l $^{-1}$ pH unit $^{-1}$). SDS–PAGE studies of the haemocyanin demonstrated the presence of 3 subunits in the upogebiids but additional subunits were observed in the other species (MW range = 70 000–90 000 Da). Studies of the association state of the haemocyanin of three of the species showed that the haemocyanin of both C.
macandreae and J. nocturna was present mainly in the form of eikositetramers (24-subunit aggregation state) with some hexameric haemocyanin also occurring. The haemocyanin of Upogebia deltaura, however, occurred as a mixture or eikositetramers and dodecamers. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Thalassinidean mud-shrimps form an important component of the macrofauna of sublittoral and some intertidal soft sediments where their burrowing life style makes a major contribution to the bioturbation of these sediments and to nutrient exchange (Pemberton and Buckley, 1976; Koike and Mukai, 1983; Nickell et al., 1996). The burrowing lifestyle adopted by these shrimps offers some protection against predation but exposes them to conditions within the burrow that may be very different from those on the sediment surface (Atkinson and Taylor, 1988; Astall et al., 1997). In particular, the water within the burrows may often be severely hypoxic and may contain significant concentrations of sulphide (Atkinson and Taylor, 1988, Johns et al., 1997).

The physiological and behavioural adaptations shown by mud-shrimps have been reviewed by Atkinson and Taylor (1988) but much less is known about the oxygen transporting properties of their haemocyanin. Although Miller and Van Holde and their co-workers have published several detailed studies of thalassinidean haemocyanins, these studies have been restricted to only three species that occur mainly in intertidal locations (Van Holde et al., 1977; Miller et al., 1977; Arisaka and Van Holde, 1979; Miller and Van Holde, 1981a,b). These studies showed that the oxygen affinities of the haemocyanins of species such as Neotrypaea (= Callianassa) californiensis, N. gigas and Upogebia pugettensis were higher than those of many other non-burrowing decapod crustaceans (Mangum, 1983a). One particularly interesting finding of the research by Miller and Van Holde and their co-workers was the fact that, although the haemocyanin molecule in many decapods exists as either hexamers or dodecamers, the haemocyanin of the thalassinideans studied exists mainly as eikositetramers (24-mers).

As part of a larger study of the physiological ecology of mud-shrimps from sublittoral locations, this paper presents the results of a comparative study of five species of mud-shrimps that occur in the same geographical location. These include three primarily deposit feeding species Calocaris macandreae (Bell), Jaxea nocturna (Nardo) and Callianassa subterranea (Montagu) as well as two suspension feeding upogebiids, Upogebia deltata (Leach) and U. stellata (Montagu) (Nickell and Atkinson, 1995; Nickell et al., 1995; Lindahl and Baden, 1997; Pinn et al., 1998). The aim of this study was to gather further comparative data on the structure and oxygen transporting properties of the haemocyanin of these species thus enabling comparisons to be made with the earlier work on intertidal species.
2. Materials and methods

2.1. Collection and maintenance of mud shrimps

_Upogebia deltaura_ were collected with an anchor dredge at depths of 6–7 m from Plymouth Sound, Devon, England (50°20′N, 4°13′W) and from the Irish Sea (54°7′N, 3°27′W). _Upogebia stellata_ were obtained at depths of 20–30 m with an anchor dredge from the north end of Isle of Cumbrae and _Calocaris macandreae_ with an Agassiz trawl, from the Main Channel (between Cumbrae and Bute), Firth of Clyde, Scotland (55°9′N, 5°11′W). Specimens of _Jaxea nocturna_ and _Callianassa subterranea_ were obtained from Loch Sween, Scotland (56°2′N, 5°36′W) by anchor dredging or by SCUBA diving. All specimens were transported back to the University of Glasgow where they were kept in a recirculating sea water aquarium (S, 3.2; T, 10°C). Individual mud shrimps were placed into small containers (1–2 l) containing sufficient mud from the sample sites to allow them to construct burrows. Using this method very low mortality rates were recorded.

2.2. Collection of haemolymph

Haemolymph was extracted from quiescent shrimps using a 100 μl syringe (Hamilton), the needle (22 g) of which was inserted either into the arthrodial membrane at the base of the walking legs, or beneath the cephalothorax and into the pericardium. Haemolymph samples (10–50 μl) were then transferred to 0.5 ml Eppendorf tubes and kept on ice. As a result of the small volume of haemolymph obtained from each individual (10–50 μl) and the irregularity of specimen capture as well as the paucity of individuals, it was necessary to pool the haemolymph samples to obtain sufficient haemolymph for some of the analyses. Pooled samples were mixed thoroughly, centrifuged at 13 000 g for 10 min to remove cells and particulate material and kept at 4°C or immediately frozen (−20°C). Storage of haemolymph at −20°C has been shown to be preferable to storage at −80°C, at which temperature the co-operativity of the haemocyanin may be affected in some crustaceans (Morris, 1988; Taylor unpubl. obs.).

2.3. Haemolymph ionic composition

Following dilution with deionised water, the concentrations of Na⁺, K⁺, Mg²⁺ and Ca²⁺ in pooled, frozen haemolymph samples from each species were determined using an Atomic Absorption Spectrophotometer (PU 9820, Philips). Lanthanum chloride was added (1:5 v/v) prior to the determination of Ca²⁺ concentrations. The total haemolymph CI⁻ concentration was determined by electrochemical titration using a chloride meter (PCLM3, Jenway). These data were used to prepare a physiological saline solution for each species.

Since lactate has been shown to be a modulator of oxygen affinity (Truchot, 1980; Bridges and Morris, 1986), the concentration of L-lactate in pooled haemolymph
samples was determined using the method of Gutmann and Wahlefeld (1974) with the modifications of Engel and Jones (1978).

2.4. Construction of oxygen dissociation curves

In vitro oxygen dissociation curves were constructed on pooled whole haemolymph samples (3 μl) using the spectrophotometric diffusion chamber technique (Sick and Gersonde, 1969). Full details of the procedure used are given in Zainal et al. (1992). Oxygen dissociation curves were constructed for the haemolymph of each species at 5, 10, 15 and 20°C by increasing the PO₂ of the gas mixture in a stepwise manner while maintaining a constant PCO₂ for each curve. Separate samples of haemolymph (100 μl) were simultaneously equilibrated, in a BMS2 (Radiometer) at 10°C, against the same gas mixture as supplied to the diffusion chamber. The pH of the haemolymph sample was determined at half saturation (P₅₀) using the microcapillary pH electrode of the BMS 2 connected to a pH meter (215 Ion Analyser, Corning). The P₅₀ value and co-operativity (n₅₀) of the haemolymph was estimated using regression analysis of the oxygen saturation values (between 25 and 75%) calculated using the Hill equation. Where possible, the pH of fresh haemolymph samples collected anaerobically from individual shrimps was also determined at 10°C using the above method.

2.5. The effect of temperature and L-lactate on haemocyanin oxygen affinity

The effect of temperature on the oxygen affinity of the haemocyanin of each species of mud-shrimp was investigated by constructing oxygen dissociation curves at 5, 10 and 15°C using the procedure described above. The effect of L-lactate on the oxygen affinity of the haemocyanin of C. macandreae was investigated by dialysing the haemolymph for 12 h at 4°C against differing concentrations of sodium lactate made up in the appropriate physiological saline. Following dialysis, oxygen dissociation curves were constructed at 10°C.

2.6. Oxygen carrying capacity

The total oxygen content of the haemolymph (C_TO₂) was measured using haemolymph samples from individual mud-shrimps. The oxygen content of duplicate 10 μl haemolymph samples, equilibrated in the BMS2 against air (to ensure full saturation) at 10°C, was determined using the method of Tucker (1967) as modified by Bridges et al. (1979). The oxygen carrying capacity of the haemocyanin (C_HCY₂) was calculated by subtracting the physically dissolved fraction of oxygen in the haemolymph from the measured total oxygen content, using an oxygen solubility coefficient of 0.002 mmol l⁻¹ Torr⁻¹ (Altman and Dittmer, 1971).

2.7. Haemocyanin sub-unit composition

The subunit composition of the haemocyanin of each species was determined using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). Pooled,
previously-frozen haemolymph samples were diluted to a final concentration of 1.0, 0.5 or 0.1% with SDS sample buffer (containing 2% SDS, 59% 2-mercaptoethanol, 10% glycerol, 0.0625 M Tris–HCl (pH 6.8) and 0.002% bromophenol blue as reference front) and heated to 100°C for 2–3 min. Aliquots of 30 µl were loaded onto a 2.5% (w/v N,N′-methylenebisacrylamide) stacking gel and resolved in a 7.5% gel. Electrophoresis was conducted at a constant current of 15 mA per gel (BioRad mini-gel System). After electrophoresis, the gels were stained for 1–2 h with 0.25% Coomassie Brilliant Blue R and then destained in 7% glacial acetic acid.

The molecular weights of the haemocyanin subunits were determined by comparing their electrophoretic mobility with known protein markers. Each gel was calibrated with SDS molecular weight markers in the weight range 29 000–205 000 (MW-SDS-200, Sigma Chemical Co.). A scanning densitometer (GS 300, Hoefer Scientific Instruments, San Francisco) was used to determine the number of bands and their relative mobility (Rm). The Rm values were plotted against the known molecular weights and the molecular weight of the unknown protein band was estimated from the calibration curve.

2.8. Association state of the haemocyanins

The association states of the haemocyanins were assessed using fast protein liquid chromatography (FPLC). Unfortunately, no analyses of the haemocyanins of *C. subterranae* and *U. stellata* could be carried out due to the limited number of animals available. A 100 µl aliquot of fresh, not frozen, haemolymph from individuals or from pooled samples, previously diluted with the appropriate physiological saline, was applied to a Superose 6 gel filtration column (HR10/30, Pharmacia). The column was eluted with the Ringer’s solution (previously filtered through a 0.22 µm filter) at a rate of 0.2 ml min⁻¹. The column was calibrated with proteins of known molecular weight; blue dextran (MW=2 000 000 Da), thyroglobulin (669 000 Da), apoferritin (443 000 Da), alcohol dehydrogenase (150 000 Da) and albumin (66 000 Da) (Sigma Chemical Co.).

The activity of naturally occurring protease enzymes in the haemolymph may lead to degradation of the haemocyanin aggregates (Mangum et al., 1987; Terwilliger et al., 1979). Therefore, the association state of the haemocyanin was also assessed for haemolymph treated with a serine protease inhibitor (phenylmethansulphonyl fluoride (PMSF) at 1 mmol l⁻¹ final concentration) and a trypsin-like serine and cysteine protease inhibitor (leupeptin at 100 µmol l⁻¹ final concentration).

3. Results

3.1. Haemolymph ionic composition

The concentrations of the major ions present in the pooled haemolymph samples of five species of thalassinidean mud-shrimps are given in Table 1. The concentrations of these ions did not differ markedly between species although the concentrations of Na⁺ and Cl⁻ ions in the haemolymph of *Calocaris macandreae* were higher than in the other species studied. All of the species examined had high concentrations of magnesium ions
Table 1
The concentrations of major ions in the haemolymph of the five species of thalassinidean mud-shrimps studied. All measurements were made from pooled, frozen haemolymph samples.

<table>
<thead>
<tr>
<th>Species</th>
<th>Na⁺ (mmol l⁻¹)</th>
<th>Cl⁻ (mmol l⁻¹)</th>
<th>K⁺ (mmol l⁻¹)</th>
<th>Mg²⁺ (mmol l⁻¹)</th>
<th>Ca²⁺ (mmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubogebia stellata</td>
<td>401</td>
<td>457</td>
<td>17.1</td>
<td>40.2</td>
<td>7.8</td>
</tr>
<tr>
<td>Ubogebia deltaura</td>
<td>394</td>
<td>446</td>
<td>14.8</td>
<td>43.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Jaxea nocturna</td>
<td>358</td>
<td>440</td>
<td>19.8</td>
<td>34.4</td>
<td>6.7</td>
</tr>
<tr>
<td>Callianassa subterranea</td>
<td>348</td>
<td>407</td>
<td>22.5</td>
<td>39.9</td>
<td>8.1</td>
</tr>
<tr>
<td>Calocaris macandreae</td>
<td>498</td>
<td>520</td>
<td>12.9</td>
<td>45.4</td>
<td>11.6</td>
</tr>
</tbody>
</table>

(34.4–45.4 mmol l⁻¹). The concentration of L-lactate in pooled haemolymph taken from quiescent individuals was low (0.17–0.58 mmol l⁻¹) for all five species of mud-shrimp (Table 2). Due to the small size and limited availability of some of the mud-shrimps, data for the in vivo pH of the haemolymph are limited. The available data indicate, however, that the pH of the haemolymph varied slightly between species (Table 2).

3.2. Oxygen carrying capacity

Values for the oxygen carrying capacity of the haemocyanin (C_O₂) of each of the five species are given in Table 2. C. subterranea had a significantly higher (p < 0.05) C_O₂ than the other mud-shrimps examined. There was no significant difference (p > 0.05) in C_O₂ between U. stellata, J. nocturna and C. macandreae although the values for U. deltaura were significantly higher (p < 0.05) than in these species.

3.3. Haemocyanin oxygen affinity

The relationships between pH and the log _P_50 and between pH and _n_50 of the haemocyanin for each of the five species are shown in Fig. 1 and Table 3. Each of the...
Fig. 1. The relationship between log $P_{50}$ and pH (A) and between $n_{50}$ and pH (B) at 10°C for the haemocyanin of the five species of mud shrimp studied. The equations of the regression lines fitted to the data are given in Table 3. Upogebia deltaura (□), Upogebia stellata (■), Jaxea nocturna (▲), Callianassa subterranea (○) and Calocaris macandreae (●).

Table 3
Regression coefficients for the relationships between log $P_{50}$ and pH (at 10°C) for the haemolymph of the five species of thalassinidean mud-shrimps presented in Fig. 3. Regression equations are given in the form of log $P_{50}=a+b$ (pH), the coefficient of determination ($r^2$) is also given. All relationships were significant at $P<0.05$. The Bohr coefficient ($\phi$) is quantified by the $b$ value. Values for calculated the oxygen affinity of the haemocyanin ($P_{50}$) at the in vivo pH (Table 2) are also given.

<table>
<thead>
<tr>
<th>Species</th>
<th>$a$</th>
<th>$b$</th>
<th>$r^2$</th>
<th>$P_{50}$ (Torr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upogebia deltaura</td>
<td>9.09</td>
<td>1.06</td>
<td>0.980</td>
<td>6.2</td>
</tr>
<tr>
<td>Upogebia stellata</td>
<td>11.56</td>
<td>1.37</td>
<td>0.941</td>
<td>9.3</td>
</tr>
<tr>
<td>Jaxea nocturna</td>
<td>11.57</td>
<td>1.48</td>
<td>0.980</td>
<td>1.6</td>
</tr>
<tr>
<td>Callianassa subterranea</td>
<td>10.02</td>
<td>1.29</td>
<td>0.960</td>
<td>1.0</td>
</tr>
<tr>
<td>Calocaris macandreae</td>
<td>8.76</td>
<td>1.10</td>
<td>0.941</td>
<td>1.5</td>
</tr>
</tbody>
</table>
mud-shrimps exhibited a reasonably large Bohr coefficient (Table 3). Comparison of the slopes of the regression lines showed, however, that although there were some differences in the Bohr values between species, these were not significant (ANCOVA, \(P > 0.05\)). The oxygen affinity of the haemocyanin from all species was quite high although there were some differences between species. In particular, the oxygen affinities of the haemocyanin of *C. subterranea*, *J. nocturna* and *C. macandreae* were significantly higher than those of the upogebiids at 10°C (ANCOVA, \(P < 0.05\)) (Table 3). There were only small differences in the co-operativity of the haemocyanin among the five species studied (\(n_{50}\) range = 2.3–3.8). For all species, pH had no significant effect on the value of \(n_{50}\) (Fig. 3).

The effect of L-lactate on the oxygen affinity of the haemocyanin of *C. macandreae* was examined by constructing oxygen dissociation curves following dialysis of the haemolymph against differing concentrations of lactate. The regression equations describing the relationships between log \(P_50\) and pH for the haemolymph of *C. macandreae* are presented in Table 4. There were no significant differences in either the slopes or the intercepts of the regression lines (ANCOVA, \(P > 0.05\)) indicating that the presence of lactate ions within this physiological range had no discernible effect of the oxygen affinity of the haemocyanin.

Values for the heat of oxygenation (\(\Delta H\)) calculated for the haemocyanins of the five species of mud-shrimp are given in Table 5. The values of \(\Delta H\) were approximately

### Table 4
Regression coefficients for the relationships between log \(P\) and pH (at 10°C) determined from oxygen dissociation curves constructed for haemolymph of *Calocaris macandreae* following dialysis against differing concentrations of sodium lactate. Regression equations are given in the form of log \(P = a + b(pH)\), the coefficient of determination \((r^2)\) is also given. For each of the regression equations \(n=8\).

<table>
<thead>
<tr>
<th>[L-Lactate] (mmol l(^{-1}))</th>
<th>(a)</th>
<th>(b)</th>
<th>(r^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.60</td>
<td>-1.11</td>
<td>0.941</td>
</tr>
<tr>
<td>5</td>
<td>9.29</td>
<td>-1.18</td>
<td>0.932</td>
</tr>
<tr>
<td>10</td>
<td>7.95</td>
<td>-1.01</td>
<td>0.917</td>
</tr>
<tr>
<td>15</td>
<td>8.72</td>
<td>-1.21</td>
<td>0.962</td>
</tr>
</tbody>
</table>

### Table 5
Values for the heat of oxygenation (\(\Delta H\)) calculated for the haemocyanins of the five species of mud-shrimps studied over the temperature ranges 5–10°C and 10–15°C. The \(P_{50}\) values used in the calculations were derived from the regression equations fitted to the relationships between pH and log \(P_{50}\) for each species at the appropriate temperature and in vivo pH.

<table>
<thead>
<tr>
<th>Species</th>
<th>(\Delta H) (kJ mol(^{-1})) 5–10°C</th>
<th>(\Delta H) (kJ mol(^{-1})) 10–15°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Upogebia deltaura</em></td>
<td>-65.4</td>
<td>-56.2</td>
</tr>
<tr>
<td><em>Upogebia stellata</em></td>
<td>-82.1</td>
<td>-63.5</td>
</tr>
<tr>
<td><em>Jaxea nocturna</em></td>
<td>-79.6</td>
<td>-66.1</td>
</tr>
<tr>
<td><em>Callianassa subterranea</em></td>
<td>-78.2</td>
<td>-53.1</td>
</tr>
<tr>
<td><em>Calocaris macandreae</em></td>
<td>-56.8</td>
<td>-43.6</td>
</tr>
</tbody>
</table>
similar between the species. It was noticeable, however, that the $\Delta H$ values were lower over the temperature range 10–15°C than between 5 and 10°C.

3.4. Haemolymph buffering capacity

The $\text{PCO}_2$ of the haemolymph could not be measured by Astrup titration because of the small amounts of haemolymph obtained from each shrimp. However, an indication of the buffering capacity can be gained by examining the change in pH with $\text{PCO}_2$ (at 10°C) determined previously during the construction of oxygen equilibrium curves. These data were used to calculate the buffering capacity of the haemolymph ($\Delta[\text{HCO}_3^-]/\Delta\text{pH}$). Values for $[\text{HCO}_3^-]$ were calculated using the Henderson–Hasselbalch equation

$$\text{pH} = pK_1 + \log\frac{[\text{HCO}_3^-]}{a\text{CO}_2 \cdot P\text{CO}_2}$$

with values for $pK_1$ and $a\text{CO}_2$ being obtained from the nomograms of Truchot (1976) at the appropriate temperature and salinity. Regression equations describing the relationships between $[\text{HCO}_3^-]$ and pH were then calculated for each of the five species and the values for the buffering capacity of the haemolymph obtained from the slopes of the regression equations. Since the values for the constants $pK_1$ and $a\text{CO}_2$ were not determined for each of the species, the calculated values for the buffering capacity must therefore be treated with some caution. The data indicate, however, that the buffering capacity of the haemolymph was generally low. The slightly higher buffering capacity of the haemolymph of *Callianassa subterranea* may be due to the greater haemocyanin content of the blood as indicated by the higher oxygen carrying capacity of the haemocyanin (Table 2).

3.5. Haemocyanin sub-unit composition

Scanning densitometer traces of thalassinidean haemocyanin run under denaturing conditions using SDS–PAGE revealed between three and five sub-units (Fig. 2). The scans for the two upogebiid shrimps showed the presence of three distinguishable sub-units, the molecular weights of which were similar for both species (Table 6). The haemocyanin of *Calocaris macandreae* and *Callianassa subterranea* were characterized by four sub-units, whereas five sub-units were present in the haemocyanin from *Jaxea nocturna* (Fig. 2). The molecular weights for each of the numbered sub-units range from 70 700 to 93 700 Da, and are summarized in Table 6.

3.6. Association state of the haemocyanins

The association states of pooled, fresh haemocyanin were measured for the mud-shrimps *Calocaris macandreae*, *Jaxea nocturna* and *Upogebia deltaura* using fast-protein liquid chromatography (Fig. 3). The haemocyanin of both *C. macandreae* and *J. nocturna* was present mainly in the form of eikositetramers (24-subunit aggregation state) ($M_t$=approx. 1 900 000 Da) with some hexameric haemocyanin also present
Fig. 2. Scanning densitometer traces of the haemocyanin run under denaturing conditions (see text for further details). A = Upogebia deltaura, B = U. stellata, C = Calocaris macandreae, D = Calianassa subterranea, E = Jasea nocturna. The individual traces have been aligned against the same \( R_m \) scale.
Table 6
The number of subunits of the haemocyanin, as determined by SDS-PAGE, for the five species of mud-shrimps. The relative mobility (Rm) was determined for each band (Fig. 2) and the molecular weight calculated from a calibration graph (for further details see text).

<table>
<thead>
<tr>
<th>Species</th>
<th>Band</th>
<th>Rm</th>
<th>mol. wt</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Upogebia stellata</em></td>
<td>1</td>
<td>0.393</td>
<td>84 700</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.428</td>
<td>76 900</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.459</td>
<td>70 900</td>
</tr>
<tr>
<td><em>Upogebia deltaura</em></td>
<td>1</td>
<td>0.393</td>
<td>84 700</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.425</td>
<td>77 400</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.455</td>
<td>71 500</td>
</tr>
<tr>
<td><em>Calocaris macandreae</em></td>
<td>1</td>
<td>0.357</td>
<td>93 700</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.386</td>
<td>86 200</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.427</td>
<td>77 000</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.454</td>
<td>71 700</td>
</tr>
<tr>
<td><em>Callianassa subterranea</em></td>
<td>1</td>
<td>0.370</td>
<td>90 300</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.416</td>
<td>79 400</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.430</td>
<td>76 500</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.460</td>
<td>70 700</td>
</tr>
<tr>
<td><em>Jaxea nocturna</em></td>
<td>1</td>
<td>0.368</td>
<td>90 900</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.381</td>
<td>87 500</td>
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<td></td>
<td>3</td>
<td>0.400</td>
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<td>4</td>
<td>0.418</td>
<td>79 000</td>
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<td>5</td>
<td>0.415</td>
<td>71 600</td>
</tr>
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$(M_r = \text{approx. 480 000 Da})$. The haemocyanin of *Upogebia deltaura* was also present mainly in the form of eikositetramers but with a significant proportion present as dodecamers. The presence of haemocyanin in the fractions corresponding to the peaks

Fig. 3. Absorbance scans (at 280 nm) of the haemolymph samples of three of the five species of thalassinidean mud-shrimps analysed by FPLC. The peaks represent the different aggregation states of the haemocyanin and indicate the presence of eikositetramers (e), dodecamers (d) or hexamers (h). A = *Calocaris macandreae*, B = *Upogebia deltaura*, C = *Callianassa subterranea*. 
eluted from the column was confirmed by the presence of the absorbance peak at 335 nm which disappeared almost completely when the fractions were treated with sodium sulphite. Because it was necessary to use pooled samples to obtain sufficient haemolymph for the FPLC analyses, the analyses were repeated using pooled samples for each species obtained from different groups of animals to ensure that the results were repeatable. The absorbance scans obtained for these different samples were almost identical within each species.

The effect of one freeze–thaw cycle at −20°C on the haemocyanin association of haemolymph from *Calocaris macandreae* was also investigated. There was little difference in the haemocyanin association state following thawing except that there was a slight decrease in the proportion of eikositetramers and a corresponding increase in the proportion of the hexamers. The addition of protease inhibitors to the haemolymph did not appear to affect the association state of the haemocyanin.

4. Discussion

4.1. Haemocyanin aggregation states

The haemocyanin molecule in many decapods exists as either hexamers or dodecamers with either form being predominant and the levels of native aggregates being species-specific (Markl, 1986). The hexamer is the typical aggregate in caridean, penaeid, pagurid and palinuran decapods (Van Holde and Miller, 1982; Mangum, 1983a). In many other decapod species, however, the haemocyanin molecules are primarily dodecamers, although in some groups both hexamers and dodecamers may co-exist in differing proportions (Markl et al., 1979; Van Holde and Miller, 1982). The haemocyanins of *Calocaris macandreae* and *Jaxea nocturna* were found to exist mainly as eikositetramers (24-mers) with a smaller amount present as hexamers. In *Upogebia deltaura*, however, the haemocyanin was present as a mixture of eikositetramers and dodecamers with the former being present in greater proportions.

The formation of eikositetramers has previously been recorded for a number of thalassinidean shrimps; *Calocaris macandreae* (Svedberg, 1933), *Neotrypaea* (as *Callianassa*) *californiensis* (Roxby et al., 1974), *N. (as Callianassa) gigas* (Miller et al., 1977) and *Upogebia pugettensis* (Miller et al., 1977). The occurrence of eikositetramers among the thalassinidean mud-shrimps appears to be almost unique within the Crustacea but any physiological significance remains to be established. The haemocyanins from these thalassinidean shrimps have been shown to exist in at least three different stable states of aggregation depending on the temperature, pH and ionic composition of the solution (Roxby et al., 1974; Miller et al., 1977; Miller and Van Holde, 1981a,b). In *U. pugettensis*, Miller et al. (1977) found evidence for a stable dodecamer at room temperature as was observed in *U. deltaura* (this study). In *N. (as Callianassa) gigas*, however, the dodecameric component was largely absent above 20°C (Blair and Van Holde, 1976). At room temperature the haemocyanin of *Callichirus* (as *Callianassa*) *major* was composed primarily of eikositetramers whereas a reduction in temperature favoured the dissociation of the eikositetramer (Miller and Van Holde, 1981a).
The association of hexamers and dodecamers requires divalent cations, either \( \text{Mg}^{2+} \) or \( \text{Ca}^{2+} \), as well as competent monomers (Mangum, 1983a). Van Holde et al. (1977) found that about 3 \( \text{Mg}^{2+} \) were required per hexamer of Neotrypaea (as Callianassa) californiensis haemocyanin. The importance of \( \text{Mg}^{2+} \) ions in the formation of eikositetramers has also been clearly demonstrated by Miller et al. (1977). It was not unexpected, therefore, to find that the concentrations of \( \text{Mg}^{2+} \) in the haemolymph were quite high (34.4–45.4 mmol l\(^{-1}\)) in the five species of mud-shrimps examined during this study but even higher values have been measured in the haemolymph of \( \text{N. californiensis} \) (48 mmol l\(^{-1}\)), \( \text{N. gigas} \) (54.5 mmol l\(^{-1}\)) and \( \text{U. pugettensis} \) (60.0 mmol l\(^{-1}\)) (Miller et al., 1977). These \( \text{Mg}^{2+} \) levels are much higher than those recorded for nearly all other decapods (Robertson, 1960; Mantel and Farmer, 1983). Although \( \text{Mg}^{2+} \) ions are required for the formation of different aggregation states, high levels of these cations are regarded as potentially disadvantageous because of the possibility of narcotizing effects (Robertson, 1960; Mangum, 1983a).

4.2. Haemocyanin subunit structural composition

SDS PAGE studies showed that the haemocyanins of Upogebia deltaura and \( \text{U. stellata} \) consist of three major mobility groups with a large part of the haemocyanin present in a single band (MW = 84 700 Da). Interestingly, Miller et al. (1977) observed a similar banding pattern for \( \text{U. pugettensis} \). The scans of the SDS–PAGE gels of Calocaris macandreae, Jaxea nocturna and Callianassa subterranea haemolymph, although not identical, exhibit a number of common components. Similarly, as many as 6 subunits were resolved for Neotrypaea (as Callianassa) californiensis and 4 subunits for \( \text{N. gigas} \) (Miller et al., 1977). Roxby et al. (1974) found that SDS–PAGE of the 24-mer of \( \text{N. californiensis} \) (termed haemocyanin C) produced only one major subunit of 70–75 000 Da. The incompetent hexamer of \( \text{N. californiensis} \) (termed haemocyanin I), as well as showing a strong band at 70–75 000, also gave 3 distinct smaller subunits (Roxby et al., 1974).

Although Miller et al. (1977) did not find any clear correlation between the subunit composition and the capacity to form 24-mers for \( \text{U. pugettensis} \), \( \text{N. californiensis} \) and \( \text{N. gigas} \), the authors did suggest that the stability of both the eikositetramer and dodecamers probably results from differences in the subunit composition. Similarly, in this study there appeared to be little correlation between the range of subunits and the ability to form aggregated structures, especially since the haemocyanin from the three species examined formed similar aggregation states. In other crustacean species, between one and eight different haemocyanin subunits have been observed (with average molecular weights ranging from 70–90 000 Da) with as many as three smaller additional non-respiratory proteins (e.g. Markl et al., 1979; Mangum, 1983a). It should be noted, however, that electrophoresis studies of crustacean haemocyanins using non-denaturing conditions frequently indicate the presence of greater numbers of bands. Such studies have demonstrated the occurrence of considerable sub-unit heterogeneity even among individuals of the same species (e.g. Markl, 1986; Markl and Decker, 1992; Reese and Mangum, 1994, Mangum and Greaves, 1996) and there is growing evidence that such
heterogeneity may influence the respiratory properties of the haemocyanin (Mangum and Rainer, 1988; Mangum et al., 1991; Reese and Mangum, 1994). For example, Mangum and Rainer (1988) showed that differences in the proportions of different subunits of the haemocyanin of the crab, *Callinectes sapidus* collected from estuarine and from fully marine habitats resulted in significantly different oxygen affinities. Further studies of this species showed that prolonged exposure (7–25 days) to hypoxia also caused changes in the subunit composition of the haemocyanin resulting in a corresponding increase in oxygen affinity (DeFur et al., 1990).

4.3. Oxygen transporting properties of the haemolymph

The oxygen carrying capacities ($C_{HbO_2}$) determined during the present study ranged from 0.22 mmol l$^{-1}$ for *Upogebia stellata* to 0.83 mmol l$^{-1}$ for *Callianassa subterranea* (Table 2), reflecting differences in haemocyanin concentration. These values are similar to that of 0.45 mmol l$^{-1}$ obtained for *Neotrypaea* (as *Callianassa*) *californiensis* (Miller et al., 1976), and are within the range recorded for many aquatic decapods (Mangum, 1983a).

The haemocyanins of all five thalassinidean species examined during the present study are characterised by having a high oxygen affinity although the oxygen affinity of the haemocyanin of the two upogebiids was significantly lower than in the other species of mud-shrimp. These differences in $P_{50}$ cannot be attributed to differences in the concentration of L-lactate or other ions in the haemolymph, since these did not differ significantly between samples. These oxygen affinities are slightly higher than those previously reported for *Neotrypaea* (as *Callianassa*) *californiensis* ($P_{50} = 2.5$ at pH 8.2, 10°C) (Miller and Van Holde, 1981a); *N. californiensis* ($P_{50} = 4$, pH 8.0, 10°C) (Miller et al., 1977); *N. californiensis* ($P_{50} = 3.7$, pH 8.0, 15°C) (Sanders and Childress, 1992); *N. gigas* ($P_{50} = 4$, pH 8.0, 10°C) (Miller et al., 1977); *Upogebia pugettensis* ($P_{50} = 11.5$, pH 7.85, 10°C) (Miller et al., 1977).

The haemocyanin oxygen affinities of burrowing crustaceans that regularly experience hypoxia appear to be consistently higher than those of decapods from normoxic habitats (McMahon, 1984; Taylor et al., 1985; Bridges, 1986; Taylor, 1988; Atkinson and Taylor, 1988; Sanders et al., 1988; Sanders and Childress, 1990). Among the thalassinideans there is growing evidence to suggest that functional differences in oxygen binding characteristics are correlated with the degree of hypoxia experienced. The higher oxygen affinity of the haemocyanin of the deposit feeding shrimps *Callianassa* spp., *Jatea nocturna* and *Calocaris macandreae* (Miller et al., 1977; this study) compared with that of the haemocyanin of the filter feeding upogebiids indicates that the haemocyanin of the deposit feeders is particularly suited for the more severely hypoxic conditions found within their burrows. For each of the thalassinidean species, the Bohr coefficient was moderately large ($\phi = -1.06$ to $-1.48$), although within the range given for a number of other burrowing and non-burrowing decapod crustaceans (Mangum, 1983a; Taylor et al., 1985; Bridges, 1986). These values are similar to those obtained in previous studies of thalassinidean decapods, e.g. $-1.12$ for *Neotrypaea* (as *Callianassa*) *californiensis* (Sanders and Childress, 1992) and $-1.15$ for *N. californiensis* (Miller et al., 1977) but a
somewhat lower value of $-0.63$ was found for *Upogebia pugettensis* (Miller et al., 1977).

Temperature is known to affect the oxygen affinity of decapod haemocyanins but the magnitude of the decrease in oxygen affinity with an increase in temperature varies greatly. There is some evidence that the haemocyanins of species that regularly experience fluctuations in environmental temperature show a reduced temperature sensitivity whereas other species that experience less temperature fluctuation have haemocyanins showing a greater temperature sensitivity (Jokumsen and Weber, 1982; Morris et al., 1985; Taylor et al., 1985). The data from this study provide some support for this theory. *Calocaris macandreae* were collected from sites at which the annual temperature variation is modest (temperature minima and maxima [1982–1996] 6°C and 12°C, Scottish Environmental Protection Agency, unpubl. data). The other species were collected from shallower waters where the annual temperature variation was greater (4–15°C; D.J. Hughes, S.J. Marrs and Scottish Environmental Protection Agency, unpubl. data). As might be expected, the haemocyanin of these mud-shrimps showed a moderate temperature sensitivity.

The differences in temperature sensitivity between species may have an alternative explanation, however. Burnett et al. (1988) have noted that there is some evidence of an inverse relationship between the magnitude of the Bohr effect and the temperature sensitivity of the haemocyanin. These authors suggested that when the effect of pH on oxygen affinity is large, a low temperature sensitivity would minimize the indirect effect of temperature due to the thermal sensitivity of haemolymph pH. Whilst this may be true for some species, there are a number of species that exhibit a large Bohr effect and a large temperature sensitivity (Jokumsen et al., 1981; Mauro and Mangum, 1982; Bridges et al., 1983; Morris and Bridges, 1985, 1986).

It is well established that, in addition to pH and temperature, the oxygen affinity of decapod haemocyanins can be modulated by other factors such as organic and inorganic ions (see review by Mangum, 1983b; Morris, 1990). A number of studies have clearly demonstrated the importance of L-lactate and also urate in the modulation of haemocyanin oxygen affinity in many, but not all, crustaceans (e.g. Truchot, 1980; Booth et al., 1982; Bridges et al., 1984; Morris et al., 1985; Lallier et al., 1987; Lallier and Truchot, 1989; Morris, 1990; Zeis et al., 1992). Although the effect of urate was not examined during the present study, no specific lactate effect could be demonstrated for *Calocaris macandreae*. Similarly, no lactate effect was observed for the haemocyanin of *Neotrypaea* (as *Callianassa*) *californiensis* (Mangum, 1983b). Further studies are needed to establish if this is a characteristic feature of the haemocyanins of thalassinidean mud-shrimps but there are data that indicate that the haemocyanins of some other decapods also lack a lactate effect or show a very small lactate sensitivity (Morris and Bridges, 1985, 1986; Johnson, 1987; Morris et al., 1988). The occurrence of a lactate effect is not restricted to decapods but has also been demonstrated in amphipod crustaceans (Spicer and McMahon, 1990; Spicer and Taylor, 1994). As in the decapods, however, not all species exhibit this effect. This appears to be true especially of the semi-/euterrestrial talitrid species (Spicer and McMahon, 1990; Spicer et al., 1990; Spicer and Taylor, 1994). Similarly, Morris (1991), in his review of factors affecting gas transport in decapods, recorded that over 20 species of terrestrial decapod showed no or
only a small lactate effect. The occurrence of a lactate effect in some, but not all, decapod (and amphipod) crustaceans has yet to be fully explained. It is interesting to note that studies using isothermal titration calorimetry have shown that lactate does not bind with the haemocyanin of species such as *C. macandreae* which do not exhibit a lactate effect, whereas in species that do, lactate binding to the haemocyanin molecule does occur (Taylor et al., unpublished data). Further work to extend the earlier studies of Graham (1985) on the mechanisms of lactate binding is required.

The haemocyanins of thalassinidean mud-shrimps therefore show some differences with those of many other decapods in terms of their aggregation state and in the apparent absence of a lactate effect on haemocyanin oxygen affinity. The high oxygen affinity of the haemocyanin appears to be an adaptation to the hypoxic conditions that regularly occur within their burrows. It is interesting to note, however, that the upogebiid shrimps have a lower oxygen affinity than the other species studied. Such differences between the oxygen affinity of thalassinidean haemocyanins may reflect adaptations to the different feeding lifestyles and burrow environments. For example, the suspension feeding upogebiids exhibit a much more continuous pattern of burrow irrigation than the mainly deposit feeding species such as *C. macandreae* and *Jaxea nocturna* (Astall et al., 1997). As a result, the PO₂ of the burrow water rarely decreases to the very hypoxic levels recorded in the burrows of species such as *C. macandreae* and *C. subterranea*. Therefore, although the extent of burrow hypoxia varies between the species investigated, all have a high affinity respiratory pigment, indicating adaptation to life in these conditions.

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**References**


