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ONTOGENY OF HEMOCYANIN FUNCTION IN THE DUNGENESS CRAB *CANCER MAGISTER*: THE INTERACTIVE EFFECTS OF DEVELOPMENTAL STAGE AND DIVALENT CATIONS ON HEMOCYANIN OXYGENATION PROPERTIES

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Summary

Calcium and magnesium ions raise the oxygen affinities of 25S hemocyanins of both first-instar juvenile and adult *Cancer magister*. A physiologically relevant change in magnesium concentration from 16 to 32mmoll^{-1} changes first-instar juvenile hemocyanin affinity by 5.6mmHg (0.7kPa) but adult affinity by only 1.1mmHg (0.15kPa). In early juvenile crabs, the higher magnesium sensitivity of the hemocyanin may be compensated for by the lower oxygen affinity, which has been shown previously to be 50% lower than that of the adult under identical experimental conditions. Furthermore, ontogeny of ionic and osmotic regulation occurs during the development of *C. magister*, with especially high concentrations of magnesium being found in the hemolymph of early juveniles. Intermediate-stage juveniles (fifth to eighth instars) have hemocyanins with subunit stoichiometries and P_{50} values approaching those of the adult. These findings are significant because they indicate that modulation of *C. magister* hemocyanin oxygen-affinity during development incorporates differences in intrinsic affinity and differences in divalent cation sensitivity of the stage-specific hemocyanins.

Introduction

The Dungeness crab, *Cancer magister*, like many crustaceans, undergoes dramatic changes in morphology, locomotion and habitat as it develops from a swimming planktonic zoea to a scuttling benthic adult. During development, both gill structure and oxygen requirements change (Guttermuth and Armstrong, 1989; Brown, 1991). It was provocative, therefore, to find that different hemocyanins are present during different developmental stages of *C. magister* (Terwilliger and Terwilliger, 1982). Hemocyanins are the copper-containing respiratory proteins found in the hemolymph of many arthropods. Both 25S two-hexamer and 16S hexamer fractions (Ellerton *et al.* 1970; Carpenter and Van Holde, 1973) occur throughout the crab's life cycle. However, adult

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hemocyanin contains a polypeptide chain that is not present in megalopa or early juvenile crab hemocyanins, and the stoichiometry of two of the other five polypeptide chains that constitute the adult 25S hemocyanin molecule (Larson *et al.* 1981) is different in the earlier stages. Preliminary data indicated that the functional properties of stage-specific hemocyanins vary as well; megalopa and juvenile hemocyanins have an oxygen affinity 50% lower than that of adult hemocyanin under identical experimental conditions (Terwilliger *et al.* 1986). Developmental changes in crustacean hemocyanin structure and function have also been reported in the lobster *Homarus americanus* (Olson *et al.* 1988, 1990; Olson and McDowell Capuzzo, 1989).

Hemolymph inorganic ions, in particular divalent cations, can affect the oxygen affinity and cooperativity of decapod hemocyanins (see Van Holde and Miller, 1982, for a review). During development from megalopa to adult *C. magister*, hemolymph levels of calcium and magnesium undergo stage-specific changes (Brown and Terwilliger, 1992). Juvenile hemolymph calcium ion activity is significantly lower in animals bathed in 50% and 75% sea water than in those bathed in 100% sea water, although megalopa and adult show no difference with changing salinity. Even more surprising, in 100% sea water, megalopa and first juvenile instar have hemolymph magnesium ion concentrations of 32mmol l^{-1}, twice that of the adult. What role might the changing divalent cation concentrations play in the respiratory physiology of *C. magister* during development?

In this paper, we document changes in oxygenation properties of purified 25S hemocyanin from megalopa, from first, second, fifth, sixth, seventh and eighth instars and from adult *C. magister*. We also investigate whether the differences in oxygen affinity that we find are caused by intrinsic functional properties of stage-specific hemocyanin molecules or by differential sensitivities of the hemocyanins to the allosteric effectors, calcium and magnesium.

Materials and methods

Animals

Cancer magister (Dana) megalopas were collected by dipnet from the surface waters of Coos Bay, Oregon, USA, near the mouth of the estuary. Because the megalopas molt within 72h of capture, they were used within 2 days. During the brief period while the megalopas were maintained in the laboratory, they were kept in running unfiltered aerated sea water at ambient seawater temperature (9–15°C) and salinity (30–33‰), pumped on an incoming tide from near the mouth of Coos Bay, and given no food. Juvenile crabs were reared from field-caught megalopas in 38l aquaria with running sea water and aeration. Adult male *C. magister* larger than 12cm in carapace width were collected with crab pots from Coos Bay and maintained in 1000l holding tanks under similar conditions to megalopas and juveniles. Adults and juveniles were fed mussels, fish and squid 3–5 times a week. All stages were maintained in holding facilities exposed to natural light/dark cycles. Juvenile molt stage was based on the hardness of the carapace and the time since the most recent molt. Only individuals judged to be in intermolt were used.

Purified 25S hemocyanin sample preparation

Hemolymph samples from adults and intermediate-instar juveniles (fifth to eighth instar) were withdrawn by needle and syringe from the sinus at the base of a walking leg. Hemolymph was allowed to agglutinate on ice for 30min and then centrifuged at 12000 ***g*** for 10min in a Sorvall RC2-B refrigerated centrifuge (4˚C). The supernatant was immediately applied to a BioGel A-5m column (1.8cm×135cm) equilibrated with 0.05 ionic strength Tris–HCl buffer (pH7.5), made 0.1mol l^{-1} in NaCl, 10mmol l^{-1} in $MgCl_2$ and 10mmol l^{-1} in $CaCl_2$ at 10˚C. The eluted 25S hemocyanin peak was concentrated using Centricon 30 tubes (Amicon).

Purified 25S hemocyanin was obtained from megalopas and first- and second-instar juveniles by cutting them across a lateral edge of the carapace, placing them in column buffer containing 1mmol l^{-1} phenylmethylsulfonyl fluoride to inhibit protease activity, and centrifuging them immediately at 3000 ***g*** for 10min at 4˚C. The supernatant was respun at 12000 ***g*** for 10min. Three hundred first-instar juveniles yielded 1–2ml of hemolymph. The supernatant was chromatographed and the 25S hemocyanin fractions were concentrated as described above for adult hemocyanin. Previous experiments had shown that this protocol prevented hemocyanin proteolysis (Terwilliger and Terwilliger, 1982; Wache *et al*. 1988; Terwilliger, 1991); immediate SDS–PAGE analysis of individual hemolymph samples obtained by micropipette from megalopas, and from first-instar to ninth-instar juveniles and adult crabs confirmed the integrity of the molecules in the pooled samples.

Buffered saline solutions for oxygen binding experiments were prepared according to the total osmolality and ion concentrations measured in the hemolymph of adult *Cancer magister* maintained in 100% sea water (Hunter and Rudy, 1975; Graham *et al.* 1983; Brown and Terwilliger, 1992). The saline used to test calcium ion effects contained (mmol l^{-1}): HCl, 50; NaCl, 454; KCl, 11.5; $MgCl_2$, 18; Na_2SO_4, 23.5 and $CaCl_2$ at 4, 8, 16 or 32mmol l^{-1}. In a second series of salines used to test magnesium ion effects, HCl, NaCl, KCl and Na_2SO_4 were used at the concentrations given above but $CaCl_2$ was present at 13.5mmol l^{-1} and $MgCl_2$ was 16, 32 or 100mmol l^{-1}. All saline solutions were titrated to the desired pH at 10˚C or 20˚C with Trizma base (Sigma). Hemocyanin samples were dialyzed against 1l of saline (four changes) for a total of 24h. Megalopa, juvenile and adult hemocyanins were treated identically for purposes of direct comparison.

Experimental calcium and magnesium ion concentrations used were based on the range of levels measured in the hemolymph of adults and first-instar juveniles exposed to 50%, 75% and 100% sea water for 8h (the approximate duration of a tidal cycle) (Brown and Terwilliger, 1992). Fresh adult and first-instar juvenile 25S hemocyanins were dialyzed as above against saline solutions containing (a) 4, 8, 16 or 32mmol l^{-1} calcium and a constant concentration of other ions, or (b) 16, 32 or 100mmol l^{-1} magnesium and a constant concentration of other ions.

Oxygen equilibria

Oxygen equilibria were determined tonometrically (Benesch *et al.* 1965) at 10˚C and

20˚C on freshly purified, dialyzed samples. Immediately after an oxygen equilibrium curve had been completed, the pH of the sample was measured at the appropriate temperature with an Orion ROSS pH electrode (model 81-03) and a Radiometer ION83 meter.

The values of P_{50} (mmHg) and cooperativity were obtained from Hill plots. Oxygen affinities and Bohr coefficients were compared by analysis of covariance (ANCOVA). Mean values of cooperativity (n_{50}) were compared by Student's *t*-test. $P<0.05$ was considered significant. Statistical analyses were carried out using SYSTAT version 4.1 (SYSTAT, Inc.).

Electrophoresis

SDS–PAGE was performed essentially according to the method of Laemmli (1970).

Results

The oxygen affinity of *C. magister* purified 25S hemocyanin increases during development from megalopa to adult crab, as shown in Fig. 1. In order to compare the intrinsic oxygen affinities of stage-specific 25S hemocyanins, all binding curves in Fig. 1 were carried out in adult *C. magister* saline (Graham *et al.* 1983). Megalopa and first- and second-instar juvenile crab 25S hemocyanin oxygen-affinities are indistinguishable and are about 50% lower than that of adult 25S hemocyanin. Oxygen affinities of hemocyanins from intermediate juveniles are higher and almost equal adult hemocyanin oxygen-affinity. There are no significant differences in either the Bohr coefficients ($\Delta\log P_{50}/\Delta\mathrm{pH}=-1.16\pm0.15$) or cooperativity in 25S hemocyanin during development.

The developmental changes in hemocyanin oxygen-affinity parallel changes in hemocyanin subunit composition. SDS–PAGE analysis of stage-specific hemocyanins (Fig. 2) shows that intermediate juvenile hemocyanins, with oxygen affinities approaching those of adult hemocyanin, contain low levels of band 6, corresponding to the subunit present in adult hemocyanin but absent from megalopa and early juvenile blood. The stoichiometry of bands 4 and 5 also changes; megalopa and early juvenile 25S hemocyanins have a preponderance of band 5, intermediate juvenile hemocyanins have equal amounts of bands 4 and 5, and adult 25S hemocyanin has more band 4 than band 5.

Increasing the calcium ion concentration raises the oxygen affinity of *C. magister* adult 25S hemocyanin (Fig. 3). The equations for regressions of $\log P_{50}$ on pH are given in Table 1. Increasing calcium ion concentration significantly decreases the cooperativity of *C. magister* adult 25S hemocyanin (N=32) (Fig. 3).

The oxygen affinity of first-instar juvenile 25S hemocyanin is about 50% lower than that of adult 25S hemocyanin at each of the calcium concentrations used. The regression equations of $\log P_{50}$ on pH are given in Table 2. The magnitudes of the calcium ion effect on both first-instar juvenile (Fig. 4) and adult hemocyanin oxygen-affinities (Fig. 3) are similar. Calcium ion concentration, however, does not have a significant effect on the cooperativity of first-instar juvenile 25S hemocyanin (N=35) (Fig. 4).

Increasing the magnesium ion concentration raises the oxygen affinity of *C. magister* adult 25S hemocyanin (Fig. 5). The equations for linear regressions of $\log P_{50}$ on pH are

given in Table 3. In contrast to calcium, increasing the magnesium concentration does not alter adult 25S hemocyanin cooperativity.

Increasing the magnesium ion concentration raises the oxygen affinity of first-instar

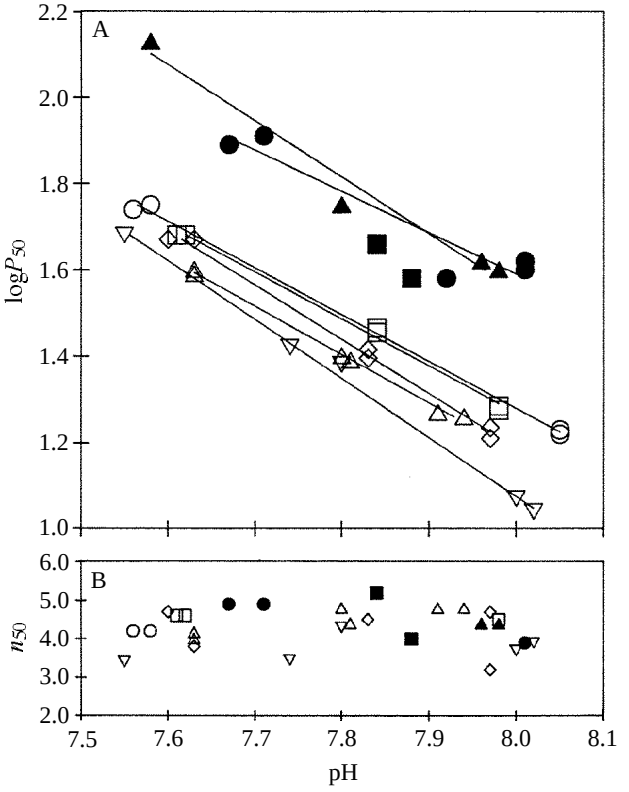

Fig. 1. Effect of developmental stage on the Bohr curve (A) and the relationship between n_{50} and pH (B), at 20˚C, for purified 25S *Cancer magister* hemocyanins in adult *C. magister* saline. ●, megalopa; ▲, first instar; ■, second instar; ○, fifth instar; △, sixth instar; □, seventh instar; ◇, eighth instar; ▽, adult hemocyanin. P_{50} in mmHg; 1mmHg=0.1333kPa.

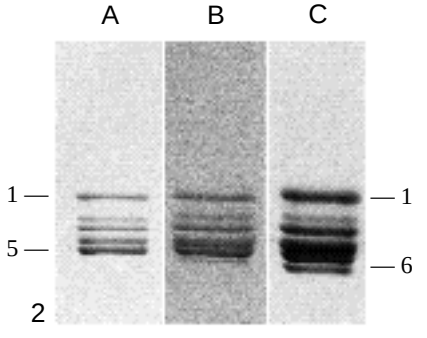

Fig. 2. SDS–PAGE of *Cancermagister* purified hemocyanin, 7.5% acrylamide. Lane A, first-instar juvenile; lane B, eighth-instar juvenile; lane C, adult hemocyanin.

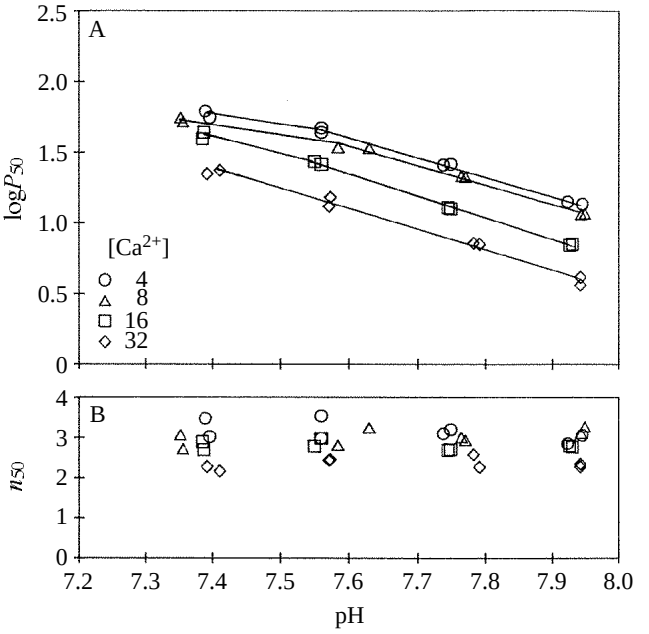

Fig. 3. Effect of calcium concentration on the Bohr curve (A) and the relationship between n_{50} and pH (B), at 10°C, for *Cancermagister* adult 25S hemocyanin. Calcium concentrations are given in mmol l^{-1}. P_{50} in mmHg; 1mmHg=0.1333kPa.

Table 1. *Equations for regressions of log*P_{50} *on pH for adult* Cancer magister *25S hemocyanin in salines with different calcium ion concentrations (10°C)*

[Ca^{2+}] (mmol l^{-1})	logP_{50}	r^2
4	−1.379pH+12.085	0.997
8	−1.368pH+11.935	0.990
16	−1.543pH+13.067	0.997
32	−1.415pH+11.846	0.988

juvenile 25S hemocyanin (Fig. 6 and Table 4). Magnesium concentration has no significant effect on the cooperativity of first-instar juvenile 25S hemocyanin (Fig. 6).

Using the regression equations given in Tables 1–4 and calculating the oxygen affinity at pH7.8, the magnitudes of the changes in P_{50} caused by the differential effects of calcium and magnesium on adult and first-instar juvenile hemocyanin are given in Table 5. Most notably, an increase in magnesium ion concentration over the physiological range 16–32mmol l^{-1} changes the affinity of adult 25S hemocyanin by only 1.1mmHg, whereas that of first-instar juvenile 25S hemocyanin is changed by 5.6 mmHg.

Discussion

Intrinsic differences in oxygenation properties clearly exist among *C. magister*

Table 2. *Equations for regressions of log*P_{50} *on pH for first-instar juvenile* Cancer magister *25S hemocyanin in salines with different calcium ion concentrations (10°C)*

$[Ca^{2+}]$ (mmol l^{-1})	logP_{50}	r^2
4	−1.541pH+13.603	0.995
8	−1.343pH+11.997	0.967
16	−1.160pH+10.409	0.971
32	−0.740pH+6.921	0.782

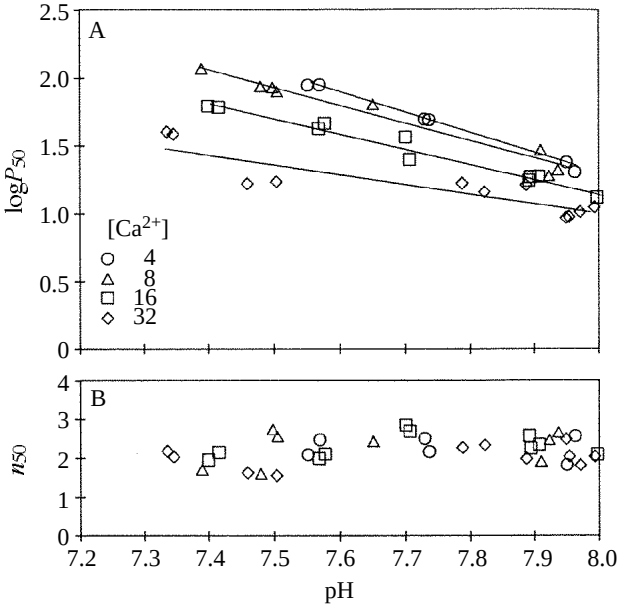

Fig. 4. Effect of calcium concentration on the Bohr curve (A) and the relationship between n_{50} and pH (B), at 10°C, for *Cancer magister* first-instar juvenile 25S hemocyanin. Calcium concentrations are given in mmol l^{-1}. P_{50} in mmHg; 1mmHg=0.1333kPa.

megalopa and early juvenile, intermediate juvenile and adult hemocyanins. In this study, the most comprehensive examination of developmental changes in hemocyanin structure and function to date, ontogenic changes in oxygen affinity directly correlate with ontogenic changes in hemocyanin subunit expression. As the concentration of subunit 5 diminishes during development, that of subunit 4 increases, and subunit 6 appears. An increase in immunologically distinguishable subunits during development was initially suggested for *Hyas araneus* and *Carcinus maenas* (Markl *et al.* 1986). They later attributed those findings to protein deterioration (Precht, 1990; Markl and Decker, 1992) and reported that, in *Carcinus maenas*, larval hemocyanin appears to be immunologically indistinguishable at the subunit level from adult hemocyanin. It is not known whether within the immunologically related groups there are several electrophoretically distinguishable subunits of similar but unique sequence that undergo stoichiometric changes during development. *Homarus americanus* hemocyanin undergoes an oligomer

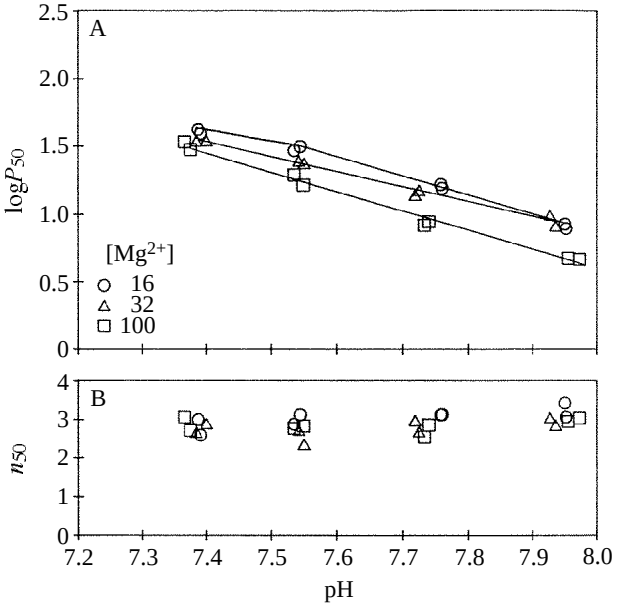

Fig. 5. Effect of magnesium concentration on the Bohr curve (A) and the relationship between n_{50} and pH (B), at 10°C, for *Cancer magister* adult 25S hemocyanin. Magnesium concentrations are given in mmol l^{-1}. P_{50} in mmHg; 1mmHg=0.1333kPa.

Table 3. *Equations for regressions of log*P_{50} *on pH for adult* Cancer magister *25S hemocyanin in salines with different magnesium ion concentrations (10°C)*

[Mg^{2+}] (mmol l^{-1})	logP_{50}	r^2
16	−1.389pH+11.961	0.992
32	−1.107pH+9.726	0.989
100	−1.428pH+12.019	0.990

shift from about 96% of the hexamer form in the stage I larva to about 35% hexamer and 65% two-hexamer in the adult (Olson *et al.* 1988). The only other report of larval and juvenile hemocyanin function concerns whole hemolymph from *Homarus americanus* (Olson *et al.* 1990).

Divalent cations have different effects on the functional properties of adult hemocyanins of many crustacean species. In only one species, *Panulirus interruptus*, has it been reported that calcium and magnesium lower the hemocyanin oxygen-affinity (Johnson *et al.* 1983). The oxygen affinity of *Procambarus simulans* hemocyanin is raised by increasing both calcium and magnesium concentrations. Calcium and magnesium also raise the cooperativity (Larimer and Riggs, 1964). Increasing calcium or magnesium concentration raises the oxygen affinity but not the cooperativity of *Callianassa californiensis* hemocyanin (Miller and Van Holde, 1974, 1981). In *Carcinus maenas*, calcium and magnesium raise the hemocyanin oxygen-affinity; the effect of

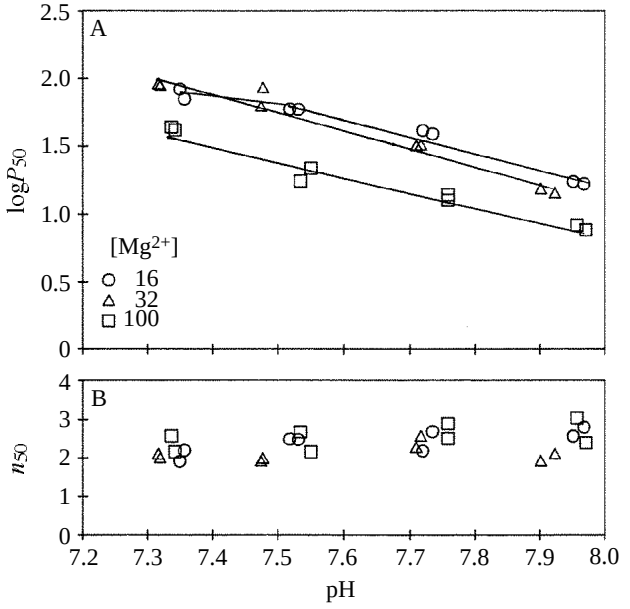

Fig. 6. Effect of magnesium concentration on the Bohr curve (A) and the relationship between n_{50} and pH (B), at 10°C, for *Cancer magister* first-instar juvenile 25S hemocyanin. Magnesium concentrations are given in mmol l^{-1}. P_{50} in mmHg; 1mmHg=0.1333kPa.

Table 4. *Equations for regressions of log*P_{50} *on pH for first-instar juvenile* Cancer magister *25S hemocyanin in salines with different magnesium ion concentrations (10°C)*

[Mg^{2+}] (mmol l^{-1})	logP_{50}	r^2
16	−1.257pH+11.258	0.971
32	−1.355pH+11.927	0.963
100	−1.125pH+9.844	0.962

magnesium on the oxygen affinity is stronger at higher pH (Truchot, 1975). This interaction between the binding of magnesium and protons has also been described for *C. californiensis* hemocyanin (Arisaka and Van Holde, 1979). Hemocyanins from *Penaeus setiferus* (Brouwer *et al.* 1978), *Callinectes sapidus* (Mason *et al.* 1983), *Austropotamobius pallipes* (Morris *et al.* 1986) and *Birgus latro* (Morris *et al.* 1988) all show an increase in oxygen affinity with increasing calcium concentration, but cooperativities do not change. Furthermore, in *B. latro*, magnesium ion concentration does not affect either oxygen affinity or cooperativity. There is, therefore, no consistent pattern of divalent cation effects on the hemocyanins in these species from a number of phylogenetically distinct groups or, for that matter, between species within phylogenetic groups. There is, however, an overall tendency for calcium, magnesium or both to increase crustacean hemocyanin oxygen-affinity and sometimes cooperativity. Each species must be examined for its unique characteristics.

Table 5. *Oxygen affinity of adult and first-instar juvenile 25S hemocyanin with varying calcium and magnesium at pH7.8 and 10°C*

Ca^{2+} ($mmol\,l^{-1}$)	Mg^{2+} ($mmol\,l^{-1}$)	P_{50} (mmHg)	
		Adult	First-instar juvenile
4	18	21.3	38.8
8	18	18.4	33.2
16	18	10.8	23.0
32	18	6.4	14.1
13.5	16	13.4	28.4
13.5	32	12.3	22.8
13.5	100	7.6	11.7

This developmental study utilizes temporally distinct populations of naturally occurring molecules synthesized *in vivo* in the same organism. Working with these genetically similar hemocyanins obviates some difficulties inherent in making interspecies comparisons of hemocyanin structure and function, difficulties exemplified in the range of individual species' responses to divalent cations, as described above. Markl *et al.* (1986) have shown that there are significant phylogenetic patterns of immunologically distinct subunits throughout the arthropods, but that pattern does not appear to correlate with divalent cation sensitivity. *Cancer magister* 25S hemocyanin, with five SDS–PAGE-distinguishable subunits in the megalopa and juvenile stages and six subunits in the adult, provides an excellent model system for delving into the relationship between structure, function and divalent cation sensitivity.

The differential functional sensitivity of the stage-specific hemocyanins of *C. magister* to divalent cations is of interest. The magnitudes of the changes in oxygen affinity of first-instar juvenile and adult *C. magister* 25S hemocyanin caused by changes in calcium and magnesium concentrations (Table 5) are well within the range reported for *Carcinus maenas* (Truchot, 1975), *Callinectes sapidus* (Mason *et al.* 1983) and *Austropotamobius pallipes* (Morris *et al.* 1988).

When different developmental stages of *C. magister* are exposed to salinity changes on the time scale of a tidal cycle, *in vivo* hemolymph ion concentrations change (Brown and Terwilliger, 1992). In juvenile hemolymph, calcium ion concentration ranges from $4.7mmol\,l^{-1}$ to $9.5mmol\,l^{-1}$ and magnesium ion concentration ranges from $14mmol\,l^{-1}$ to $32mmol\,l^{-1}$ depending on ambient salinity. Adult hemolymph calcium and magnesium levels are strongly regulated and remain around $10mmol\,l^{-1}$ and $14mmol\,l^{-1}$, respectively, even after 8h in 50% sea water (Brown and Terwilliger, 1992). The magnitude of the change in oxygen affinity of juvenile 25S hemocyanin (about 5mmHg) is similar when either calcium or magnesium concentration is altered over its respective physiological range (4–$8mmol\,l^{-1}$ calcium or 16–$32mmol\,l^{-1}$ magnesium). These same divalent cation concentrations cause a smaller change in adult 25S hemocyanin oxygen-affinity (Table 5). This stage-specific difference in hemocyanin divalent cation sensitivity, particularly the large difference in magnesium sensitivity, may be important in whole hemolymph oxygen transport *in vivo*. The decrease in

cooperativity of the adult 25S hemocyanin as calcium ion concentration is increased (Fig. 3) is unusual. First, there is no change in *C. magister* juvenile 25S hemocyanin cooperativity with increasing calcium concentration. Second, magnesium does not alter cooperativity of either adult or juvenile *C. magister* 25S hemocyanin. Third, in those crustacean species for which data are available, the hemocyanin cooperativity seems to be either unchanged or increased by increasing divalent cation concentrations. The stage-specific effects of calcium on cooperativity raise intriguing questions about the role of individual *C. magister* subunits in the binding of divalent cations.

Although we have previously noted that megalopa and adult hemocyanins differ with respect to subunit characteristics and oxygen affinities (Terwilliger and Terwilliger, 1982; Terwilliger *et al.* 1986), the basis for the difference in affinity was unclear. Fetal–maternal oxygen affinity differences in the red blood cells of most viviparous vertebrates are the result of either different hemoglobins or different concentrations of allosteric effectors. Stripped fetal human hemoglobin has a slightly lower oxygen affinity than that of the adult. The higher affinity of maternal hemoglobin for the organic phosphates 2,3-diphosphoglycerate and adenosine triphosphate and the consequently greater lowering of maternal hemoglobin oxygen-affinity, however, result in the higher oxygen affinity of fetal whole blood (for a review, see Ingermann, 1992). Conversely, the seaperch *Embiotoca lateralis* contains fetal hemoglobin which, in the stripped condition, has an oxygen affinity greater than that of the adult; i.e. there is a large intrinsic difference in oxygen affinity between fetal and adult hemoglobin chains (Ingermann and Terwilliger, 1981). Seaperch fetal and adult hemoglobins respond indistinguishably to allosteric modifiers, both pH and ATP. *Cancermagister* megalopa and early juvenile crab have a low-affinity unique hemocyanin. Although megalopa, juvenile and adult hemocyanins share similar, if not identical, responses to two allosteric effectors, H^+ and lactate (Terwilliger *et al.* 1986), they show stage-specific differences in response to both calcium and magnesium. The marked differences in both intrinsic affinity of the proteins (analogous to the seaperch hemoglobins) and differential sensitivity to certain allosteric effectors (analogous to human fetal and adult hemoglobins) play roles in modulation of oxygen affinity during *C. magister* development.

In summary, the purified 25S hemocyanins from different developmental stages of *C. magister* have intrinsically different oxygen affinities. There is also a stage-specific differential response to calcium and magnesium: the juvenile 25S hemocyanin is more sensitive to calcium, and especially to magnesium, than is the adult 25S hemocyanin. The lower affinity of juvenile hemocyanin may be compensated for *in vivo* by the high hemolymph magnesium levels in juvenile crabs (Brown and Terwilliger, 1992). Modulation of *C. magister* oxygen affinity during development incorporates changes in hemocyanin structure and functional properties and is integrated with the ontogeny of hemolymph ion regulation.

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