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ONTOGENY OF HEMOCYANIN FUNCTION IN THE DUNGENESS CRAB *CANCER MAGISTER*: HEMOLYMPH MODULATION OF HEMOCYANIN OXYGEN-BINDING

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Summary

The *in vivo* oxygen-binding characteristics of *Cancer magister* whole hemolymph were compared across developmental stages with those of purified hemocyanin. When the 25S hemocyanins from first-instar juvenile and adult *C. magister* were dialyzed against first-instar juvenile saline, the P_{50} values at pH 7.8 differed by 54 %: 2.16 kPa for the adult and 4.68 kPa for the first-instar juvenile. Since both purified proteins were examined under identical conditions, this represents an intrinsic stage-specific difference in hemocyanin O_2-affinity. When the two types of hemocyanin were dialyzed against their respective stage-specific salines, the oxygen affinities differed by only 28 %: 3.39 kPa for the adult and still 4.68 kPa for the first-instar juvenile. Thus, the intrinsic difference in hemocyanin O_2-affinity was reduced by the stage-specific differences in hemolymph ion concentrations. Even more significant is the fact that the whole-hemolymph P_{50} values of the juvenile and adult were indistinguishable at *in vivo* pH and divalent cation levels specific for each stage. Thus, despite significant differences in the intrinsic oxygen affinity of the purified 25S hemocyanin during development, the whole-hemolymph oxygen-binding properties are conserved. In the juvenile crab, it appears that the low-affinity hemocyanin serves to modulate the effects of a weak renal regulation of $[Mg^{2+}]$. As ion regulation is enhanced during development and divalent cation levels decrease, the crab synthesizes higher-affinity hemocyanin.

Key words: *Cancer magister*, crab, hemocyanin, oxygen binding, hemolymph, development, ion regulation.

Introduction

Oxygen transport in crustaceans is affected by a myriad of factors. The gill ventilation rate, the gill and tissue perfusion rates and the oxygen-binding characteristics of hemocyanin can all be altered in response to changes in external and internal factors that the organism experiences. A change in environmental salinity can cause changes in hemolymph inorganic ion concentrations (for a review, see Mantel and Farmer, 1983). These changes in ion concentrations in turn affect the oxygen-binding characteristics of crustacean hemocyanins. Increases in Ca^{2+} and Mg^{2+} concentrations cause increases in both the affinity and cooperativity of hemocyanin from the crayfish *Procambarus simulans* (Larimer and Riggs, 1964). In the green crab *Carcinus maenas*, Mg^{2+} and Ca^{2+} increase the oxygen affinity of the hemocyanin and Mg^{2+} has an interaction with H^+ that increases the effect of Mg^{2+} at higher pH (Truchot, 1975). In the blue crab *Callinectes sapidus*, Ca^{2+} increases hemocyanin oxygen-affinity but has no effect on cooperativity over the physiological range of Ca^{2+} concentration (Mason *et al.* 1983). Similarly, changes in environmental temperature cause changes in the function of hemocyanin in crustaceans. One of the many species in which this has been clearly demonstrated is *Carcinus maenas* (Truchot, 1975).

The products of metabolism and other naturally occurring constituents of crustacean hemolymph are internal factors that affect the oxygen-binding properties of hemocyanin. Crustaceans produce L-lactate as a result of anaerobic energy metabolism. L-Lactate raises the hemocyanin oxygen-affinity of many crustacean species, including *Carcinus maenas*, *Cancer pagurus* (Truchot, 1980), *Callinectes sapidus* (Booth *et al.* 1982) and *Cancer magister* (Graham *et al.* 1983). Not all crustacean hemocyanins are affected by the presence of lactate, however (reviewed in Mangum, 1983). Other naturally occurring organic effectors of hemocyanin oxygen-affinity in some species are urate (Morris *et al.* 1985, 1986), a purine product accumulating in crustaceans exposed to environmental hypoxia (Lallier *et al.* 1987; Lallier and Truchot, 1989), dopamine and related cardiac neuroamines (Morris and McMahon, 1989; Morris, 1990) and nitrogen metabolites, ammonium and trimethylamine (Sanders *et al.* 1992).

Developmental changes also affect crustacean oxygen transport. First, hemocyanin structure changes during

ontogeny of *Cancer magister* (Terwilliger and Terwilliger, 1982) and *Homarus americanus* (Olson and McDowell Capuzzo, 1989). Terwilliger and Terwilliger (1982) and Terwilliger and Durstewitz (1996) have shown that hemocyanin from adult *Cancer magister* contains a polypeptide chain, subunit 6, that is not present in megalopa or early juvenile hemocyanins. These stage-specific hemocyanins of *C. magister* have different intrinsic oxygen affinities and yet have the same sensitivity to lactate and pH (Terwilliger *et al.* 1986).

Second, ionic regulation also shifts during development from megalopa to adult in *C. magister*, especially with respect to stage-specific differences in Ca^{2+} and Mg^{2+} regulation (Brown and Terwilliger, 1992). *Cancer magister* adults strongly hyporegulate [Mg^{2+}]. Megalopas, first- and fifth-instar juveniles in 100 % sea water have hemolymph Mg^{2+} activities approximately twice as high as in the adult. The Ca^{2+} activities in hemolymph from the different life stages are not significantly different in 100 % sea water, but as ambient salinity decreases the adult and megalopa regulate [Ca^{2+}], and the first- and fifth-instar juveniles hyperconform. Physiologically relevant changes in Ca^{2+} and particularly in Mg^{2+} concentrations cause differential effects on the oxygen affinity of the hemocyanin from first-instar juvenile and adult crabs (Terwilliger and Brown, 1993). Information on oxygen affinity and the effects of organic and inorganic effectors on purified hemocyanin, and studies on changes in osmoregulation during crustacean development, have often been analyzed to determine whether there are trends relating to environment or activity (see Young, 1972*a*,*b*; Mangum, 1983; McMahon, 1986; Charmantier and Charmantier-Daures, 1991). In *C. magister*, developmental changes in hemocyanin structure and function appear to be more closely related to ontogeny of ionic and osmotic regulation than to habitat selection.

Crustacean hemolymph functions as an important interface between the organism and the environment and is critical to the animal's internal transport, not only of oxygen-carrying hemocyanin but also of metabolites that may influence oxygen transport. By studying different stages of a single species, such as *C. magister*, that share an identical evolutionary history, we can explore both developmentally linked and habitat-related modifications in respiratory physiology without many of the complications of interspecific comparisons. In the present paper, we investigate whether the intrinsic differences in oxygen affinity between juvenile and adult crab hemocyanins are reflected in the oxygen-binding characteristics of whole hemolymph under *in vivo* conditions. We bring together the developmental changes in (1) ion regulation, (2) sensitivity of hemocyanin oxygen-affinity to divalent cations and (3) hemocyanin structure and function to examine how the combined effects of these changes are integrated to modulate the oxygen-transport properties of whole hemolymph from various life stages of *C. magister*. We find that the P_{50} values of whole hemolymph, measured at what might be *in vivo* pH, and stage-specific divalent cation levels are surprisingly similar across developmental stages.

Materials and methods

Animals

Cancer magister megalopas, juveniles and adults were caught and maintained as previously described (Terwilliger and Brown, 1993).

Whole hemolymph sample preparation

Hemolymph samples were obtained from the infrabranchial sinus of fifth-instar juvenile and adult crabs with a needle and syringe. Hemolymph samples were taken from the same place in first-instar juveniles, but the sampling was performed using a microcapillary pipette. Megalopa hemolymph was obtained by puncturing the heart with a microcapillary pipette. In order to avoid puncturing the yellowish-brown digestive tract, sampling of megalopas and first-instar juveniles was performed under a dissecting microscope. Hemolymph from five intermolt adults, 10 intermolt fifth-instar juveniles, 150 intermolt first-instar juveniles or 250 premolt megalopas was pooled for each whole hemolymph sample.

The fresh whole hemolymph samples were allowed to clot on ice for 30 min and then centrifuged at 12 000 ***g*** for 10 min at 4 °C to remove the aggregate. Samples to be used for tonometric oxygen equilibria were stored at 4 °C and used within 24 h. Samples of whole hemolymph for determination of oxygen equilibria using a diffusion method (Sick and Gersonde, 1969) were immediately centrifuged, stored at –73 °C and transported on dry ice to the University of Calgary, Alberta, Canada.

Purified 25S hemocyanin sample preparation

Samples of purified 25S hemocyanin from first-instar juveniles and adults were prepared by chromatography of fresh whole hemolymph on BioGel A5-m, as previously described by Terwilliger and Brown (1993).

Oxygen-carrying capacity

The fresh whole hemolymph samples used in oxygen equilibrium determinations contained an unidentified component with a low broad absorbance peak at approximately 325 nm. This peak was apparent in the deoxygenated spectra of whole hemolymph but not in the deoxygenated purified hemocyanin (Fig. 1). Owing to the presence of this interfering component, the absorbance used to calculate the hemolymph oxygen-carrying capacity was based on the difference between the oxyhemocyanin and the deoxyhemocyanin absorbances at 340 nm (10 °C), adjusted for the altered baseline absorbance caused by the unidentified component. Examination of 47 paired sets of *C. magister* 25S oxyhemocyanin and deoxyhemocyanin absorbances at 340 nm revealed that deoxyhemocyanin absorbance for whole hemolymph containing the interfering component should be: $OD_{deoxy,adjusted}=0.09(OD_{oxy}-OD_{deoxy,measured})$ ($r^2=0.86$). Given this relationship, the difference in absorbance of oxygenated and adjusted deoxygenated whole hemolymph was used in oxygen-carrying capacity calculations as the absorbance of fully oxygenated hemocyanin in the sample.

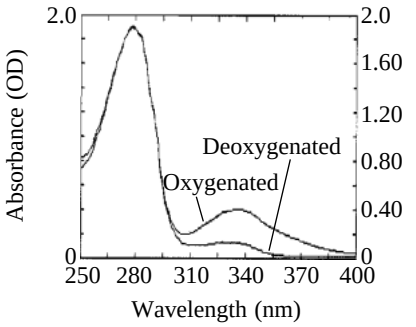

Fig. 1. Representative spectra of oxygenated and deoxygenated whole hemolymph at pH 7.5 at 10 °C from adult *Cancer magister*. The absorbance at 278 nm is 1.9 for both spectra, the absorbance of the fully oxygenated sample at 337 nm is 0.403, and the absorbance of the low broad peak at approximately 325 nm in the deoxygenated spectrum is 0.131.

The extinction coefficient at 280 nm of a 1 % solution of *C. magister* hemocyanin in a 1 cm pathlength cuvette, $E^{1\%}_{1cm}$, is 15 (Nickerson and Van Holde, 1971). Based on a ratio of 0.2 for the absorbance of purified *C. magister* oxyhemocyanin at 340 nm and 280 nm, the value of the extinction coefficient at 340 nm would be 3 (Graham, 1983). Since each hemocyanin subunit combines reversibly with one molecule of oxygen, the hemocyanin oxygen-carrying capacities of the whole hemolymph samples were calculated from the absorbance at 340 nm of a fully oxygenated sample, a value of $E^{1\%}_{1cm}$=3 at 340 nm, and an average subunit molecular mass of 75 kDa. The total oxygen-carrying capacity of the hemolymph includes the amount of oxygen physically dissolved in solution when ambient P_{O_2}=19.94 kPa.

Lactate and urate assays

L-Lactate concentrations in the whole hemolymph samples were assayed enzymatically (Boehringer-Mannheim, no. 139 084). Urate concentrations in the samples were also assayed by enzymatic assay (Sigma Chemical Co., procedure no. 685).

Oxygen equilibria

Fresh whole hemolymph samples for oxygen binding were buffered in stage-specific saline solutions (Table 1). The ion concentrations for each stage-specific saline corresponded to the concentrations measured in the hemolymph of megalopas, first-instar juveniles and fifth-instar juveniles in 100 % sea water, as reported by Brown and Terwilliger (1992). The saline for the adult hemolymph was made to match the formulation used by Graham *et al.* (1983). Titrating with Trizma base (Sigma Chemical Co.) rather than HCl provides a more consistent level of chloride for ion studies and/or analytical ultracentrifugation.

Oxygen equilibria of whole hemolymph samples were determined by two methods. First, oxygen equilibria were determined tonometrically (Benesch *et al.* 1965) at 10 °C using 0.4–0.5 ml of diluted whole hemolymph. Fresh whole hemolymph was diluted, 1:2 or 1:3, in appropriate stage-specific saline in order to obtain the desired pH and an absorbance at 340 nm near 0.3 in a 1 mm pathlength cuvette. Second, oxygen binding was also measured on previously frozen samples of undiluted whole hemolymph at 10 °C at the University of Calgary, using the diffusion chamber method described by Sick and Gersonde (1969). The pH of these samples was adjusted by using gas-mixing pumps to vary P_{CO_2}.

Oxygen equilibria for purified 25S hemocyanin of first-instar juvenile and adult were also determined tonometrically at 10 °C in stage-specific salines: first-instar juvenile 25S hemocyanin in first-instar juvenile saline; adult 25S hemocyanin in both first-instar saline and adult saline. Purified 25S hemocyanin samples were dialyzed against the appropriate salines at 4 °C for 24 h prior to oxygen equilibrium determinations.

Table 1. *Composition of stage-specific* C. magister *salines for 100 % sea water at 10 °C*

Salt	Megalopa	First instar	Fifth instar	Adult
NaCl	322	322	353	454
KCl	5.4	5.4	4.7	11.5
$CaCl_2$	7.4	7.4	8.5	13.5
$MgCl_2$	32	32	23.2	18
Na_2SO_4	23.5	23.5	23.5	23.5

Concentrations given in mmol l^{-1}. HCl (50 mmol l^{-1}) was added to each saline solution, which was then titrated to the desired pH at 10 °C with Trizma base (Sigma Chemical Co.).

Data analysis

The Bohr coefficients (slope of logP_{50} *versus* pH) and the oxygen affinities 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 performed using SYSTAT version 4.1 (SYSTAT, Inc.).

Results

Oxygen-carrying capacity

The concentration of hemocyanin in the hemolymph changes with developmental stage (Table 2). The intermolt first-instar juvenile had the lowest hemocyanin concentration, and the adult had the highest. The intermediate level seen in the megalopa is probably due to its premolt condition, the time in the molt cycle when hemocyanin levels reach a maximum (Terwilliger and Otoshi, 1994). These differences in concentration have a very pronounced effect on the total oxygen-carrying capacities of the four stages.

Lactate and urate assays

No urate was detected in any of the whole hemolymph samples from any of the four stages examined. L-Lactate levels varied among individual samples and among stages. The

Table 2. *The hemocyanin concentrations and oxygen-carrying capacities of four life history stages of* Cancer magister

Stage	Hemocyanin concentration (mg ml^{-1})	Oxygen-carrying capacity (ml O_2 dl^{-1})
Megalopa	20.6±0.8	1.26
First-instar juvenile	6.2±0.4	0.83
Fifth-instar juvenile	11.1±2.2	0.97
Adult	38.0±2.1	1.75

Hemocyanin concentrations are means ± S.D.

Oxygen-carrying capacity includes the amount of oxygen dissolved in solution at ambient P_{O_2} of 19.94 kPa.

lowest detectable level of L-lactate was 0.05 mmol l^{-1}. L-Lactate concentrations in the megalopa hemolymph samples (*N*=7; 250 megalopa per pooled sample) for tonometry ranged from the limit of detection to 0.57 mmol l^{-1}. Only one first-instar juvenile hemolymph sample (*N*=6; 150 first-instar juveniles per pooled sample) for tonometry had a detectable L-lactate level, 0.47 mmol l^{-1}. There was no detectable L-lactate in any of the fifth-instar juvenile samples (*N*=8; 10 fifth-instar juveniles per pooled sample) for tonometry. L-Lactate concentration in adult hemolymph samples (*N*=12; five adults per pooled sample) for tonometry ranged from 0.18 to 3.44 mmol l^{-1}.

Among the samples for diffusion chamber oxygen equilibrium determination, the hemolymph L-lactate concentrations were 1.10 mmol l^{-1} in the megalopa hemolymph (*N*=1; 250 megalopas), 0.69 mmol l^{-1} in the first-instar juvenile hemolymph (*N*=1; 150 first-instar juveniles), 0.30 mmol l^{-1} in the fifth-instar juvenile hemolymph (*N*=1; 10 fifth-instar juveniles) and 0.49 mmol l^{-1} in the adult hemolymph sample (*N*=1; five adults).

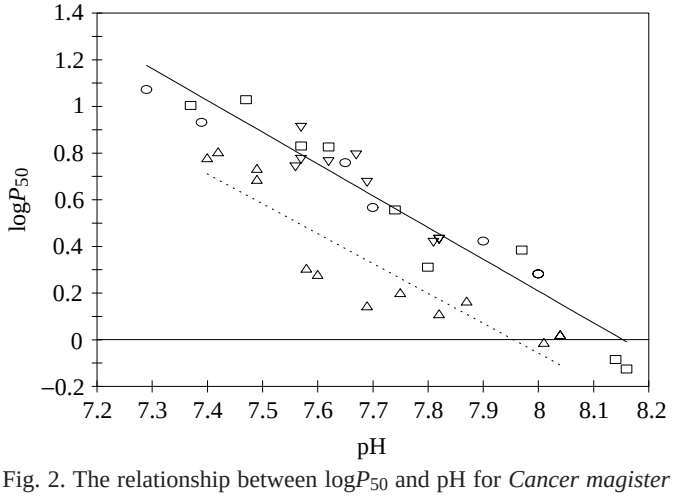

Fig. 2. The relationship between logP_{50} and pH for *Cancer magister* whole hemolymph with endogenous L-lactate and inorganic ions at 10 °C (P_{50} in kPa). Oxygen equilibria were determined tonometrically. The solid line is the regression for the combined data from megalopas, first-instar and fifth-instar juvenile stages, log P_{50}=–1.36(pH)+11.12, r^2=0.92; the dashed line is the regression for the adult data, log P_{50}=–1.28(pH)+10.19, r^2=0.83 (megalopa, □; first-instar juvenile, ○; fifth-instar juvenile, ▽; adult, △). The slopes are not significantly different (*P*=0.666).

Whole hemolymph oxygen equilibria

The oxygen affinities (measured tonometrically) of megalopa, first-instar juvenile and fifth-instar juvenile fresh whole hemolymph samples with endogenous L-lactate and ion concentrations are indistinguishable (Fig. 2). The oxygen affinity of adult fresh whole hemolymph, with endogenous L-lactate and ion concentrations, is higher than those of the the megalopa and juvenile whole hemolymph samples (Fig. 2). There are no significant differences in the slopes of the regressions of logP_{50} *versus* pH of the whole hemolymph from the four stages (see Fig. 2 legend).

The P_{50} of each sample in Fig. 2 was adjusted to what it would be if each sample contained an identical concentration of L-lactate, equal to the lowest detectable level of 0.05 mmol l^{-1}. The value used to adjust the P_{50} values of all four stages was $\Delta\log P_{50}/\Delta\log$[L-lactate]=–0.29. This value is based on the coefficients for the relationship of logP_{50} *versus* log[L-lactate] for adult *C. magister* hemocyanin (–0.287) and for juvenile hemocyanin (–0.291) (Terwilliger *et al.* 1986). When the P_{50} values of the whole hemolymph samples were adjusted for the effect of L-lactate, there were no significant differences in oxygen affinity or Bohr coefficient among any of the four stages (Fig. 3).

When the affinities of the frozen whole hemolymph samples (diffusion chamber method) were adjusted to 0.05 mmol l^{-1} L-lactate, they overlapped entirely with the P_{50} values of the tonometer samples in the physiological pH range, pH 7.2–8.2 (Graham *et al.* 1983; Brown and Terwilliger, 1992) (Fig. 4). Whether the slight trend in increasing P_{50} for tonometric analyses is indicative of a specific effect of CO_2 is unknown.

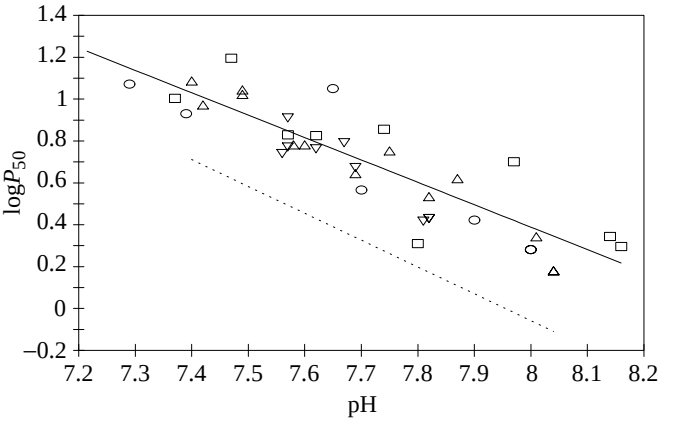

Fig. 3. The same data set as presented in Fig. 2; however, the P_{50} (kPa) values have been adjusted as though each sample contained 0.05 mmol l^{-1} L-lactate (the lowest level of detection in these experiments). The solid line is the regression for the data from all four stages with P_{50} adjusted, log P_{50}=–1.17(pH)+8.95, r^2=0.77. The dashed line is the regression for the adult data in Fig. 2 (megalopa, □; first-instar juvenile, ○; fifth-instar juvenile, ▽; adult, △). The slopes are not significantly different (*P*=0.685).

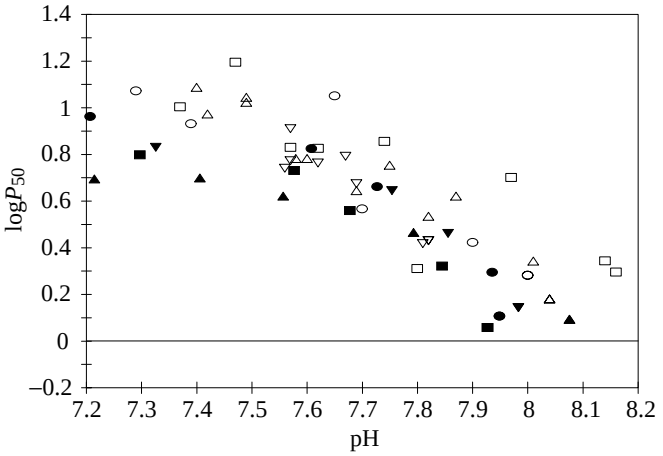

Fig. 4. The relationship between logP_{50} and pH for the whole hemolymph of four life history stages of *Cancer magister*. Filled symbols are for diffusion chamber determinations on frozen samples and open symbols are for tonometric determinations on fresh samples; symbols for stages as in Figs 1 and 2. Oxygen equilibria were determined at 10 °C, and affinities have been adjusted to 0.05 mmol l^{-1} L-lactate. P_{50} is in kPa.

Some crustacean hemocyanins exhibit a specific CO_2 effect while others do not (see Mangum, 1997). The Bohr coefficients and the statistically adjusted mean values for logP_{50} were not significantly different between the tonometer data and the diffusion chamber data.

The cooperativities of the fresh whole hemolymph samples among the four stages from the tonometric method were not significantly different (n_{50}=3.08±0.48, mean ± S.D.; Fig. 5). The mean cooperativities of the diffusion chamber oxygen equilibria were significantly lower than those of the tonometric samples (n_{50}=2.24±0.24, mean ± S.D.; P<0.05; Fig. 5). This decrease in cooperativity in the frozen samples is consistent with previous reports on the effects of freezing hemocyanin (Mangum, 1983; Morris, 1988).

Purified 25S hemocyanin oxygen equilibria

The oxygen affinities of purified 25S hemocyanins from first-instar juvenile and adult crabs were compared under different salinity regimes. The relationship between logP_{50} and pH for purified 25S hemocyanin from first-instar juveniles and adults in stage-specific salines is shown in Fig. 6. When both hemocyanins were dialyzed against first-instar juvenile saline, the P_{50} values at pH 7.8 differed by 54 %: 4.68 kPa for the first-instar juvenile and 2.16 kPa for the adult. When the two types of hemocyanin were dialyzed against their respective stage-specific salines, the affinities differed by only 28 %: 3.39 kPa for the adult and still 4.68 kPa for the first-instar juvenile. Thus, a major part of the intrinsic difference in stage-specific hemocyanins could be reduced by stage-specific differences in hemolymph ion concentrations. Cooperativity values for both hemocyanins in first-instar juvenile saline were significantly lower than cooperativity values for adult hemocyanin in adult-type saline (Fig. 7).

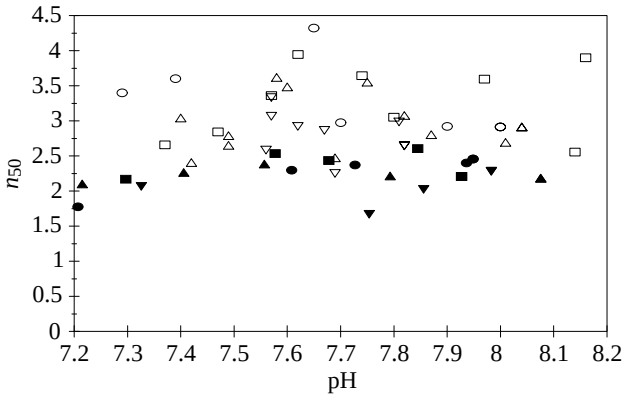

Fig. 5. The relationship between n_{50} and pH for whole hemolymph samples of four life stages of *Cancer magister*. Filled symbols are for diffusion chamber determinations on frozen samples and open symbols are for tonometric determinations on fresh samples; symbols for stages as in Figs 1 and 2. Oxygen equilibria were determined at 10 °C.

Discussion

The oxygen affinities of hemolymph from juvenile and adult *Cancer magister* are indistinguishable at *in vivo* pH and cation levels. These results are initially surprising in the light of the significant differences in intrinsic oxygen affinities of 25S hemocyanins from the various life stages of *C. magister* (Terwilliger and Terwilliger, 1982; Terwilliger and Brown, 1993). The stage-specific hemocyanins also have different sensitivities to the allosteric effectors Ca^{2+} and Mg^{2+}. While both ions increase hemocyanin oxygen-affinity, the P_{50} of juvenile hemocyanin is more sensitive to [Ca^{2+}], and especially to [Mg^{2+}], than is adult hemocyanin (Terwilliger and Brown, 1993). In addition, *C. magister* undergoes ontogenetic changes

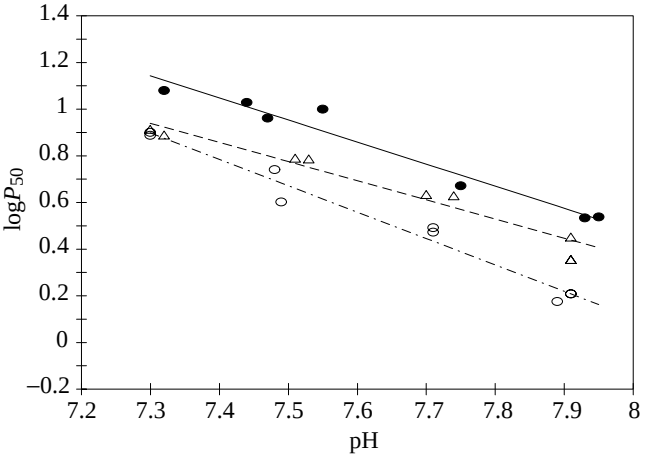

Fig. 6. The relationship between logP_{50} and pH at 10 °C for purified 25S hemocyanin from first-instar juvenile and adult *Cancer magister* in stage-specific salines. First-instar juvenile 25S hemocyanin in first-instar juvenile saline, ●, solid line, log P_{50}=–0.95(pH)+8.05, r^2=0.96; adult 25S hemocyanin in adult saline, △, dashed line, log P_{50}=–0.82(pH)+6.93, r^2=0.95; adult 25S hemocyanin in first-instar juvenile saline, ○, dash-dot line, log P_{50}=–1.13(pH)+9.16, r^2=0.97. The slopes are significantly different (P=0.035). (P_{50} is in kPa).

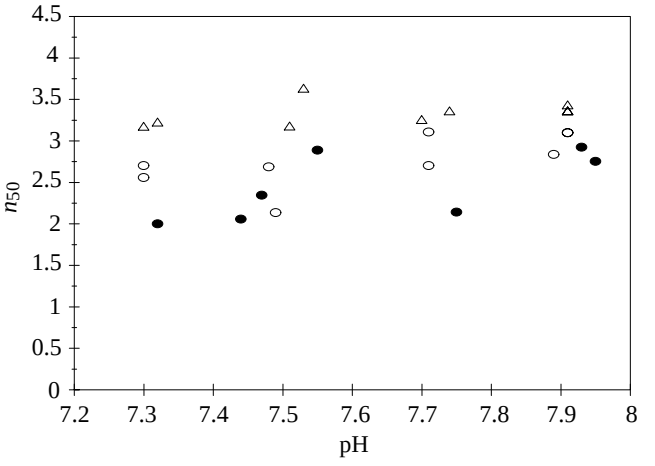

Fig. 7. The relationship between n_{50} and pH for purified hemocyanin from first-instar juvenile and adult *Cancer magister* (first-instar juvenile 25S hemocyanin in first-instar juvenile saline, ●; adult 25S hemocyanin in adult saline, △; adult 25S hemocyanin in first-instar juvenile saline, ○).

in ionic and osmotic regulation (Brown and Terwilliger, 1992) such that both megalopas and first-instar juveniles have more than twice the adult hemolymph Mg^{2+} level. We suggest that the intrinsically low-affinity hemocyanin of the first-instar juvenile serves to counterbalance the increase in oxygen affinity caused by both high hemolymph Mg^{2+} levels and increased sensitivity to [Mg^{2+}]. As the ionic regulatory capabilities of the young crab develop and it becomes better able to maintain low Mg^{2+} levels in the hemolymph, the synthesis of higher-affinity adult hemocyanin is initiated, and it gradually replaces juvenile hemocyanin. Expression of adult-type hemocyanin, as indicated by the appearance of hemocyanin subunit 6 mRNA, begins at approximately the sixth juvenile instar (Durstewitz and Terwilliger, 1997). It is the combination of developmental differences in (1) hemocyanin intrinsic affinity and (2) hemocyanin sensitivity to allosteric effectors, in parallel with (3) developmental changes in capacities for ion regulation, that interact and allow the crab to maintain a constant oxygen affinity of its blood as ontogeny progresses. Divalent cations are clearly significant effectors of crustacean hemocyanin oxygen-binding properties. The importance of the differences in hemolymph Mg^{2+} levels and in sensitivity to [Mg^{2+}] as an allosteric modifier are emphasized when we compare the results of oxygen-binding experiments on first-instar juvenile 25S hemocyanin and adult 25S hemocyanin in stage-specific salines. When functional studies were carried out on the two types of hemocyanin in identical salines, the affinity of the first-instar juvenile hemocyanin was 54 % lower than that of the adult (Fig. 5), consistent with earlier reports on purified hemocyanin. Half of this intrinsic difference in oxygen affinity at pH 7.8 between the two stages was eliminated when first-instar juvenile and adult hemocyanins were each tested in their own stage-specific saline. The most notable differences between the salines are the Ca^{2+} and Mg^{2+} concentrations.

Both urate and lactate increase the oxygen affinity of hemocyanin from a variety of crustaceans (Truchot, 1980; Graham *et al.* 1983; Mangum, 1983; Morris *et al.* 1985, 1986). We can rule out the influence of urate and L-lactate in these experiments, however. First, no urate was detected in any of the hemolymph samples. Second, the effects of L-lactate on the oxygen affinity of *C. magister* hemocyanin are pronounced (Graham *et al.* 1983), and circulating L-lactate levels can vary widely, depending on the activity of an individual crab (Booth *et al.* 1982; McDonough, 1990). We have accounted for L-lactate differences among samples, however, in order to compare the function of the hemolymph. It should be noted that there may be stage-specific differences in the capacity of *C. magister* to produce L-lactate. Sastry and Ellington (1978) reported that the larval stages of *Cancer irroratus* differ in the activity of lactate dehydrogenase, which may imply stage-specific differences in anaerobic capacity and L-lactate production.

The similarities in whole-hemolymph P_{50} are due in large part to the interplay between the lower-affinity hemocyanin and high Mg^{2+} levels of the juvenile *versus* the higher-affinity hemocyanin and lower Mg^{2+} levels of the adult crab. Additional stage-dependent factors in the hemolymph also contribute to equalizing the oxygen affinities of juvenile and adult hemolymph. We did not measure hemolymph concentrations of dopamine, other cardiac neuroamines or ammonium metabolites, and there have been suggestions of unidentified compounds in other crustacean hemolymphs that affect oxygen affinity (see Bridges *et al.* 1997; Lallier and Truchot, 1997).

The cooperativity of adult *C. magister* 25S hemocyanin was higher in adult saline than in juvenile saline. This may be due to independent or additive effects of higher concentrations of both Ca^{2+} (a specific divalent cation effect) and sodium chloride (an ionic strength effect) in adult saline. Ionic effects on cooperativity have been shown for other crustacean hemocyanins (for a review, see Mangum, 1997).

It has been known for some time that the oxygen-carrying capacity of crustacean hemolymph varies with the stage of the molt cycle (Drach, 1939), partly because of the increased water uptake just before ecdysis and partly because of a change in biosynthesis of hemocyanin (Mykles, 1980; Mangum *et al.* 1985; Spindler *et al.* 1992). The present study indicates that the oxygen-carrying capacity of *C. magister* hemolymph varies with developmental stage as well, increasing as the animal gets older. The low oxygen-carrying capacity of the hemolymph of juvenile crabs may impose constraints on oxygen transport and, therefore, on the activity of the crabs under certain conditions; this remains to be determined. The oxygen-carrying capacity calculated for the adult crab (1.75 ml O_2 dl^{-1}) is comparable to values reported by other researchers for adult *C. magister* (McMahon *et al.* 1978) and *Callinectes sapidus* (Booth *et al.* 1982).

In summary, the functional importance of the low-affinity hemocyanin in juvenile *C. magister* is to compensate for immature renal development and weak ion regulation during

early development. This study demonstrates that the inherent functional differences in the oxygen transport proteins of this crab are modulated by stage-specific constituents of the native hemolymph. There are additional unknown factors in the hemolymph of megalopas, first-instar juvenile, fifth-instar juvenile and adult crabs that shift the intrinsic affinities and cooperativities of the stage-specific hemocyanins so that these respiratory properties are indistinguishable in the whole hemolymph of these stages. Coordinated changes in ion regulation and hemocyanin during development maintain homeostasis of oxygen affinity.

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