

## ONTOGENY OF DECAPOD CRUSTACEAN HEMOCYANIN: EFFECTS OF TEMPERATURE AND NUTRITION

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### Summary

Hemocyanin is present throughout the decapod crustacean's life, usually as one-hexamer and two-hexamer oligomers. Hemocyanins of some decapod crustaceans undergo changes in subunit composition and oxygen affinity during development. Maternal hemocyanin is taken up from the hemolymph *via* endocytosis by the oocyte. Embryo hemocyanin differs in subunit composition from hemocyanin of oocyte and adult crab and may represent the onset of hemocyanin synthesis. Complex changes in expression of hemocyanin subunits occur through megalopa and early juvenile stages of the crab *Cancer magister*, culminating in the pattern of adult hemocyanin. The influences of food availability and temperature on development, growth and hemocyanin ontogeny in early juvenile *C. magister* have been studied. Crabs were raised in warm or cold sea water and fed high or low levels of food for 6 months. While intermolt period

was shorter in crabs fed high food levels, especially those raised in warm water, crabs reared in cold water with high food levels attained the largest sizes. Thus increased food availability affects growth more than increased temperature. Adult hemocyanin appeared at about the same number of weeks after the start of the experiment for crabs in the warm water/high food, warm water/low food and cold water/high food groups, even though warm water/low food crabs had molted fewer times. Crabs in the cold water/low food group expressed adult hemocyanin much later than the other groups. Molt stage and maturation from juvenile to adult are not absolutely coupled, and food availability has a greater influence than temperature on hemocyanin ontogeny.

Key words: crustacean, hemocyanin, oocyte, embryo, zoea, megalopa, temperature, nutrition, ontogeny, development

### Introduction

Hemocyanin gene expression changes during development of decapod crustaceans. The copper-containing respiratory protein is present throughout the life cycle of a decapod crab, but the kinds of hemocyanin polypeptides that self-assemble into one-hexamer (16S) and two-hexamer (25S) oligomers change, and the oxygen affinity of the hemocyanin also changes. In this publication, we will review changes in the crustacean hemocyanin molecule that occur with different developmental stages, from oocyte to adult crab. We will also examine the relative influences of food limitation and temperature on growth and hemocyanin ontogeny in juvenile crabs.

### Ontogenetic changes in decapod hemocyanin

#### *Oocyte hemocyanin*

We were interested in determining whether the crab oocyte contains hemocyanin, and if so, whether the hemocyanin is produced by the mother and deposited in the oocyte or is synthesized by the oocyte. Hemocyanin had been identified in ovarian tissue extracts of several crustaceans, including the fiddler crab *Uca pugilator* (Fielder et al., 1971), the mole crab

*Emerita analoga* (Gilchrist and Lee, 1972) and the crayfish *Astacus leptodactylus* (Durliat, 1984). Crustacean ovaries are highly vascularized, however, so it was possible that the hemocyanin was actually in the circulating hemolymph and not in the oocytes. We examined oocytes of three species of cancrivore crab *Cancer productus* (Wache et al., 1988), *C. magister* and *C. gracilis* (Terwilliger, 1991), and oocytes of a grapsid crab *Hemigrapsus nudus* (Terwilliger et al., 1983), under conditions designed to obviate contamination of the oocytes by maternal hemolymph. Hemocyanin was present in unfertilized, ripe oocytes of all species examined. Chromatography of ripe oocytes of each species on a BioGel A-5m column resulted in similar patterns, shown here for *Cancer magister* (Fig. 1A). Analysis of the peaks by polyacrylamide gel electrophoresis (PAGE) at pH 7.4 showed that both 25S two-hexamer and 16S one-hexamer hemocyanin molecules were present in chromatograph elution positions corresponding to those of adult 25S and 16S hemocyanin (Fig. 1B). The quaternary structures were confirmed by transmission electron microscopy. Hemocyanin subunit patterns are uniquely specific for each species of crab (for a review, see Van Holde and Miller, 1995). Within each species,

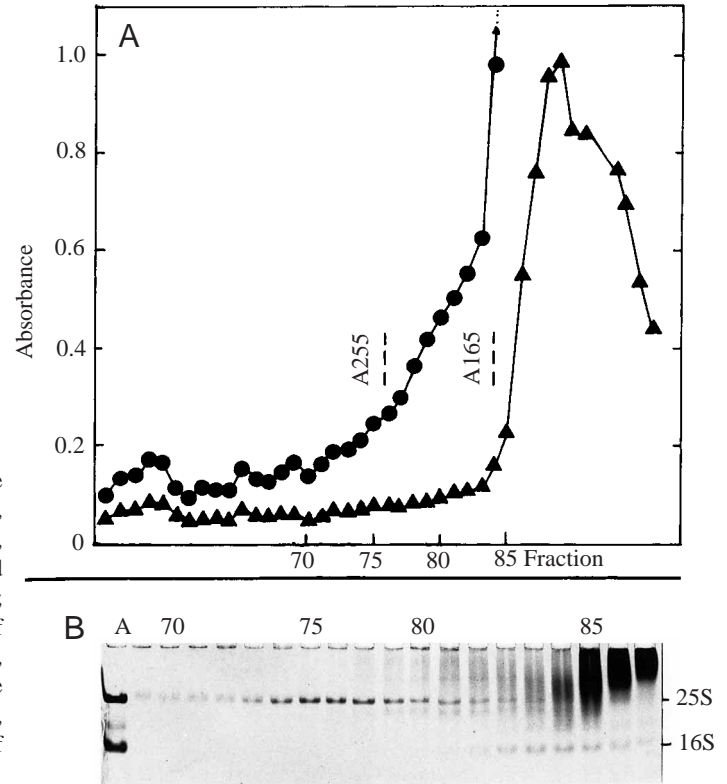


Fig. 1. (A) BioGel A-5m chromatography of *Cancer magister* oocyte supernatant. The buffer consisted of 0.1 M Tris-HCl, 0.1 M NaCl, 10 mmol $l^{-1}$  CaCl $_2$ , 50 mmol $l^{-1}$  MgCl $_2$ , pH 7.5. Column, 100 cm $\times$ 1.5 cm. Absorbance was measured at 280 nm (●) and 340 nm (▲). Calibrants: A25S, adult *C. magister* 25S hemocyanin; A16S, adult *C. magister* 16S hemocyanin. (B) pH 7.4, 5% PAGE, of fractions from A. Calibrant: lane A, adult *C. magister* hemolymph, containing 25S hemocyanin (upper band), cryptocyanin (middle band) and 16S hemocyanin (lower band). (From Terwilliger, N. B., 1991, *Crustacean Egg Production*, Plate 1, with permission of Balkema.)

we found that the oocyte hemocyanin subunits were indistinguishable from the corresponding maternal hemocyanin subunit pattern as identified by SDS-PAGE.

The amount of oocyte hemocyanin increased during the vitellogenic stage of oocyte development, a time when brachyuran crab oocytes are known to be engaged in active endocytosis of maternal vitellogenins from the hemolymph (Charniaux-Cotton, 1985). As the cancrroid and grapsid oocytes increased in volume and changed from small, pale cream oocytes to large, deeply pigmented ones filled with carotenolipoproteins, the concentration of hemocyanin increased also. When isolated *C. magister* oocytes were incubated in *C. productus* hemolymph and *vice versa*, the oocytes from each species took up the foreign hemocyanin (Wache et al., 1988). Thus, the evidence indicates that oocytes do contain hemocyanin, the hemocyanin is indistinguishable from maternal hemocyanin, and it can be obtained *via* endocytosis from maternal hemolymph.

#### Embryo hemocyanin

When a female crab spawns, the oocytes are fertilized as they move through the oviduct past the sperm storage sacs and out of the ovipores on the ventral thorax. The female scoops up the newly fertilized embryos with her abdominal pleopods, and the sticky embryos adhere to the hairlike setae on the pleopods. The embryos remain attached to the female until hatching as zoeae, but the embryos are external and separate from the female's circulatory system. Examination of developing embryos of the *Cancer* and *Hemigrapsus* species

by transmission electron microscopy and PAGE at pH 7.4 revealed that they contained one-hexamer and two-hexamer hemocyanin molecules, quaternary structures similar to those of adult and oocyte hemocyanin. The purified embryo hemocyanin contained several additional subunits, however, suggesting that the onset of hemocyanin synthesis begins in the embryo (Terwilliger et al., 1983; Wache et al., 1988; Terwilliger, 1991). The commencement of hemocyanin synthesis at this stage is consistent with many other systems of protein synthesis that are initiated after fertilization.

Embryos of other crustaceans also contain hemocyanin. Busselen had noted its presence in embryos of *Carcinus maenas*, *Eriocheir sinensis* and *Portunus holsatus* (Busselen, 1971), and hemocyanin was also present in embryos of *Emerita analoga* (Gilchrist and Lee, 1972) and *Astacus leptodactylus* (Durliat, 1984). The embryonic hemocyanin differed electrophoretically from the maternal hemocyanin subunit pattern, consistent with our findings on *C. productus*, *C. magister*, *C. gracilis* and *H. nudus*. The embryo protein may represent the earliest stage-specific expression of hemocyanin synthesis.

#### Zoea hemocyanin

Along the coast of Oregon, zoeae of *C. magister* are released during the winter months into the nearshore waters of the Pacific Ocean and progressively move offshore. During their oceanic phase, they molt through five zoeal stages before metamorphosing into a megalopa, beginning in April and May (Lough, 1976). Hemocyanins purified from the swimming

zoeal stages of *C. magister* and *H. nudus* have the one-hexamer and two-hexamer quaternary structures typical of hemocyanin from adult crabs, oocytes and embryos. Whether the subunit pattern is zoeal stage-specific has not been clearly determined. Zoea hemocyanin is capable of reversible O<sub>2</sub> binding, as shown in Fig. 2.

#### *Megalopa and juvenile hemocyanin*

As the decapod crab matures, its patterns of hemocyanin gene expression change. The ontogeny of hemocyanin structure and function from megalopa to adult crab has been studied most extensively in *C. magister*. The megalopa metamorphoses to a first-instar juvenile crab shortly after returning to nearshore and estuarine waters, where it continues to develop into a benthic juvenile and then an adult crab. Hemocyanin of both megalopa and early juvenile crabs consists of one-hexamer and two-hexamer fractions (Terwilliger and Terwilliger, 1982). The hexameric hemocyanin contains four different kinds of subunits, Cmag 1, 2, 4 and 5, while the two-hexamer hemocyanin contains these four plus an additional subunit, Cmag 3, that is probably involved in linking the two hexamers together. Initially, subunit Cmag 5 is present in greatest amount, but Cmag 4 gradually increases in concentration until it is the predominant subunit in the hemocyanin of older juveniles and adult crab.

#### *Adult hemocyanin*

The onset of adult hemocyanin synthesis usually occurs by the sixth juvenile instar. Its presence is indicated by the appearance of another unique polypeptide subunit, Cmag 6, in both one-hexamer and two-hexamer fractions of hemocyanin in the hemolymph (Terwilliger and Terwilliger, 1982). Thus, hemocyanin of the adult *C. magister* is composed of six different subunits, 1–6, in the two-hexamer and five subunits, 1, 2, 4, 5 and 6, in the one-hexamer. The appearance of hemocyanin containing Cmag 6 in the hemolymph is preceded by the presence of subunit 6 mRNA in hepatopancreas tissue (Durstewitz and Terwilliger, 1997b).

We have obtained the complete sequences of *C. magister* subunits 1 and 6 and partial sequences of subunits 3 and 4, as well as the N-terminal sequences of all six subunits (Durstewitz and Terwilliger, 1997a; N. B. Terwilliger and M. C. Ryan, unpublished data). Some subunits from other crustacean hemocyanins, including *Panulirus interruptus* (Bak, 1987) and *Penaeus vannamei* (Sellos et al., 1997), have also been sequenced. While the different sequences are closely related and show numerous highly conserved residues, especially at the active sites, they are unique gene products. The expression of hemocyanin phenotypic plasticity that occurs during development of *C. magister* appears to cease once adult hemocyanin appears (A. Adamczewska and N. B. Terwilliger, in preparation). The subunit composition of the adult hemocyanin is remarkably stable compared with adult hemocyanins from other species such as *Callinectes sapidus*, where synthesis of certain subunits seems to increase under environmental stressors such as hypoxia (Mangum, 1997).

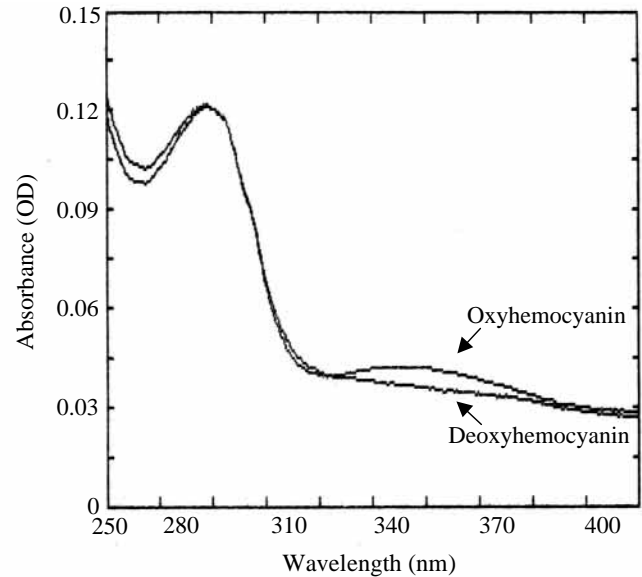


Fig. 2. Spectra of oxy- (upper curve) and deoxyhemocyanin from *Cancer magister* zoeae. Hemocyanin samples were purified by BioGel A-5m column chromatography, deoxygenated and reoxygenated tonometrically. Buffer as in Fig. 1.

#### *Hemocyanin function changes during development*

Megalopa and early juvenile hemocyanin of *C. magister* have an intrinsic O<sub>2</sub> affinity 50% lower than that of adult hemocyanin under identical experimental conditions (Terwilliger and Terwilliger, 1982). This lower O<sub>2</sub> affinity counterbalances the higher Mg<sup>2+</sup> levels found in the blood of the young crab. Mg<sup>2+</sup> is an allosteric effector that raises the O<sub>2</sub> affinity of the hemocyanin. Consequently, the O<sub>2</sub> affinity of whole blood of the juvenile crab is similar to that of the adult crab with its high affinity hemocyanin and low Mg<sup>2+</sup> levels (Brown et al., 1998; Terwilliger and Brown, 1993). The balance between onset of adult hemocyanin and the development of osmoregulatory capability as well as the potential role of Mg<sup>2+</sup> in regulating the ontogeny of adult hemocyanin are currently under investigation.

#### **Effects of nutrition and temperature on growth and hemocyanin ontogeny in juvenile crabs**

Hemocyanin gene expression clearly changes during crustacean development. What regulates this change in expression? The switch from juvenile to adult hemocyanin synthesis could be part of a tightly regulated developmental trajectory. Alternatively, the onset of synthesis of adult hemocyanin, in conjunction with growth of the juvenile crab, might be subject to seasonal environmental variables.

When the megalopas of *C. magister* return to the coast to metamorphose into juveniles, some remain in the nearshore waters while others enter the estuaries of the Pacific Northwest. Armstrong and Gunderson noted that estuarine juveniles were larger than their oceanic cohorts (Armstrong and Gunderson, 1985). They attributed this phenomenon to an increase in growth

due to warmer estuarine waters (Gunderson et al., 1990). Preliminary data from our laboratory indicated a strong effect of food levels on the rate of growth of *C. magister*. We wondered whether the increased growth of estuarine crabs could be due to enhanced food levels rather than increased temperature, or maybe a combination of both. Environmental variables including food availability, temperature and salinity have been shown to modify rates of development and growth in several species of decapod crustacean larvae (for a review, see Anger, 1998). Studies on free-living *C. magister* juveniles linked post-settlement growth to seasonal water temperature but did not examine variations in food level or diet (cf. McMillan et al., 1995). To better determine the factors that cause or affect growth and the developmental change in hemocyanin expression, juveniles of *C. magister* were reared from megalopas under controlled conditions in the laboratory. Salinity was held constant to isolate the relative effects of food availability and temperature on crab growth and hemocyanin ontogeny.

Megalopas were collected from the plankton by dipnet from the Coos Bay estuary and transported in cold sea water to running seawater aquaria at the Oregon Institute of Marine Biology. Within 24 h of collection, 60 *C. magister* megalopas were placed into four treatment groups, cold water/high food, cold water/low food, warm water/high food and warm water/low food. Crabs were housed individually in flowthrough containers in seawater tables maintained at average temperatures of either 14 °C or 21 °C, temperatures corresponding to measured summer estuarine water temperatures at the mouth of Coos Bay (14 °C) and at the head of the South Slough in Coos Bay (21 °C). The seawater tables had a continuous supply of running, unfiltered aerated sea water at a salinity of 30–33 ‰, pumped on an incoming tide from near the mouth of Coos Bay. The high-food crabs were fed fresh chopped mussel daily, while the low-food crabs were allowed to feed on the same diet for only 4 h every fourth day for the duration of the study.

The crabs were monitored for 6 months to determine the effects of food level and temperature on size, growth rate and hemocyanin ontogeny. Carapace width at each instar was measured with calipers to 0.1 mm, and growth rate was recorded as the number of days between successive molts. Hemocyanin concentration and the first appearance of adult hemocyanin were determined by analysis of hemolymph proteins. Hemolymph samples were subjected to non-dissociating, non-denaturing, pH 7.4 PAGE (Terwilliger and Terwilliger, 1982). Amounts of 25S and 16S hemocyanin in each sample were quantified using video image analysis software (JAVA, Jandel Scientific). Images of pH 7.4 gels were captured using identical camera settings and magnifications. Each protein band was outlined, the background subtracted and the area and average intensity measured. Average intensity units were calculated using the equation:  $(255 - \text{average intensity}) \times \text{area} / 10,000$ , which inverts the grayscale so that larger numbers equal high intensities. Hemolymph samples were also subjected to dissociating, denaturing SDS-PAGE (Laemmli, 1970) to analyze subunit

composition. Statistical analyses (normality, homogeneity of variances, multiple *t*-tests) were performed between food groups. Apparent differences between cold- and warm-treatment groups are discussed below, but statistical analyses were not performed because of potential pseudoreplication resulting from the necessary separation of temperature treatments into different seawater tables.

#### *Effects of food level and temperature on size and intermolt duration*

Crustacean growth appears incremental, since the external carapace increases in size only at ecdysis. During postmolt and intermolt, however, synthesis of muscle and internal organs proceeds at a high rate, and the premolt crab directs much metabolic activity towards formation of the new exoskeleton. The impact of food availability on growth is directly reflected in both carapace size-at-instar and intermolt duration.

During the first two instars, there was no appreciable difference in size among groups (Fig. 3). Crabs fed high food levels were significantly larger than those fed low food levels during third through fifth instar. Crabs raised in cold water were larger than those raised in warm water; the difference in carapace width was most dramatic after fourth instar.

Intermolt duration (number of days between successive molts) in crabs fed high food levels was significantly shorter than in crabs whose diets were limited (Fig. 4). Data are absent for the later instars in the cold water/low food and warm water/low food groups because the experiment was stopped after 6 months, before they had reached the next instar, not because mortality rates were high for these groups. Intermolt duration became progressively longer with each instar within each treatment group. Within the high food group, crabs in warm water had a shorter intermolt duration at a given instar than those in cold water. While one might expect warm water, a shorter molt cycle and ample food to result in larger crabs, that is not the case with this species. Larvae of *C. magister* also tended to grow larger in colder than in warmer water (Shirley et al., 1987; Sulkin and McKeen, 1994). Maximal growth of crustaceans probably occurs near a species-specific optimal range of temperatures. At temperatures of 9 and 18 °C, for example, the relationship between intermolt duration and instantaneous growth rate in *Carcinus maenas* zoea I changed, and overall growth was lower than at 12 °C (Anger, 1998; Dawirs et al., 1986).

Within the low food group of *C. magister*, no difference in intermolt duration with temperature was evident. Thus, with sufficient nutrition, the rate of molting was enhanced in warmer water. When food reserves were low, temperature had no effect.

#### *Effects of food level and temperature on hemocyanin concentrations*

Hemocyanin levels in the blood of *C. magister* rise during premolt, decrease just before ecdysis, and then quickly return to levels found in the previous instars, a pattern typical of decapod crustaceans (Terwilliger et al., 1999). For the 25S two hexamer hemocyanin, this molt cycle pattern was relatively unaffected by differences in temperature and food levels



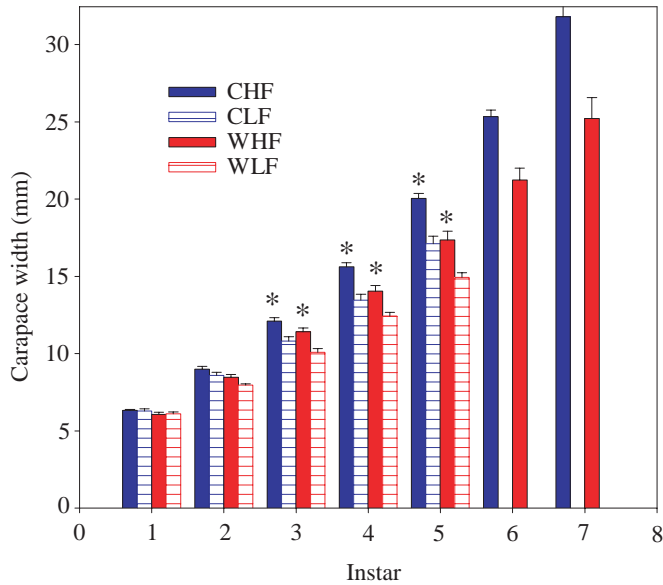


Fig. 3. Size (carapace width, in mm; mean + S.E.M.) of *Cancer magister* juveniles by instar for each treatment group (CHF: cold water/high food; CLF: cold water/low food; WHF: warm water/high food; WLF: warm water/low food). Numbers of surviving crabs at fourth instar were CHF (15), CLF (13), WHF (9) and WLF (11). The effect of food on size within each temperature group was determined for each instar (two-sample  $t$ -test or Mann–Whitney  $U$  statistic); asterisks indicate significant differences at  $P < 0.05$ .

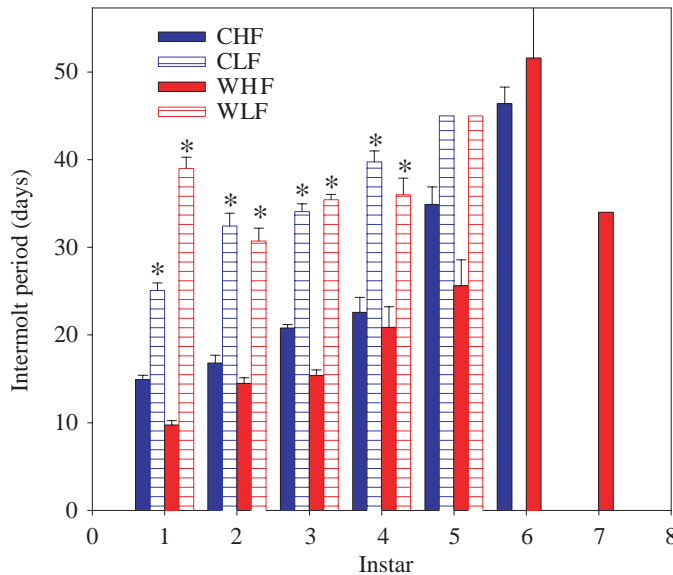


Fig. 4. Intermolt duration (days; mean + S.E.M.) of *Cancer magister* juveniles by instar for each treatment group (CHF: cold water/high food; CLF: cold water/low food; WHF: warm water/high food; WLF: warm water/low food). Numbers of surviving crabs at fourth instar were CHF (15), CLF (13), WHF (9) and WLF (11). The effect of food on intermolt length within each temperature group was determined for each instar (two-sample  $t$ -test or Mann–Whitney  $U$  statistic); asterisks indicate a significant difference at  $P < 0.05$ .

(Fig. 5). In contrast, levels of 16S one-hexamer hemocyanin were higher in response to increased temperature. The warm

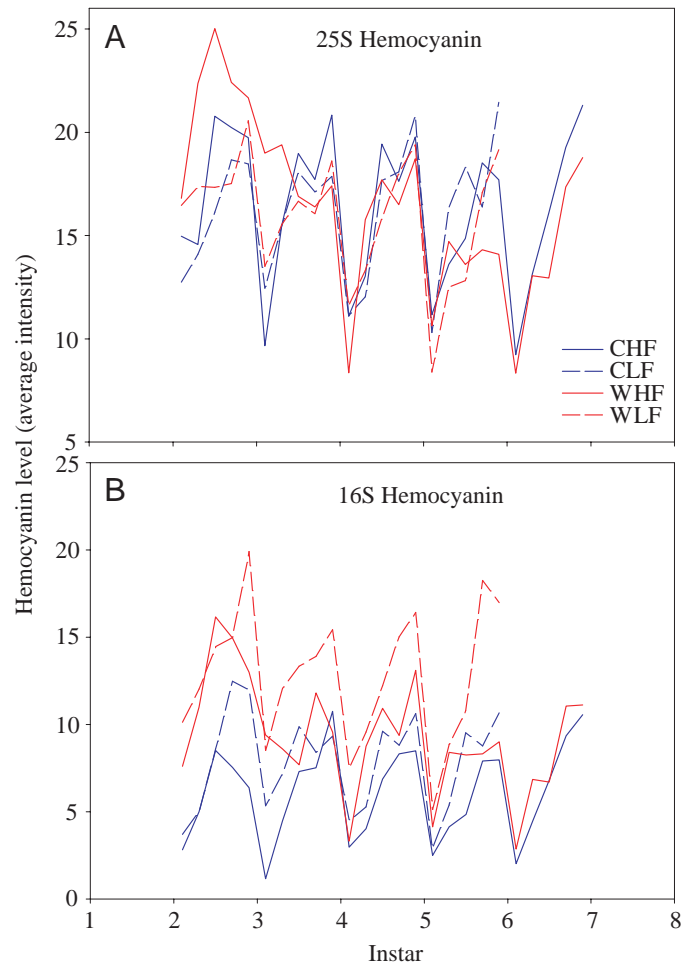


Fig. 5. Concentration expressed as mean average intensity (arbitrary units) for 25S two-hexamer hemocyanin (A) and 16S one-hexamer hemocyanin (B) of *Cancer magister* at each instar for each treatment group (CHF: cold water/high food; CLF: cold water/low food; WHF: warm water/high food; WLF: warm water/low food).

water/low food group had noticeably higher 16S hemocyanin levels than all three other groups.

These results on hemocyanin concentration changes in juvenile hemocyanin are interesting for several reasons. First, the increase in 16S one-hexamer hemocyanin level observed in juvenile *C. magister* raised under warm temperature conditions could possibly be an effect of hypoxic conditions resulting from the warmer water, similar to a hypoxic response seen in adult *Callinectes sapidus* hemocyanin (Mangum, 1997). There would be little change in  $O_2$  concentration between  $14^\circ\text{C}$  and  $21^\circ\text{C}$ , however; the difference would be from 150 mmHg down to about 130 mmHg (1 mmHg = 133.3 Pa). Furthermore, the response was strongest in juvenile *C. magister* subjected to both warm water and low food, suggesting that changes in the rate of synthesis of two-hexamer versus one-hexamer hemocyanin are a more general stress response.

A second point is that in adult *C. magister*, hemocyanin synthesis does not appear to respond to environmental change.

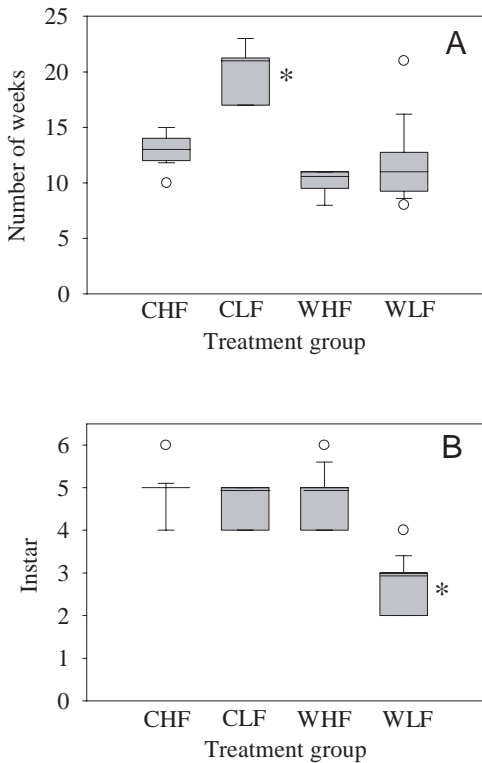


Fig. 6. Median onset of adult hemocyanin (dark bars on boxplots) (A) by the number of weeks from the initiation of the study and (B) by instar, in juvenile *Cancer magister* for each treatment group (CHF: cold water/high food; CLF: cold water/low food; WHF: warm water/high food; WLF: warm water/low food). Onset of adult hemocyanin was detected by appearance of subunit 6. Boxes encompass the 25<sup>th</sup> to 75<sup>th</sup> percentile and open circles denote single outliers. Comparison between food groups of adult hemocyanin onset, measured by number of elapsed weeks and by instar, were determined (Mann–Whitney *U* statistic); asterisks indicate a significant difference at  $P < 0.05$ .

The hemocyanin concentration and the ratio of two-hexamer to one-hexamer are unresponsive to hypoxic conditions (A. Adamczewska and N. B. Terwilliger, in preparation). Preliminary observations indicate that adult *C. magister* also shows no change in hemocyanin subunit composition or ratio of one-hexamer to two-hexamer molecules in response to variable salinity, temperature or food levels (N. Terwilliger, personal communication).

Juvenile *C. magister* hemocyanin is phenotypically plastic, responding both to intrinsic ontogenetic stimuli and to extrinsic environmental changes. The response of juvenile hemocyanin to hypoxia has not yet been determined. In contrast, adult *C. magister* hemocyanin is less plastic, at least under the conditions that have been experimentally tested. It may be that the opportunity for phenotypic plasticity is available only through the young instar stages. This pattern is different from species such as *Callinectes sapidus*, where phenotypic change persists in the adult crab; a change in the ratio of two-hexamer to one-hexamer hemocyanin has been observed in response to hypoxia (Mangum, 1997). The one-hexamer hemocyanin has a higher O<sub>2</sub> affinity than does the two-hexamer fraction, and the one-hexamer hemocyanin concentration in the hemolymph increases when adult *C. sapidus* is exposed to hypoxia.

*Effects of food level and temperature on onset of adult hemocyanin*

The combined effects of food level and temperature on hemocyanin synthesis demonstrate that hemocyanin ontogeny is not coupled to molt stage. The appearance of adult hemocyanin, defined by the presence of subunit 6 in the hemocyanin, occurred at about the same number of weeks from the beginning of the study in both warm-water groups, high food and low food, and only slightly later for the cold water/high food crabs (Fig. 6A). It is important to note that the onset occurred at a considerably more immature instar in the

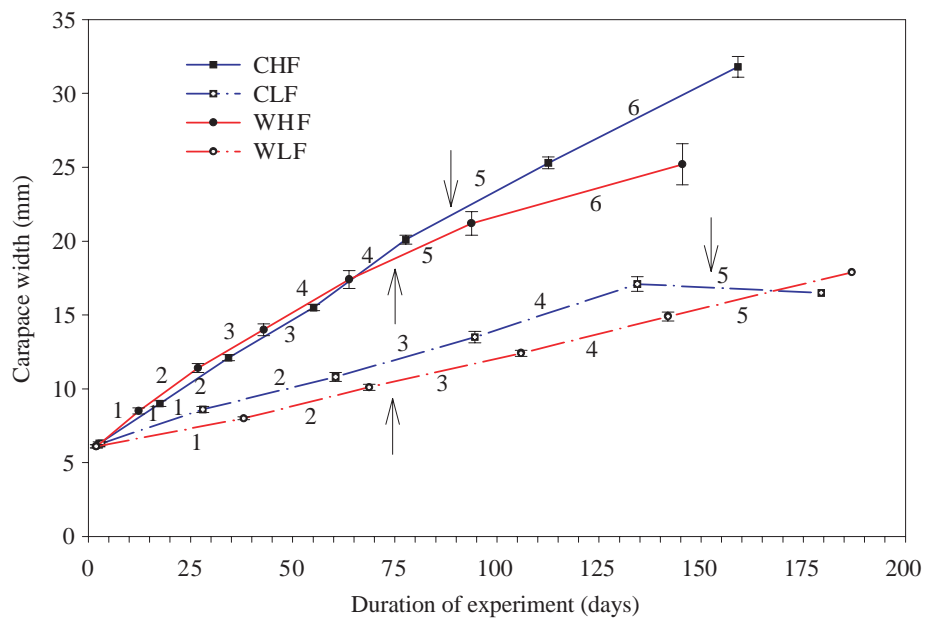


Fig. 7. Size (carapace width, in mm; mean  $\pm$  S.E.M.) at mean cumulative number of days to each instar from the initiation of the study for each *Cancer magister* treatment group (CHF: cold water/high food; CLF: cold water/low food; WHF: warm water/high food; WLF: warm water/low food) by instar. Instars are denoted by numbers between data points. The arrows indicate mean onset of adult hemocyanin.

warm water/low food group than in the other two groups because of the depressed rate of molting in that group (Fig. 6B).

The appearance of subunit 6 was notably delayed by about 7 weeks in the cold water/low food group, although the crabs were at the same fifth-instar stage of development as the warm water/high food and cold water/high food groups (Fig. 6). The cold water/low food crabs were probably in a state of temperature- and low-food-induced metabolic depression (Whiteley et al., 1997), as shown by their slower rate of molting, and had a lowered O<sub>2</sub> demand. Conversely, the warm water/low food crabs would have experienced an increased O<sub>2</sub> demand because of an increase in metabolic rate. Despite limited nutritional resources, early appearance of adult hemocyanin would provide them with a higher-affinity hemocyanin.

### Conclusions

Increased food availability rather than elevated temperatures may confer a competitive advantage to estuarine *C. magister* juveniles over their coastal conspecifics, as summarized in Fig. 7. Juvenile crabs fed high food levels grew larger and faster than those fed low food levels. *C. magister* juveniles raised in cold water attained larger sizes, even though their molting rate was slower than crabs raised in warm water.

Levels of two-hexamer hemocyanin were similar for each group, while levels of one-hexamer hemocyanin were higher in juveniles fed low food and in those raised in warm water. Juvenile *C. magister* may respond to sublethal stress by synthesizing more one-hexamer hemocyanin and by accelerating onset of adult hemocyanin with a higher O<sub>2</sub> affinity. Recent studies have attributed a number of functions, in addition to O<sub>2</sub> transport, to hemocyanin. These include heavy metal detoxification (Martin and Rainbow, 1998; Rtal et al., 1996), phenoloxidase activity and sclerotization (Decker and Terwilliger, 2000) and stress response. It is worth considering that the two hemocyanin oligomers may serve different roles. Perhaps the two-hexamer hemocyanin fraction of decapod crustaceans functions primarily in O<sub>2</sub> transport while the one-hexamer hemocyanin fraction participates in multiple functions, including O<sub>2</sub> transport, and regulation of its biosynthesis is more sensitive to physiological and environmental change.

Finally, the onset of adult hemocyanin was found to be neither stage-specific nor time-dependent. This indicates that molting and hemocyanin ontogeny are not strictly coupled but respond differently to external environmental stressors.

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