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Ontogeny of Crustacean Respiratory Proteins¹

Nora B. Terwilliger^{2,1} and Margaret Ryan¹

¹Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon 97420

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▶ SYNOPSIS

The respiratory proteins hemoglobin and hemocyanin share the function of oxygen transport, but the proteins belong to separate gene families, and their active sites and the metal ions that bind the oxygen differ. Either hemoglobin or hemocyanin, but not both, is expressed in the hemolymph of many arthropod crustaceans. Hemoglobin is present in Branchiopoda, Ostracoda, Copepoda, rhizocephalan Cirripedia and one suborder of amphipodan Malacostraca, while hemocyanin has been described in Malacostraca. Recent work by several laboratories have provided new information on the gene structure, exon-intron patterns, site of synthesis and expression of hemoglobins in the branchiopods *Artemia* and *Daphnia*. These studies suggest the branchiopods are excellent model organisms for studies of oxygen sensors and hypoxia inducible transcription factors during developmental and adult stages. The focus in our laboratory on the ontogeny of hemocyanin in the Dungeness crab, *Cancer magister*, has demonstrated that both structure and function of hemocyanin change from megalopa to adult crab. The hemocyanin of an oceanic megalopa contains four subunits. Another subunit appears about the time of metamorphosis to first juvenile instar, and expression of a sixth subunit begins four or five molts later. The timing of onset of adult hemocyanin can be altered experimentally by food levels and temperature. Gene expression and functional properties of both red and blue oxygen transport proteins of crustaceans change during ontogeny to insure oxygen delivery appropriate for each developmental stage.

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▶ INTRODUCTION

As an organism develops, it must obtain sufficient oxygen from the environment to fuel its changing metabolic needs for growth and survival. With an increase in size from embryo to larva to juvenile, the surface to volume ratio decreases, simple diffusion of oxygen becomes limited, and oxygen transport becomes progressively important. Ontogenetic requirements for oxygen are supplied in part by circulating oxygen transport proteins that change in structure and function as the organism changes.

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Among the Arthropoda, a spectrum of oxygen transport proteins is expressed (Terwilliger, 1998¹). Hemocyanin is the only respiratory protein available to the Chelicerata and the Myriapoda, while in the Crustacea, either hemocyanin or hemoglobin occurs. No respiratory protein is found in most of the Insecta. A few insects express hemoglobin, especially in larval aquatic stages. Certain blood-sucking insects produce hemoglobin-containing oocytes but do not synthesize the hemoglobin themselves (Wigglesworth, 1943²). One grasshopper embryo has a protein that closely resembles hemocyanin in sequence (Sánchez *et al.*, 1998³), but no oxygen binding studies to confirm its function have yet been done. The fact that genes for both hemocyanin and hemoglobin are present in the Arthropoda and in the Mollusca, the only other phylum in which a copper-oxygen transport hemocyanin is found, is noteworthy.

The differences between hemoglobins and hemocyanins are marked. Hemoglobins are composed of multiple polypeptide chains or subunits called globins (Terwilliger, 1992, 1998¹). Usually there is one heme with one Fe per 18 kDa protein; this combination is referred to as a functional unit or a monodomain subunit, and it binds reversibly with one oxygen molecule. Sometimes a hemoglobin subunit contains two or more covalently linked globin units, each with a heme group. These didomain or multidomain subunits are probably the result of ancestral gene duplications. Each globin unit within the polypeptide chain has a heme group and therefore multiple oxygen-binding sites per subunit are expressed. Multidomain hemoglobin subunits are especially prevalent in the Arthropoda. Hemoglobin subunits, whether monodomain or multidomain, self assemble into dimers, tetramers or huge aggregates. Some hemoglobins are retained in the cell of synthesis that circulates in the vascular system (erythrocytes) or the coelomic fluid (coelomocytes). Others are released as extracellular proteins into the vascular system.

Hemocyanins are also composed of subunits, but the amino acid sequences do not resemble those of globins (Van Holde and Miller, 1995⁴). Hemocyanins contain neither heme nor iron, but rather covalently bind two copper atoms at specific

and Kamei, 1992). Hemocyanins contain neither heme nor iron, but rather covalently bind two copper atoms at specific sites per subunit. Arthropod hemocyanins are always monodomain and contain one oxygen-binding site (two copper atoms) per subunit. The arthropod hemocyanin subunit is about five times as large as a monodomain globin subunit, and the subunits self assemble into hexamers and multiples of hexamers. Hemocyanins are extracellular in the vascular system.

Arthropod hemoglobins and hemocyanins differ from one another, therefore, in size, shape, sequence and metal ligand. They are products of different gene families, but they do share a common function of oxygen transport. Both arthropod proteins also undergo ontogenetic changes in expression, structural changes that result in adjustments in function as developmental demands change.

▶ DISTRIBUTION OF CRUSTACEAN OXYGEN TRANSPORT PROTEINS

The focus of this paper is on the Crustacea, the class of Arthropoda that expresses both hemoglobin and hemocyanin. An overview of the distribution and structures of crustacean oxygen transport proteins will enhance an appreciation of the sometimes perplexing patterns of expression of these proteins.

Hemoglobins have been described in a select number of crustacean groups, including the Branchiopoda, Ostracoda, Copepoda, Cirrhipedia, and Decapoda (Terwilliger, 1992). All Branchiopoda express hemoglobin, and the hemoglobins are assembled from either didomain or multidomain subunits. The more primitive of the Branchiopoda, the Anostraca (brine shrimp, *Artemia*) have a hemoglobin subunit made of nine covalently linked globin units, each with one heme (Manning *et al.*, 1990). The circulating molecule, 320 kDa, contains two of these giant subunits. The three other groups of Branchiopoda, the Notostraca (tadpole shrimp, *Lepidurus*), the Conchostraca (clam shrimp, *Caenestheriella setosa*), and the Cladocera (*Daphnia*) each have a hemoglobin assembled from 32 kDa didomain subunits. The sizes of the circulating hemoglobins vary from 220 to 800 kDa among the groups, with oligomers assembled from different numbers of didomain subunits.

Ostracod hemoglobin has not been well characterized, although its presence was demonstrated by Fox (1948). Information on ostracod hemoglobin would be useful for several reasons, including a better understanding of the phylogenies of crustaceans and hemoglobin.

Fox also noted a hemoglobin in Copepoda (Fox, 1948). Few details were known about copepod hemoglobin until recently, when the structure of hemoglobin from *Benthoxymus spiculifer*, a copepod from a hydrothermal vent habitat, was described (Hourdez *et al.*, 2000). In contrast to Branchiopoda hemoglobins, that of *B. spiculifer* has only a monodomain subunit, although it assembles to a 208 kDa oligomer.

Most Cirrhipedia do not contain an oxygen transport protein. Even the giant barnacle, *Balanus nubilus*, is apparently able to obtain sufficient oxygen for its tissues without the assistance of a circulating hemoglobin or hemocyanin. Only the parasitic rhizocephalan barnacles are known to contain hemoglobin. *Briarosaccus callosus*, a rhizocephalan that parasitizes several species of king crab, contains copious amounts of an extracellular hemoglobin (Shirley *et al.*, 1986). The hemoglobin has a single domain subunit and assembles to a giant oligomer of 1000–4000 kDa (Terwilliger *et al.*, 1986).

Cyamid amphipods, obligate ectocommensals on the surface of whales, are the only described examples within the malacostracan Decapoda that contain hemoglobin (Terwilliger *et al.*, 1986). The extracellular hemoglobin found in *Cyamus scammoni* is a giant oligomer of 1800 kDa; the smallest subunit is a ten-domain globin (Terwilliger, 1991a). The cyamids and the hemoglobin are present in concentrations high enough to be seen from a distance as red patches on the surface of the gray whale, *Eschrichtius robustus*.

Hemoglobin expression in the crustaceans reflects many successful evolutionary experiments. The subunits range from mono to di to multidomain, and the circulating molecules are all giant, extracellular assemblies of the subunits.

Among the Crustacea, hemocyanin has been described only in the malacostracan Decapoda, a group including many of the larger crabs, shrimps, and lobsters, as well as smaller peracaridans such as isopods and most amphipods. Crustacean hemocyanins are always composed of monodomain subunits and are found in the hemolymph as multiples of six subunits. The size of the hexameric assemblage is species specific, ranging from single hexamers in shrimp and some peracaridans to two and four hexamers in brachyurans and thalassinids.

In this paper, recent findings about patterns of ontogeny and synthesis of two branchiopod hemoglobins, one in the anostracan *Artemia* and the other in the cladoceran *Daphnia*, and experimental results testing the regulation of onset of adult hemocyanin in the malacostracan *Cancer magister* will be described. These will illustrate how gene expression and functional properties of both red and blue oxygen transport proteins of crustaceans change during ontogeny to insure stage-specific oxygen delivery.

▶ ARTEMIA HEMOGLOBIN

When is hemoglobin first expressed in *Artemia*, and does it change during development? The presence in *Artemia* of three circulating hemoglobins, Hb I, Hb II and Hb III, having different oxygen affinities has been known since the 1970s (D'Hondt *et al.*, 1978). Studies on the ontogeny of *Artemia* hemoglobin were carried out by Heip *et al.* (1978a). They found that the two-hour post hatch nauplius contained Hb II, with a moderate oxygen affinity of 3.7 mm Hg at pH 8.5, 25°C. The higher affinity Hb III, with a P₅₀ of 1.8 mm Hg, appeared in the 8-hr nauplius, and the lower affinity Hb I, P₅₀ = 5.3, was present 7–8 days after hatching, a time coinciding with the appearance of functional gills. For the next 20 days, all three hemoglobins were present, then Hb III disappeared by 30–40 days post hatch. The effect of salinity on the pattern of hemoglobin concentration or composition during ontogeny was tested, but a 20-fold

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effect of salinity on the pattern of hemoglobin concentration or composition during ontogeny was tested, but a 20-fold variation in salinity had no effect (Heip *et al.*, 1978b).

When adult *Artemia* was cultured under hypoxic conditions, Hb III reappeared after 4 days, and the concentrations of all three hemoglobins increased (Heip *et al.*, 1978b). When the oxygen levels were returned to normal levels, Hb III gradually decreased as it had in the nauplius and disappeared after 10 days. These results were exciting, because they demonstrated first, that *Artemia* hemoglobin is inducible, and second, that Hb III is the most sensitive to oxygen levels. *Artemia* hemoglobin thus becomes an important model for studies on the mechanism and regulation of globin gene expression.

More recently, the effects of oxygen levels on hemoglobin ontogeny in *A. franciscana* were examined by raising nauplii at 28°C in normoxia or hypoxia (Spicer and El-Gamal, 1999). The authors found an increase in concentration of hemoglobin in nauplii during chronic hypoxia, similar to the studies described above on adult *Artemia*, and they saw a decrease in oxygen affinity during normoxic development (Fig. 1). This is consistent with loss of the high affinity Hb III 30 days after hatching in *Artemia* (Heip *et al.*, 1978a). Interestingly, those nauplii raised in hypoxic conditions did not show a change in oxygen affinity. Although Spicer and El Gamal did not examine the hemoglobin species present, a reasonable explanation is that the hypoxic nauplii continued to produce Hb III and therefore their blood maintained the same high affinity as early nauplii.

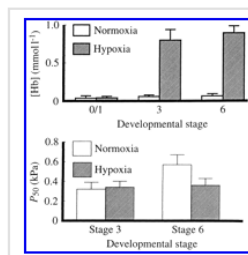


FIG. 1. Hemoglobin ontogeny in *Artemia franciscana* cultured under normoxia or chronic hypoxia. Hemoglobin concentration in the hemolymph (upper graph) and P₅₀ values for pooled hemoglobin samples (lower graph). (From Spicer and El Gamal [1999], Figs. 5 and 6.)

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How do the gene structures of *Artemia* hemoglobin agree with information on the ontogeny of hemoglobins of *Artemia*? The hemoglobin of *Artemia* is made up of two different subunits, C and T. These assemble into two homodimers, CC and TT, equivalent to the two circulating hemoglobins, Hb I and III, and a heterodimer, CT, corresponding to Hb II. As described above, each subunit is a continuous chain of nine globin domains. Two of the domains were sequenced at the amino acid level (Moens *et al.*, 1988, 1990). The sequence of the complete cDNA of the first chain was presented in 1990 (Manning *et al.*, 1990) and confirmed the multidomain nature of the subunit. This was followed by the cDNA sequence of the second subunit (Jellie *et al.*, 1996). The gene structures of these large hemoglobins have also been determined, which has allowed analysis of the exon and intron locations in the multidomain gene (Jellie *et al.*, 1996; Trotman *et al.*, 1994). The domains are 17–35% identical at the amino acid level. This relatively low level of identity led to the suggestion that the domains probably evolved from a monomeric globin by an ancient series of gene duplication events (Jellie *et al.*, 1996). In contrast, the two multidomain subunits, T and C, are 88% identical at the amino acid level. Thus the second subunit probably has resulted from a duplication of the entire 9-domain gene much more recently. A scenario for the evolution of the two, nine-domain subunits has been proposed (Fig. 2) (Matthews and Trotman, 1998).

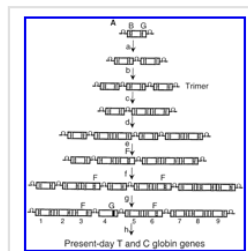


FIG. 2. A scenario for the evolution of the two, nine-domain subunits of *Artemia* hemoglobin. A, the ancestral single-domain globin gene. The open box represents the globin domain and positions of intradomain introns are indicated by smaller shaded boxes (helix position indicated above); potential interdomain introns are represented by loops. a, b, Domain duplication events, facilitated by the presence of interdomain (linker) introns. c, Loss of a linker intron. d, Duplication of the trimer. e, Linker intron loss and movement of the G helix intron in the third domain to an F helix position. f, Duplication of the amino-terminal three domains to yield a nine-domain globin gene with two domains containing an F helix intron. A single base movement of one of the F helix introns. g, Loss and gain of an intron in the G helix of the fourth domain and then loss of the B helix intron in the same domain. Movement (by loss/gain) of the amino terminal intron and the linker intron between domain 6 and domain 7. h, Duplication of the entire nine-domain gene to produce two genes for T and C polymers. (Modified from Matthews and Trotman [1998], Fig. 2.)

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The concentration of all *Artemia* hemoglobins, I, II and III increases in response to hypoxia. Only the homodimer TT (Hb III), however, is specifically induced or repressed in response to oxygen levels. The combination of information on functional properties, chain identification and gene sequence makes the hemoglobin of *Artemia* a potent system for future studies.

► DAPHNIA HEMOGLOBIN

Hemoglobins of cladocerans, like those of anostracans, are sensitive to oxygen levels and show a drastic increase in concentration in response to a decrease in ambient oxygen. Adult *Daphnia* raised under hypoxic conditions are red, and the hemoglobin in red *Daphnia* has a higher oxygen affinity than hemoglobin from white *Daphnia* raised under normoxic conditions (Trotman *et al.*, 1996; El-Gamal and

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from pale *Daphnia* raised under normoxia (Goslin *et al.*, 1967), Kobayashi and Tanaka, 1991). Both concentration and oxygen affinity change in response to oxygen levels.

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Cladocerans are considered to be more advanced branchiopods than the anostracans like *Artemia*, and they have no free-swimming larval stage. Embryos are brooded in the mother's carapace and hatch out as miniature *Daphnia*, rather than as nauplii. Hemoglobin is present in embryos in the brood pouch (Fox, 1948); its concentration is the same as or higher than in maternal hemolymph, as evidenced by the fact that red *Daphnia* have red embryos and pale *Daphnia* have pale embryos (Kobayashi and Takahashi, 1994). To date, there is no evidence for *de novo* synthesis of hemoglobin in the embryo, and the oxygen affinities are apparently the same in mother and embryo. The females transfer hemoglobin before and/or during egg laying.

When and why the *Daphnia* hatching begins to synthesize new hemoglobin is not known, nor is it known whether there is an ontogenetic switch from a juvenile to an adult hemoglobin. Two current areas of research promise to enhance developmental studies on cladoceran hemoglobins, however. The first includes new information on the structure of hemoglobin from adult *Daphnia* at the protein, mRNA and gene level. Previous studies had indicated the circulating hemoglobin was composed of twelve to sixteen subunits, with each subunit containing two globin domains (Dangott and Terwilliger, 1980; Ilan *et al.*, 1982). The size of the quaternary assembly is twice that of *Artemia* hemoglobin. Recently six major subunit types, all didomains, have been identified in *D. magna* (Kimura *et al.*, 1999; Tokishita *et al.*, 1997). The same six subunits are present in hypoxia and normoxia, and all six chains are upregulated by hypoxia. Significantly, the concentrations of some chains increase more than others: three of the subunits show a 15–20 fold increase, while three other subunits only show a 5–10 fold increase. Again, differential regulation of expression of hemoglobin subunits is occurring in the Branchiopoda.

The complete nucleotide sequences of three of the four identified hemoglobin genes and the cDNAs of *D. magna* have been sequenced (Kimura *et al.*, 1999), as have one hemoglobin gene and the cDNA of *D. pulex* (Dewilde *et al.*, 1999). The structure of all four subunits is similar; a unique secretory signal, then two globin domains connected by a linker homologous to those seen in other nonvertebrate globin chains (Dewilde *et al.*, 1999). Each gene has a seven-exon, six-intron structure (Dewilde *et al.*, 1999; Kimura *et al.*, 1999). Within each globin domain, the exon/intron pattern is similar to that of a domain of *Artemia* hemoglobin. Analysis of the intron sequences suggests that the mechanism of gene duplication that led to a didomain globin in *D. pulex* is more similar to that of the didomain globin of *Barbatia reeveana*, a mollusc, than that of the multidomain globin of *Artemia* (Dewilde *et al.*, 1999).

Details of the gene sequence provide important links with oxygen responsive genes found in many other organisms. The four hemoglobin genes of *D. magna* are clustered on the same chromosome (Fig. 3) (Kimura *et al.*, 1999). Multiple HIF-1 binding motifs are found in the intergenic regions of the hemoglobin gene cluster. HIF-1 is a trans-acting factor important in hypoxic induction of several physiologically important genes in mammalian cells (Bunn and Poyton, 1996). The *Daphnia* hemoglobin gene and its differential up-regulation in response to hypoxia is an excellent model to study oxygen sensors, oxygen responsive genes and the mechanisms involved with their regulation.

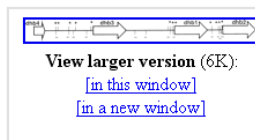
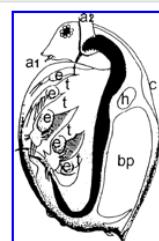


FIG. 3. Organization of cluster and structures of the hemoglobin genes of *Daphnia magna*. Open arrows indicate the positions and directions of the hemoglobin genes. Vertical lines indicate sites of HIF-1 binding motifs (marked + or -, depending on the strand they are located on) in the regions between the genes. (From Kimura *et al.* [1999], Fig. 5A.)

The second recent discovery enhancing studies of hemoglobin ontogeny in *Daphnia* is identification of the sites of hemoglobin synthesis. Using the cDNA sequence of *Daphnia* hemoglobin (Tokishita *et al.*, 1997), Goldmann *et al.* (1999) localized the sites of hemoglobin mRNA and therefore hemoglobin synthesis in adult *D. magna* by *in situ* hybridization. They found two sites of hybridization. One site involved "fat cells" located near the gut and in the thoracopodites. These cells have previously been implicated in the synthesis of glycogen and fat to be delivered to the developing oocytes via the hemolymph (Jager, 1935). The other site of hemoglobin mRNA was in the epithelial cells of the epipodites, small balloon shaped structures on the base of the thoracic appendages (Fig. 4), that were originally thought to function as gills based on their location and the fact that they were filled with red hemolymph. The epithelium of the epipodites is 4–5x thicker than the rest of the limb epithelia, however, a design criterion that would diminish their usefulness as a gas exchange region (Kikuchi, 1983). The ultrastructure and chloride staining of one of the two cell types in the epipodite epithelium indicate it functions in osmoregulation (Kikuchi, 1983). Perhaps the other cell type is responsible for hemoglobin synthesis, but the cell identification has not yet been resolved. Interestingly, in another branchiopod, *Artemia*, immunoreactive hemoglobin has been localized in surface tissues of embryos and 16 hr nauplii (Trotman, 1986). The site of hemoglobin synthesis in *Artemia* is yet to be determined.



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FIG. 4. Lateral view of *Daphnia magna* showing essential features of the anatomy. E, epipodite, t, thoracopodite, a1, first antenna, a2, second antenna, h, heart, c, carapace, bp, brood pouch. (Modified from Goldmann *et al.* [1999], Fig. 2A.)

Daphnia should surely be a target for studies on hypoxia-induced gene control, especially since the site of synthesis has been localized. This new information will also advance our knowledge about the ontogeny of hemoglobin in cladocerans.

▶ **CANCER MAGISTER HEMOCYANIN**

The ontogeny of crustacean hemocyanin, the copper-based oxygen transport protein, has been studied in relatively few species, including *Nephrops norvegicus* (Spicer, 1995) and *Homarus americanus* (Olson, 1991). The most thoroughly studied system is the ontogeny of hemocyanin structure and function in *Cancer magister*, the Dungeness crab.

Hemocyanin is present in oocytes, embryos and zoeae of a number of decapod crustaceans. In our lab, we have examined these stages in *Cancer magister*, *C. productus*, *C. gracilis*, and in the grassid crab, *Hemigrapsus nudus*. As previously reported, oocyte Hc appears to be obtained from maternal hemolymph, whereas embryo hemocyanin has a unique subunit composition and probably reflects the earliest expression of hemocyanin in these crab species (Terwilliger, 1991b; Terwilliger and Dumler, 2001). Figure 5 summarizes how hemocyanin gene expression changes during development of *C. magister* from megalopa to adult. It is important to note that both two-hexamer and one-hexamer hemocyanin are present in all stages of *C. magister*, oocyte to adult, and furthermore, there is always one extra subunit type in the two-hexamer oligomer. This subunit, C mag 3, is probably a linker subunit involved in the assembly of the two-hexamer.

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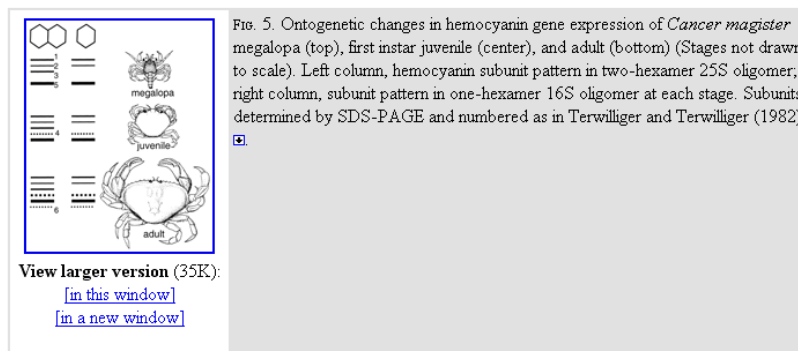


Fig. 5. Ontogenetic changes in hemocyanin gene expression of *Cancer magister* megalopa (top), first instar juvenile (center), and adult (bottom) (Stages not drawn to scale). Left column, hemocyanin subunit pattern in two-hexamer 25S oligomer; right column, subunit pattern in one-hexamer 16S oligomer at each stage. Subunits determined by SDS-PAGE and numbered as in Terwilliger and Terwilliger (1982).

A change in hemocyanin expression occurs during the megalopa phase of development. In Oregon, zoeae of *C. magister* metamorphose to megalopas during their oceanic phase, up to 200 miles off the coastline. The megalopas return in large swarms to the nearshore waters and estuaries in the spring, where they metamorphose into first instar juvenile crabs. We recently found that oceanic megalopas that were caught offshore before they migrated back to the estuaries and nearshore waters had a hemocyanin that lacked two of the subunits, 4 and 6, seen in adult hemocyanin (Fig. 5). The hemocyanin subunits still formed two hexamer (subunits 1, 2, 3, 5) and one hexamer (subunits 1, 2, 5) oligomers, however. Newly metamorphosed first instar juveniles from this period also lacked the two subunit types, 4 and 6. By 3 days post metamorphosis, subunit 4 began to appear and gradually increased in concentration. Megalopas that complete their oceanic return to the estuary usually arrive with subunit 4 and the pattern typical of early juvenile crabs: two-hexamer (subunits 1, 2, 3, 4, 5) and one-hexamer (subunits 1, 2, 4, 5) (Terwilliger and Terwilliger, 1982). Perhaps the addition of subunit 4 during the megalopa stage is a reflection of duration since zoea stage, and a sign of delayed metamorphosis due to ocean currents or wind that prolonged onshore movements of the megalopas. Whether this change in expression during the megalopa stage is caused by an endogenous factor or triggered by a difference in an external factor such as salinity from the plume of the estuary is not known.

As the early juvenile crab molts and grows, subunit 4 gradually increases in concentration until it is greater than that of subunit 5. Adult hemocyanin is defined by the appearance of subunit 6. Under controlled conditions of temperature and diet, this usually occurs by the 6th juvenile instar. We have determined the cDNA sequence of subunit 6 (Durstewitz and Terwilliger, 1997). The expression of subunit 6 mRNA becomes detectable as subunit 6 begins to appear in circulating hemocyanin of the young crabs.

The structural changes in expression of hemocyanin in *C. magister* are accompanied by functional changes. Hemocyanin of the estuarine megalopa and early juvenile crab has an intrinsic oxygen affinity 50% lower than that of adult crab (Terwilliger and Brown, 1993). Hemolymph of the juvenile crab has higher Mg^{2+} levels than adult crab (Brown and Terwilliger, 1992). This is important, because Mg^{2+} is an allosteric effector that causes an increase in hemocyanin oxygen affinity in *C. magister*. The effect of high Mg^{2+} raising the juvenile hemocyanin oxygen affinity is counterbalanced by the low intrinsic oxygen affinity of the juvenile hemocyanin. This combination of effects results in a juvenile hemolymph P_{50} that is indistinguishable from that of the adult hemolymph (Brown and Terwilliger, 1998). As subunit 6 appears and as the ratio of subunits 4 and 5 change in the hemolymph of the juvenile crab, the oxygen affinity of the hemocyanin increases. This occurs at the same time that the crab becomes better able to regulate its hemolymph Mg^{2+} to lower levels. The intrinsic oxygen affinity of the hemocyanin changes in concert with changes in ion regulation. We see both a conservation of P_{50} *in vivo* during development from juvenile to adult and an example of enantiostasis between hemocyanin oxygen affinity and ion regulation.

Is the timing of hemocyanin ontogeny tightly fixed to a precise developmental stage or can regulation of the shift in gene expression during development be altered by environmental conditions? We examined the effects of temperature and food level on timing of hemocyanin expression and growth in *C. magister* (Terwilliger and Dumler, 2001). A cohort of megalopas caught in the estuary molted into 1st instars within 24 hr in our running seawater aquaria at the Oregon Institute

of Marine Biology. We immediately placed them into four treatment groups, cold water-high food, cold water-low food, warm water-high food and warm water-low food. The experimental temperatures were 14° and 21°C, temperatures the organisms would normally encounter in the nearshore waters and at the head of the Coos Bay estuary during spring and summer. As expected, crabs fed high food were significantly larger than those fed low food (Fig. 6). The intermolt period was shorter in crabs fed high food levels, especially those in warm water. Despite the faster growth and molt rate of crabs in warm water-high food, the crabs in cold water-high food attained the largest sizes after six months. Thus it seems that food quantity affects growth more than temperature. Concentration of two-hexameric hemocyanin levels was relatively unresponsive to differences in temperature and food, while the one-hexameric hemocyanin concentration rose in response to increased temperature, especially in the low food treatment. The different food levels and seawater temperatures also affected hemocyanin ontogeny. The first appearance of adult hemocyanin, based on the presence of subunit 6, occurred at a significantly more immature instar in the crabs raised in warm water and low food than in the other treatment groups (Terwilliger and Dumler, 2001). Adult hemocyanin appeared at a significantly later time, based on number of days since metamorphosis into first instar juvenile, in the cold water-low food treatment group. These results demonstrate that molt stage and onset of adult hemocyanin are not strictly coupled. The ontogeny of hemocyanin, furthermore, can be altered by environmental conditions and nutritional state.

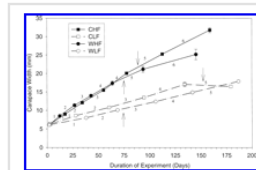


Fig. 6. Size (carapace width, in mm, mean \pm SEM) at mean cumulative number of days to each instar from the initiation of the study for each *Cancer magister* treatment group (CHF: cold water-high food; CLF: cold water-low food; WHF: warm water-high food; WLF: warm water-low food) by instar. Initial number of crabs, 15 per treatment group. Number of surviving crabs on day 175 are: CHF (15), CLF (13), WHF (9), WLF (1). Instars are denoted by numbers between data points. The arrows indicate mean onset of adult hemocyanin. (From Terwilliger and Dumler [2001], Fig. 7.)

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CONCLUSIONS

The two kinds of proteins, hemoglobin and hemocyanin, that transport oxygen in crustaceans, are structurally unrelated to one another except perhaps for a shared folding motif, a four α -helix bundle which provides the dioxygen binding pocket. Based on x-ray structures of hemoglobins and hemocyanins, it has been suggested that the first oxygen-binding proteins evolved from a pair of antiparallel α -helices that probably provided the histidines or cysteines that bound the iron or copper ions (Volbeda and Hol, 1989). Gene duplications and fusions of this ancestral pattern may have resulted in today's dioxygen-binding proteins, hemoglobins, hemerythrins and hemocyanins (Decker and Terwilliger, 2000; Volbeda and Hol, 1989). Similar functions of cooperative oxygen binding and transport among these proteins are due to shared characteristics of a dioxygen binding pocket and multisubunit oligomerization.

Another common feature among crustacean hemoglobins and hemocyanins is the selective expression of subunits during development that alters the oxygen affinity of the protein to meet the changing demands of the larva, juvenile or adult, as described in this paper. Research on the synthesis of *Artemia* and *Daphnia* hemoglobins has demonstrated that branchiopod hemoglobins are responsive to oxygen levels during development and during adult life. The ontogeny of hemoglobin and hemocyanin are sensitive to environmental factors as well as endogenous patterns.

It is intriguing that crustaceans seem to express either hemoglobin or hemocyanin but not both proteins. The molecular phylogenies of these oxygen transport proteins and the complex systematics of the phylum Arthropoda may be better understood after a thorough exploration for hemoglobin and hemocyanin genes in selected arthropods.

Finally, branchiopod hemoglobins promise to be excellent model systems for studying oxygen-dependent gene regulation. In mammals, the hormone erythropoietin stimulates red blood cell proliferation, and the upregulation of erythropoietin in response to hypoxia is mediated by several DNA-binding transcription factors. The presence of HIF-1 binding motifs in the branchiopod hemoglobin gene cluster suggests that these invertebrate systems will yield important information about oxygen sensors and the regulation of oxygen transport proteins. Whether regulation of hemocyanin synthesis is under similar control remains to be discovered.

FOOTNOTES

¹ From the Symposium *Ontogenetic Strategies of Invertebrates in Aquatic Environments* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 3–7 January 2001, at Chicago, Illinois. [*](#)

² E-mail: Nterwill@oimb.uoregon.edu [*](#)

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