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The molecular heterogeneity of hemocyanin: Its role in the adaptive plasticity of Crustacea

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Abstract

Crustacean hemocyanin (Hc) represents a unique case of molecular heterogeneity among oxygen-carrying proteins. The existence of different genes, encoding single polypeptide chains, constitutes the genetic basis for the inter- and intra-specific polymorphism. In addition, the large number of Hc subunits within crustacean species, together with their flexible expression, provides an efficient intrinsic mechanism of modulation of oxygen transport. This review presents a description and classification of the various aspects of crustacean Hc heterogeneity and defines its role in a perspective of crustacean adaptive physiology.

Keywords: Hemocyanin; Quaternary structure; Subunit; Crustacea; Oxygen binding; Molecular Heterogeneity; Phenotypic polymorphism

1. Introduction

The evolution of Metazoa is realized with a progressive increase of complexity in structures and functions and a consequent development of physiological networks for their integrated regulatory processes. In this context, the evolution of reliable systems for efficient gas exchange and O_2 transport allowed for an increase of body size and tissue differentiation. Furthermore, the development of higher aerobic capacity and advanced metabolisms increased the power availability of the evolving species.

The essential role of O_2 transport proteins is clearly underlined by the independent evolution of analogous carriers. Hemoglobins, hemerythrins and hemocyanins (Hcs), although extremely different in structure and origin, share convergent functions across *phyla*. Moreover, they contribute to the radiation of species diversity through several mechanisms of functional adaptations like regulation of expression, variation of synthesis and sensitivity to extrinsic modulators (Terwilliger, 1998).

Among the O_2 binding proteins, the Hcs of Arthropoda, and those of Crustacea in particular, show the largest extent of heterogeneity, with a great variety of subunits expressed within the same oligomeric proteins and a wide degree of regulatory mechanisms.

To describe the structural features and the quaternary architecture of Hc oligomers, to disclose the balance between functional constraints and adaptive modulation of the O_2 binding physiology, and to trace the evolutionary pathway that have led to the current complexity, several reviewers have differently explored the arthropod Hc heterogeneity. Detailed analyses of structural heterogeneity have led to the recognition of distinct subunit types which differ in immunogenicity and in their specific roles in complex oligomerization (Markl et al., 1979a,b, 1986; Markl, 1986; Markl and Decker, 1992). Hcs sequencing has provided a clear identification of distinct subunits in several species allowing for a precise definition of subunit families and of their phylogenetic relationships (Burmester, 2001). Thus, possible ambiguities on subunit identification by chromatographic or electrophoretic approaches are avoided. Numerous studies have investigated the origin and the architecture of the three protein domains that represent the characteristic fold of the subunits (Linzen et al., 1985; Salvato and Beltramini, 1990; van Holde et al., 2001; Decker and Terwilliger, 2000). The

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quaternary arrangement and the different oligomeric assemblages have been reviewed to delineate reliable models for the different Hc structures (Markl and Decker, 1992; Magnus et al., 1994). Other authors have reviewed the functional properties of Hcs with particular consideration of the narrow links between physiological plasticity and environmental selection (Truchot, 1992; Terwilliger, 1998). These studies have dealt with the functional heterogeneity of Hcs, considering their primary role as oxygen carriers as well as their non-respiratory functions such as phenoloxidase and antimicrobial activity (Terwilliger, 1998; Bridges, 2001; Decker and Jaenicke, 2004; Jaenicke and Decker, 2004). Finally, the subunit heterogeneity has been considered to draw the evolutionary dynamics and to trace the ancestral origin (Hughes, 1999; Decker and Terwilliger, 2000; van Holde et al., 2001; Burmester, 2004) of arthropod Hcs. The wide sequence database (about 50 sequences of crustaceans, insects, chelicerates, myriapods, and onychophorans) has allowed for comparison of the phylogenetic relationships among the different subunit types and the diverse molecular evolution among the *taxa* (Voit et al., 2000; Burmester, 2001; Terwilliger and Ryan, 2006; Terwilliger et al., 2006).

Hc heterogeneity contributes to the adaptive potential in the respiratory physiology of Crustacea through several mechanisms which can be classified as extrinsic and intrinsic. Extrinsic mechanisms depend upon integrated responses of Hc to different external modulators that affect the protein oxygen-binding properties as allosteric effectors: the roles of inorganic and organic ions, pH, sulphide, thiosulphate, and neurohormones as Hc affinity modulators have been reviewed in detail (Morris, 1990; Burnett, 1992; Bridges, 2001). Intrinsic mechanisms, on the contrary, are realized through shifts in protein structure, such as changes in the ratios between different Hc oligomers and phenotypic modifications through the regulation of expression levels for distinct Hc subunits.

The present contribution provides an overview on the importance of the intrinsic plasticity of Hc of Crustacea for the adaptive physiology of these organisms in response to both ontogenetic processes and environmental stimuli. The wide structural and functional variability of crustacean Hc, referred to as crustacean Hc heterogeneity, will be described along three hierarchical levels:

(i) the level of subunit diversity. This refers to the existence of distinct subunit types, which contribute differently to the structural and physiological properties of the whole Hc molecule. The adaptive role of subunit heterogeneity will be discussed in relation to its role in the complex oligomerization and oxygen-binding plasticity.

(ii) the level of phenotypic polymorphism. This refers to the occurrence of various phenotypes, intended as assemblages of different subunits, both at intra and inter specific level. The adaptive role of distinct subunit patterns will be discussed for populations of the same species, by reference to the ontogenetic development and life cycle, as well as for the species specificity in the case of different species.

(iii) the level of protein expression modulation. This concerns the plastic modulation of protein synthesis in response to internal or external stimuli. Changes in Hc expression can affect the total concentration of Hc in the hemolymph and/or the level of expression of a single subunit with respect to the others. A major consequence of Hc heterogeneity is the modulation of O_2 capacity which in turn affects the adaptive potential of Crustacea.

2. Subunit diversity

2.1. Subunit diversity and oligomerization

The precise arrangement of the different subunits within the quaternary structure plays a key role in the generation of oligomers above the hexameric state. In the presence of a single subunit type the resulting aggregation state is hexameric. This has been observed both *in vivo* and *in vitro*. The Hc of the isopod *Bathynomus giganteus* contains *in vivo* only one subunit referred to as "α-type subunit" in its hexameric structure (Markl, 1986). *In vitro* dissociations of the native oligomers from a number of species can be obtained at alkaline pH and by removing divalent cations by EDTA. The reversibility of this process has been studied by changing the buffer conditions of isolated subunits or of a pool of subunits returning to neutral pH in the presence of divalent cations. There are numerous examples of isolated subunits that form homohexamers in reassociation experiments (Jeffrey et al., 1976; Stöcker et al., 1988; Dainese et al., 1998; Molon et al., 2000). The dissociation of dodecameric Hc of the portunid crab *Carcinus aestuarii*, followed by reassociation of the pool of subunits, results in hexamers. However, the reassociation is not quantitative since one monomeric fraction (referred to as CaeSS2) remains in the monomeric state (Dainese et al., 1998). These data are in line with the concept of the "linker" subunit namely one subunit, present in each hexamer of the dodecameric oligomer, that is essential for the inter-hexameric interactions responsible for building up the higher oligomer. In the hemolymph of *Portunus trituberculatus*, Hc is found in the dodecameric and hexameric form; the former contains four antigenically distinct subunits (I–IV), the latter lacks subunit IV. Thus, the aggregation state is differentiated by a specific component (Yoo et al., 1988). The expression level of the linker subunits may vary in natural populations, thus changing the dodecamers-to-hexamers ratio in the hemolymph of individual specimens (Mangum, 1994). In some species, the formation of dodecameric Hcs involves covalent interactions of subunits that form dimers through a disulphide bridge. In this case, each subunit of the dimeric structure participates in the formation of a distinct hexamer in combination with other monomeric subunits; the aggregation of the hexamers into dodecameric structures, therefore, depends on such an inter-hexameric covalent bridge (Murray and Jeffrey, 1974; Jeffrey et al., 1976; Stöcker et al., 1988).

The subunit–subunit interactions that stabilize the monomers in the hexameric oligomers have been described with high precision in the case of the hexameric Hc of *Panulirus interruptus* that has been crystallized (Linzen et al., 1985;

Volbeda and Hol, 1989; Markl and Decker, 1992). Furthermore sequence comparisons have demonstrated that the residues involved in such intra-hexameric contact areas are strongly conserved in evolution (Linzen et al., 1985). By homology modeling it has been recently proposed that the remarkable resistance to dissociation of peneid Hc hexamers is attributable to an increase of stabilization in the conserved contact areas due to specific amino acid substitutions compared to other Hcs that are more prone to dissociation (Beltramini et al., 2005). Unfortunately, there is no crystallographic information on oligomers that result from aggregation of hexamers. Thus, the description of the inter-hexameric interactions and the structural determinants that define the "linker" nature of one subunit are still lacking.

2.2. Subunit diversity and oxygen-binding modulation

The total oxygen-binding affinity of a whole Hc mixture in the hemolymph results from the sum of individual affinities of single subunits and from their relative interactions (Mangum, 1997; Rickey et al., 1985; Decker and Sterner, 1990). The functional role of Hc heterogeneity at subunit level depends, therefore, on the balance among different intrinsic affinities, enhancing the complexity and the integration of the oxygen-carrying process.

Numerous studies have shown that different sets of subunits determine distinct oxygen-binding affinities as evidence of the extensive functional variability (Mangum and Rainer, 1988; DeFur et al., 1990; Mangum, 1994; Spicer and Hodgson, 2003a,b). Also a plastic regulation of cooperative transition is provided by subunit heterogeneity as shown in *P. interruptus*. This Hc is naturally composed by a mixture of three distinct subunits, namely a, b and c, (Neuteboom et al., 1992), but the last does not participate in the cooperativity of the hexamers (Soeter et al., 1986). Homohexamers prepared *in vitro* from isolated subunits have in general different affinity or cooperativity as compared with the native heterohexamers (Dainese et al., 1998; Molon et al., 2000).

The adaptive potential of subunit diversity in crustacean Hc is also revealed by the different sensitivity to allosteric effectors shown by diverse monomers (Nies et al., 1992). Natural selection could, therefore, drive the evolution of Hc heterogeneity, enhancing or diminishing the consequential sensitivity to allosteric regulation. Research on *Callinectes sapidus* Hc, studying lactate as allosteric modulator, showed the presence of various lactate binding sites among distinct subunits and, as a consequence, differential sensitivity to its effect. The *C. sapidus* Hc studies revealed that the number of lactate binding site is smaller than the number of subunits in each hexamers. Moreover, in analyzing the lactate effect on artificial homohexamers obtained from purified subunits of *C. sapidus* Hc, a different response was detected (Johnson et al., 1984). The results suggest that, in native Hc, the effector binding site occurs on specific subunits rather than on the inter-subunit contact regions. Similar results have been found analyzing *P. interruptus* Hc where monomers are differently affected by lactate. While c subunits are markedly influenced, a and b polypeptides appear only marginally sensitive to allosteric regulation (Johnson et al., 1987). An isothermal titration calorimetric study performed on dodecameric Hc of *Astacus leptodactylus* has revealed the presence of two distinct binding sites for urate (Hellmann et al., 2001). This metabolite constitutes an organic allosteric modulator of Hc affinity (Morris et al., 1985) and its concentration, in *A. leptodactylus* hemolymph, is increased when animals undergo acute hypoxia (Bridges, 1990). Hellmann et al. (2001) demonstrated that the two urate binding sites differ in their specificity toward the ligand and might be located in different subunits (β and γ type). These works are examples of how the oxygen-binding properties depend on the behavior of distinct subunits and support the role of Hc subunit diversity as plastic substrate in physiological adaptive processes.

3. Phenotypic polymorphism

3.1. Intra-specific polymorphism

The intra-specific heterogeneity of Hc implicates differences in phenotype composition and subunit expression levels that occur both among populations and among individuals of the same population within a species. A large number of different subunits constitute the genetic base of this phenotype complexity.

The clearest cases of within-species variation of Hc subunit composition are represented by the decapods Ocypodidae (Mangum, 1993b, 1996; Callicott and Mangum, 1993; Mangum and Greaves, 1996) and amphipods (Hodgson and Spicer, 2001; Spicer and Hodgson, 2003a). In the genus *Uca* up to 17 polypeptide chains and 39 different subunit compositions in the oligomers have been detected among individuals of the same species, providing strong example of the extent of intra-specific phenotype heterogeneity (Mangum and Greaves, 1996). Analyzing the functional significance of this variability, an O_2 binding dependence of phenotypes has been demonstrated. The various phenotypes of oligomers resolved in *Uca pugilator*, characterized by different subunit compositions, show significant functional differences and reveal that the more prominent effect on the O_2 affinity is attributable to the variation of few specific subunits rather than to the whole phenotype (Mangum, 1993b). However, a clear relation between phenotypes and environmental regimes has not yet been established, and physico/chemical factors like temperature and salinity do not totally account for the subunit composition variability. The results on *U. pugilator* reveal more pronounced differences at the edge of the geographic distribution, underlying an environmental effect in a latitudinal gradient as a selective force for phenotypic differentiation in disjunct populations (Callicott and Mangum, 1993).

The intra-specific heterogeneity has been described also in some species of Amphipoda. A clear relationship between environmental salinity, Hc subunit composition and O_2 affinity, was described for *Chaetogammarus marinus* (Spicer and Hodgson, 2003b) (more details are presented in Section 4.2). In contrast, any functional difference and any salinity dependence were recognized for the Hc phenotypes of *Orchestia gammarellus* (Spicer and Hodgson, 2003a).

The nature of the Hc subunits as different transcripts of several genes, or products of different alleles, or even same transcripts of few genes with different post-transcriptional and post-translational modifications, has not been determined in most species. However, an extensive investigation on the relative occurrence of subunit phenotypes in Ocypodidae showed a more complex scenario than that expected in relation to simple allele frequencies (Mangum and Greaves, 1996; Mangum, 1997). Furthermore, experimental evidence on the Dungeness crab *Cancer magister* Hc shows the presence of six distinct genes, each coding for a specific subunit (Terwilliger et al., 2006).

Not all Crustacea show similar phenotype variability, and in some cases the Hc subunits are constitutively and equally expressed in the entire species (Larson et al., 1981). During ontogenetic transitions and molting processes, however, these species demonstrate remarkable cases of intra-specific heterogeneity in Hc composition. *C. magister* and the American lobster *Homarus americanus* have been intensely investigated to disclose the sequential expression of different subunits and changes in dodecamers/hexamers ratio during ontogenesis (Terwilliger and Terwilliger, 1982; Terwilliger et al., 1986; Olson et al., 1988; Olson and Mcdowell Capuzzo, 1989; Terwilliger and Brown, 1993; Brown and Terwilliger, 1998; Terwilliger and Dumler, 2001; Durstewitz and Terwilliger, 1997). The progressive recruitment of Hc chains during development demonstrated in *C. magister* shows four constitutive subunits at the stage of megalopa, an additional fifth subunit in the juvenile instars, and the definitive six bands phenotype in adult stages (Terwilliger and Brown, 1993). This ontogenetic recruitment of Hc subunits reveals its adaptive potential when considering the integrated regulation of O_2 binding physiology. In earlier developmental stages, in fact, the four and five subunits phenotypes show lower O_2 affinity with respect to the adult Hc. Megalopas and juveniles present higher levels of Mg^{2+} concentration in the hemolymph, due to the immature renal development with consequent weak ion regulation. Mg^{2+} constitutes an essential allosteric effector that raises the O_2 affinity of *C. magister* Hc, and its high concentration in immature crabs hemolymph compensates for the intrinsic low Hc O_2 affinity. In adult stages the effect of the lower Mg^{2+} concentration is counterbalanced by a Hc with an intrinsically higher O_2 affinity. The resulting effect of developmental Hc heterogeneity is the maintenance of a whole hemolymph O_2 affinity from megalopa to adult (Brown and Terwilliger, 1998; Terwilliger and Ryan, 2001).

A pronounced heterogeneity both in the total Hc concentration and in the subunit phenotypes is exhibited by specimens of *A. leptodactylus* and *C. magister* at different molting stages (Spindler et al., 1992; Terwilliger and Otoshi, 1994; Terwilliger et al., 2005). In these cases, Hcs, together with the non-respiratory protein cryptocyanin, show high concentration during pre-molt stage, while a subsequent decrease occurs during ecdysis, although more marked for cryptocyanin. Cryptocyanin shares with Hc the hexameric quaternary structure and might be difficult separated from the hemolymph preparations. The concomitant presence of Hc and cryptocyanin may cause ambiguities in evaluating intra-specific variability of subunit patterns.

Finally, a particular case of intra-specific Hc heterogeneity is provided by the polymorphism at the level of protein quaternary structure in *A. leptodactylus* in response to different thermal regimes. In this species, when acclimatized at different temperatures, a shift in subunit expression occurs. The thermal response involves the subunits linkers which covalently connect single hexamers in dodecameric molecules, and the concentration of the dodecameric form is reduced at higher temperature (Decker and Föll, 2000).

3.2. Inter-specific polymorphism

The great heterogeneity in Hc composition among crustacean species, both in the types of subunit and in the complexity of phenotypes, has led to the concept of species specificity of subunit patterns (Reese and Mangum, 1994; Mangum and McKenney, 1996) and to its use as a discriminatory taxonomic tool for cryptic species (Mangum, 1996; Hodgson and Spicer, 2001). These studies were aimed at analyzing the Hc subunit composition in several species with particular emphasis on congeneric, recently speciated, sibling species, and their hybrids (Mangum, 1993a, 1996; Reese and Mangum, 1994; Mangum and Greaves, 1996; Mangum and McKenney, 1996). From these studies a highly complex picture emerges. However, some general findings on the inter-specific Hc heterogeneity led the authors to suggest possible evolutionary processes shared among the different *taxa*.

Each species [with the exception of *Uca musica musica* (Mangum and Greaves, 1996)] showed several invariant subunits, constitutively expressed in all specimens of the same species. These monomers, generally, represent the major fraction of the whole Hc and are the main contributors to the respiratory properties (Mangum and McKenney, 1996). A shift in the expression of invariant subunits has been suggested to be a major adaptive change in the respiratory physiology, and a genetic divergence that occurs early in speciation (Mangum, 1993a,b; Reese and Mangum, 1994). Sibling species, or species that are believed to be in the process of speciation, would share analogous phenotypes which generally differ in at least one constitutive subunit. These differences could produce a prominent variation in oxygen-binding properties, allowing the new species to cope differently with environmental stimuli with respect to the ancestral species. Several physico-chemical and biological factors have been implicated as selective forces which could drive the phenotype variation (Reese and Mangum, 1994), such as temperature in the case of disjunct population of *Uca mimax* and *Sesarma reticulatum* (Mangum and McKenney, 1996).

In addition to the constitutive, other subunits, variably expressed, are present in lower amount in the Hc oligomers and are responsible for the species specificity of subunit composition. The functional significance of these variable chains still remains unclear, even if the study on *C. sapidus* provides interesting directions (Mangum et al., 1991). In this species, the variable chains modulate the O_2 transport properties of Hc

because either different subunit compositions result in different oxygen-binding properties of the oligomer or the ratio dodecamers/hexamers is modified (more details are presented in Section 4.2). Moreover, a wide assortment of variable subunits in inter-specific context supports a role of Hc during speciation. The early divergence of subunit expression during species differentiation may occur by enhancing or silencing the expression of some subunits with respect to the others and producing a functional shift in the whole monomer assemblages. The heterogeneity of variable and low expressed subunits may provide, therefore, the genetic basis and a simple mechanism for adaptive changes in Hc phenotype. In conclusion, the evolution of inter-specific Hc heterogeneity could be related to the successful modulation of existing structures, rather than to the development of *ex novo* molecules.

Even if the subunits banding pattern could constitute a reliable taxonomic tool for discriminating cryptic species, it does not seem to provide any distinguishable characteristics for a specific taxon (Reese and Mangum, 1994). The differences among phenotypes of closely related species are so much pronounced, that no one relationship within the taxon appears easily to be inferred. Combining the analyses on the subunit patterns with the O_2-binding properties of the phenotypes, an interesting scenario emerges. Reese and Mangum (1994), in fact, described a more evident relationship between the O_2-affinities and the environmental characteristic, with respect to the subunit composition. They suggested a prominent selective effect of environmental factors on the respiratory properties, which, consequently, acts on the available subunit array and generates different phenotypes without evident correspondence with those of related species. The species specificity of subunit pattern is differently evident among crustacean families. As an example, a recent comparative analysis performed on portunid crabs shows that closely related species share a common subunit pattern (Giomi et al., 2007).

The concepts of species specificity of subunit composition include Hc from amphipods and isopods as well as decapod crustaceans (Hodgson and Spicer, 2001). The authors further support the reliability of Hc subunit patterns as taxonomic character and its physiological/biochemical significance for the study of cryptic events of speciation. These findings highlight the significance of inter-specific Hc heterogeneity as a general phenomenon common to all crustaceans, while suggesting some uncertainty on the adaptive potential of inter-specific Hc heterogeneity. In this context any evident correlation between subunit composition and functional properties of the protein was found within amphipod species. The functional modulation of these Hcs is suggested to depend from allosteric effectors rather than from changes in subunit array.

4. Molecular heterogeneity in protein synthesis

4.1. Absolute variations: changes of concentration

Change in Hc concentration, hence in oxygen-carrying capacity of the hemolymph, represents a general physiological response to cyclic or stochastic environmental variations of oxygen concentration. However, it represents one among several factors, such as allosteric regulation of the oxygen-binding properties, involved in the integrated physiology of oxygen transport adaptation. Thus, a marked difference exists between and within species as far as the direction and the magnitude of the observed concentration variation are concerned (Truchot, 1992; Taylor and Anstiss, 1999; Spicer and Baden, 2001; Baden et al., 2003).

Hc concentration in the hemolymph is affected by intrinsic and extrinsic factors (Table 1). The former normally occur during the animal lifecycle, the latter take place in response to various environmental solicitations.

Ontogenic variations have been observed for many species such as *Palaemon elegans*, *P. longirostris*, *Palaemonetes varians*, *Crangon crangon*, *Systellaspis debilis*, *Penaeus japonicus*, *Nephrops norvegicus*. For these species a positive correlation between body size and hemolymph Hc concentration has been established (Abdennour, 1997; Rainbow and Abdennour, 1989; Chen and Cheng, 1993; Spicer and Eriksson, 2003). This correlation suggests that the increase of body complexity and metabolic demand, associated with the ontogenetic development, are supported by the dynamics of Hc expression during the different life stages or at different body weight. In addition, qualitative changes (*i.e.* changes in subunit composition), contribute, together with the quantitative variations, to modify the oxygen-carrying capacities in each developmental stage, as shown for *C. magister* (Terwilliger and Brown, 1993; Brown and Terwilliger, 1998). The spider crab *Hyas aranaeus* shows lower Hc concentration in larger specimens and represents one case of negative correlation between oxygen-carrying capacity and body weight (Spicer and Baden, 2000).

Cases of gender-related differences in Hc concentration have been described for *Palinurus elephas*, *Nephrops norvegicus* and *Penaues japonicus*, where males present higher contents of Hc than females (Bellelli et al., 1988; Baden et al., 1990; Chen and Cheng, 1993), probably in dependence of different behaviors or life styles. Molting represents an important life phase that affects the Hc concentration in many species. The general trend shows the higher Hc concentration values during inter-molt and pre-molt stages, followed by a drastic decline of Hc concentration when animals undergo ecdysis and a subsequent recovery during post-molt phase (Spicer and Strömberg, 2002; Terwilliger et al., 2005). The initial Hc decline is a secondary effect of the marked water uptake that leads to the shedding of the old exoskeleton but produces a general dilution of hemolymph (Skinner, 1985; Taylor and Kier, 2003). Secondarily, as in other circumstances of forced starvation, Hc is metabolically recycled and employed as source of energy and amino acids (Zuckerkandl, 1960; Hagerman, 1983). The magnitude of the Hc concentration decrease is not only species dependant, but also related to the metabolic rate and the physiological conditions of animals (Zuckerkandl, 1960; Mangum et al., 1985). The fine integration between oxygen capacity demand and feeding status appears to be essential to assess the adaptive role of Hc during molt.

The marked variation in Hc synthesis and catabolism, naturally occurring in several Crustacea, probably contributes to

Table 1
Intrinsic and extrinsic causes determining modifications of Hc concentration in the hemolymph of crustacean species

Species	[Hc] modification	Condition	Reference	Species	[Hc] modification	Condition	Reference
	Intrinsic causes of variation				***Extrinsic causes of variation***		
Palaemon elegans	Positive relation	Size	Abdennour (1997)	*Nephrops norvegicus*	Differences	Collecting site	Spicer and Baden (2000)
Palaemon longirostris	Positive relation	Size	Abdennour (1997)	*Liocarcinus depurator*	Differences	Collecting site	Spicer and Baden (2000)
Palaemonetes varians	Positive relation	Size	Abdennour (1997)	*Callinectes sapidus*	Differences	Collecting site	Engel et al. (1993)
Crangon crangon	Positive relation	Size	Abdennour (1997)	*Palinurus elephas*	Differences	Season	Bellelli et al. (1988)
Systellaspis debilis	Positive relation	Size	Rainbow and Abdennour (1989)	*Nephrops norvegicus*	Increase [b]	Hypoxic exposure	Spicer and Baden (2001)
Penaeus japonicus	Positive relation	Size	Chen and Cheng (1993)	*Nephrops norvegicus*	Increase	Hypoxic exposure	Hagerman et al. (1990)
Hyas aranaeus	Negative relation	Size	Spicer and Baden (2000)	*Saduria entomon*	Increase	Hypoxic exposure	Hagerman and Oksama (1985)
Cancer magister	Changes [a]	Life stages	Brown and Terwilliger (1998)	*Nephrops norvegicus*	Increase	Hypoxic exposure	Hagerman and Baden (1988)
Cancer magister	Changes [a]	Life stages	Terwilliger and Brown (1993)	*Nephrops norvegicus*	Increase	Hypoxic exposure	Baden et al. (1990)
Nephrops norvegicus	Differences ♂>♀	Gender	Baden et al. (1990)	*Crangon crangon*	Increase	Hypoxic exposure	Hagerman (1986)
Palinurus elephas	Differences ♂>♀	Gender	Bellelli et al. (1988)	*Homarus americanus*	Increase	Hypoxic exposure	Senkbeil and Wriston (1980)
Callinectes sapidus	Decrease	Molt cycle	Engel (1987)	*Nephrops norvegicus*	Decrease [c]	Hypoxic and Mn exposure	Baden et al. (2003)
Meganyctiphanes norvegica	Decrease	Molt cycle	Spicer and Strömberg (2002)	*Callinectes sapidus*	Increase	Hypoxic exposure	DeFur et al. (1990)
Homarus gammarus	Decrease	Molt cycle	Hagerman (1983)	*Nephrops norvegicus*	Increase	Hypoxic exposure	Hagerman and Uglow (1985)
Carcinus maenas	Decrease	Molt cycle	Truchot (1978)	*Carcinus maenas*	Decrease	Starvation	Uglow (1969)
Maya squinado	Decrease	Molt cycle	Zuckerkandl (1960)	*Nephrops norvegicus*	Decrease	Starvation	Baden et al. (1994)
Callinectes sapidus	Decrease	Molt cycle	Mangum et al. (1985)	*Meganyctiphanes norvegica*	Decrease	Starvation	Spicer and Strömberg (2002)
Cancer magister	Decrease	Molt cycle	Terwilliger et al. (2005)	*Homarus gammarus*	Decrease	Starvation	Hagerman (1983)
Penaeus japonicus	Decrease	Molt cycle	Chen and Cheng (1993)	*Crangon vulgaris*	Decrease	Starvation	Djangmah (1970)
Penaeus duorarum	Decrease	Molt cycle	Bursey and Lane (1971)	*Eriocheir sinensis*	Increase	Low salinity	Gilles (1977)
Crangon vulgaris	Decrease	Molt cycle	Djangmah (1970)	*Carcinus maenas*	Increase	Low salinity	Boone and Schoffeniels (1979)
				Callinectes sapidus	Increase	Low salinity	Mason et al. (1983)

[a] The [Hc] levels are higher in megalopa and adult stages, and lower during juvenile instars.
[b] The magnitude of increase is negatively related with the initial [Hc].
[c] The hypoxia-induced increase of [Hc] is reduced by the simultaneous exposure to Mn.

adaptive physiological processes also involved in the successful colonization of extremely diverse habitats. The modulation of the oxygen-carrying capacity among the developmental stages, and during molting process could have played an important role for the larval dispersal and juvenile settlement. Moreover, a rapid change in Hc concentration provides an adaptive potential toward gradual or sudden environmental stimuli, that are classified as extrinsic causes of variation (Table 1).

Different values of Hc concentration have been measured in separated populations of *N. norvegicus*, *Liocarcinus depurator* (Spicer and Baden, 2000) and *C. sapidus* (Engel et al., 1993). Modification of Hc concentration, together with the variation of heart and ventilatory rates, provides an integrated compensation to cope with both acute and chronic hypoxia (McMahon, 2001). However, hypoxic-induced modifications in oxygen-carrying capacity present a considerable species specific variability. When *N. norvegicus* is subjected to different degrees of oxygen depletion, an increase of Hc synthesis occurs, but significant differences in the magnitude of such variation have been recorded both in field and laboratory analyses (Hagerman and Uglow, 1985; Hagerman and Baden, 1988; Baden et al., 1990; Hagerman et al., 1990). On the basis of these observations, Spicer and Baden (2001) investigated intra-specific variation of Hc concentration, following hypoxic exposure, unraveling marked differences in individual responses. A higher increment of Hc synthesis was recorded in specimens with an initial low Hc concentration, while individuals with initial higher contents presented no significant increase or even a reduction of Hc

concentration. These results have been further confirmed by the finding that the initial level of Hc is also involved in the response timescale after release from hypoxic exposure (Baden et al., 2003). Other environmental factors cause variations in Hc concentration, highlighting a broad involvement of this physiological mechanism. Exposure to low salinities generally increases Hc concentration (Gilles, 1977; Boone and Schoffeniels, 1979; Mason et al., 1983), while pollutants, such as an excess of Mn, can significantly impair an increase of the oxygen capacity following a hypoxic exposure (Spicer and Weber, 1991; Baden et al., 2003).

Individual variability in the extent of Hc concentration modifications is clearly detectable. This could be in general related with the physiological conditions of the specimen but in particular it appears more strictly dependant on the nutritional status (Uglow, 1969a,b; Hagerman, 1983; Baden et al., 1990, 2003; Spicer and Strömberg, 2002). Fasting, both natural and experimental, markedly reduces the Hc concentration.

The induced changes of Hc concentration have been demonstrated at very different timescales ranging from annual variation as in adults *P. elephas* (Bellelli et al., 1988); to monthly as in *C. magister* juvenile instars (Terwilliger et al., 2005). These differences can be accounted for by the different molting frequencies in the two cases. Recent studies have, in addition, revealed significant changes within the day. The Nordic krill *Meganyctiphanes norvegica*, like other Euphausiacea, adopts diel vertical migration moving from 100 m depth during the day to the surface layers during the night (Spicer et al., 1999). This organism, therefore, is cyclically exposed to environments that are characterized by different temperature, salinity, dissolved oxygen and food availability (Strömberg and Spicer, 2001). With field sampling of *M. norvegica*, Spicer and Strömberg (2002) have accurately described a negative correlation of Hc concentration with depth. In laboratory experiments the effects of the single physical variables on the Hc changes have been quantified. These authors have clearly demonstrated that starvation, as naturally encountered in the deeper waters, among other experimental variables strongly reduces Hc concentration and that the Hc turnover in this species takes very short time. These phenomena, considering the high metabolic rate registered in krill, underline the potential of Hc as metabolic reserve.

In conclusion, changes of Hc concentration have a great physiological significance. The plasticity of Hc content constitutes an efficient mechanism for Crustacea to cope with metabolic changes or environmental stimuli, although more data on the turnover rate of Hc are needed. The great variability of Hc concentration, indeed, points to a fine balancing between oxygen capacity and amino acid storage.

4.2. Relative variation: change in subunit composition

The physiological adjustment through small changes in Hc subunit composition represents a paradigmatic example of how physiological complexity supports adaptive processes in Crustacea.

Unlike changes in Hc concentration, the differential expression of distinct subunits suggests gene-specific transcriptional activation in response to a set of environmental stimuli. Moreover, while variations in Hc concentration are common to almost all Crustacea, the regulation of expression of individual monomers appears to be strictly species-related, and it conclusively accounts for the intra-specific Hc heterogeneity. In particular, it has been demonstrated that a change in Hc phenotype by means of differential expression of one or more subunits are a consequence of a defined physico-chemical stimuli and produces a shift in O_2-binding affinity (Mason et al., 1983; Mangum and Rainer, 1988; DeFur et al., 1990; Mangum, 1994; Spicer and Hodgson, 2003b). This phenomenon has been intensely investigated in two species: the blue crab *C. sapidus* and the amphipod *C. marinus*. In *C. sapidus* three of the six polypeptides are almost invariantly expressed and correspond to the constitutive fraction of the whole Hc; the other three subunits are highly variable (Mangum and Rainer, 1988). *C. marinus* shows a similar pattern with a single subunit highly variable and the other 7 not significantly different among specimens (Spicer and Hodgson, 2003b). These fine variations in polypeptide expression establish the regulation of O_2-binding affinity through changes of the stoichiometric ratio between subunits. However, the effect on the oxygen-binding properties of the whole Hc is different in the two species. The transition from a lower to a higher O_2 affinity phenotype in *C. sapidus* is achieved through a decreased expression of the three variable subunits (Mangum, 1994). This shift is reflected by a change in the proportion of the two native oligomers with an increase of the hexamer with respect to the dodecamer. The six polypeptide chains apparently contribute differently to the quaternary conformation. While the three invariant subunits are equally involved in hexamer and dodecamer, the three variable ones play a role as linker-subunit and are mainly responsible in promoting higher aggregation. The hexameric form has a higher O_2 affinity and lower cooperativity than the dodecameric (Mangum et al., 1991). Thus, following an external stimulus, a decrease of the three variable linker subunits occurs; this results in a decrease of dodecameric aggregates and provides a Hc mixture in the hemolymph with the higher affinity hexamer as the main component. (Mangum, 1997). The amphipod *C. marinus* also responds to environmental stimuli with appreciable changes of the differential expression of Hc subunits. Here, three out of the eight subunits remain almost unchanged, the others present significant variation. Not all variable subunits account for functional shift of the phenotype on consideration that few of them constitute a very small amount of the total Hc. A possible role of subunit 2, which is present in all specimens analyzed with a mean expression of 24% (at high salinity) and 26% (at low salinity) and a range of 6–36% of the total Hc, was proposed. This hypothesis has been verified with oxygen-binding experiments of the different phenotypes, revealing a significant correlation between expression amount and O_2 affinity only for subunit 2 (Spicer and Hodgson, 2003b). Thus, in *C. marinus*, in contrast to *C. sapidus*, an increase of expression of a specific subunit provides an increase in O_2 affinity, but its role on oligomer aggregation and a possible correlation between structure and function in amphipod Hc is still unknown.

Few physico-chemical stimuli have been identified as the environmental causes which could determine the shift between

different functional phenotypes. Exposure to different salinity regimes, both in field and in laboratory, have been proved to modify the subunit composition in *C. sapidus* (Mason et al., 1983) and *C. marinus* (Spicer and Hodgson, 2003b), respectively. Further studies on *C. sapidus*, have pointed out the effect of environmental hypoxia as determinant of Hc phenotype (DeFur et al., 1990; Mangum et al., 1991; Mangum, 1994; Brouwer et al., 2004). However, in addition to the clear evidences on the individual effect of salinity and hypoxia, a possible synergic effect of the two environmental parameters, as well as the influence of other factors (*e.g.* temperature, pH, CO_2) can be hypothesized. The great potential of this adaptive process is finally achieved with the prompt timing of the response. All studies underlined the quick modification of subunit patterns. Both, *C. sapidus* and *C. marinus*, show a significant variation in subunit pattern starting from 1–4 days after the experiment stimulus, revealed by changes of both protein expression (Mason et al., 1983; Spicer and Hodgson, 2003a,b) and gene activation (Brouwer et al., 2004; Brown-Peterson et al., 2005).

In conclusion several requirements appear essential to the functional adaptation through variations of the Hc subunit phenotype: a marked heterogeneity of Hc subunits provides the basis for individual adaptation; constitutively expressed subunits organize the scaffolding of oligomeric structure; variable subunits, from one to several, influence the functional properties by changing the proportion among monomers or between oligomers; a prompt change of Hc phenotypes occurs as a response to the environmental stimuli or developmental signals.

5. Conclusion and implications

Crustacean Hc heterogeneity provides a model system to investigate the evolutionary biochemistry and physiology in crustacean species and represents an interesting paradigm for the description of the integrated complexity of respiratory physiology adaptability. Phylogenetic analysis on the sequence relationships between different subunits, and between the same subunit in closely related species, could provide a precise insight on the evolution of Hc heterogeneity. The divergence of Hc subunit expression and the consequent change of phenotype in early differentiation of species provide interesting features of physiological implications during speciation. In this context, Hc heterogeneity, and the consequent functional differentiation of divergent phenotypic traits, may represent a focal aspect to be analyzed also in a view of ecological selection (Schluter, 2001).

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