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Molecular approach to the study of hemocyanin in stoneflies (Plecoptera): a biologic and phylogenetic interpretation.

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Coordinator

Dr. Roberta Cimmaruta

Tutor

Prof. Romolo Fochetti

PhD student

Dr. Valentina Amore

Abstract

(in English)

Contrary to what was assumed regarding the presence of respiratory proteins in insects, a functional hemocyanin was recently found in larvae and adults of the stoneflies species *Perla marginata* in *P. grandis*. In order to verify if the presence of this ancient trait is widespread within the order and to investigate why stoneflies have maintained it, we have extended the search for hemocyanin within Plecoptera families. In particular, we assessed the presence of hemocyanin in the larval and adult stage the seven families of the European stonefly fauna, and we tested some taxa of Oriental and Southern African fauna living in environments with different ecological features respect to those of the Palaearctic streams.

We cloned and sequenced cDNAs corresponding to the conserved hemocyanin fragment (in domain II) and studied their expression with PCR technique. To evaluate hemocyanin presence as translated product, highly sensitive method based on liquid chromatography tandem mass spectrometry, was used. Phylogenetic analyses were inferred.

On the basis of our outcomes, we have revealed that hemocyanin seems to be not uniformly distributed within this order. Phylogenetic relations, environmental induction and biological aspects, such as larval size, life cycle length and trophic roles, are discussed as possible factors that may be correlated with the presence or absence of hemocyanin in the studied species. Moreover, we take into account the potential multifunctionality of hemocyanin. In this work we presented the current knowledge about the hemocyanin in the Plecoptera as outcomes of our research.

Moreover, we performed

Abstract

(in italiano)

Diversamente dalle conoscenze attuali sulla presenza delle proteine respiratorie negli insetti, è stata recentemente ritrovata emocianina in larve ed adulti delle specie di plecotteri *Perla marginata* in *P. grandis.* Allo scopo di verificare se la presenza di questo carattere antico è diffuso all'interno dell'ordine e per indagare sulle motivazione per cui i plecotteri lo hanno mantenuto, abbiamo esteso la ricerca dell'emocianina all'interno di questo gruppo. In special modo, abbiamo comprovato la presenza in larve ed adulti nelle sette famiglie della fauna europea, ed inoltre abbiamo saggiato qualche taxa della fauna orientale e sudafricana, caratterizzate da condizioni ecologiche differenti rispetto a quelle dei fiumi paleartici.

Abbiamo clonato e sequenziato cDNA corrispondenti al frammento conservato di emocianina (nel dminio II), ed abbiamo studiato la loro espressione con tecniche di PCR. Per accertare la presenza di emocianina come prodotto di traduzione, si è utilizzato un metodo altamente sensibile basato sulla spettrometria di massa. Infine la presenza dell'emocianina è stata analizzata in chiave filogenetica.

Sulla base dei risultati ottenuti, abbiamo messo evidenza che l'emocianina sembra non essere uniformemente distribuita all'interno dell'ordine. Le relazioni filogenetiche, l'induzione ambientale ed alcuni caratteri biologici, come la grandezza delle larve, la durata del ciclo biologico ed il ruolo trofico, sono state discusse come possibili fattori che possono essere correlati con la presenza o l'assenza dell'emocianina nelle specie studiate. Inoltre sono state considerati le possibili multifunzioni dell'emocianina. Nel presente lavoro viene raccolto lo stato di attuale conoscenza sul tema dell'emocianina nei plecotteri, frutto di questa ricerca.

Index

Molecular approach to the study of hemocyanin in stoneflies (Plecoptera): a biologic and phylogenetic interpretation.

Summary

This summary has the intention to trace a global view to the whole research I done during the three years of my European PhD. The thesis I present consists of an introductive and conclusive chapter and of some scientific articles (published and in progress). Scientific articles had to be independent sections that point out a complete step of the investigation. This is the reason why, in a general view, the introductive parts of each article might seem a little similar. The last paragraph of scientific articles part (new data on the presence of hemocyanin in Plecoptera: recomposing a puzzle) is comprehensive of all data we have at the present time. Here a wide analysis is done considering all outcomes obtained during the study and taking into account hypothesis to test in a future perspective.

The present research is on hemocyanin, so called respiratory protein, in the Plecoptera order (Hexapoda). The essential role of O_2 transport proteins is clearly underlined by the independent evolution of analogous carriers. Hemoglobins, hemerythrins and hemocyanins, although extremely different in structure and origin, share convergent functions across animal phyla. Several reviewers have differently studied Arthropod hemocyanin in order to describe different aspects, such as structural features and the quaternary architecture of hemocyanin oligomers; to disclose the balance between functional constraints and adaptive modulation of the O_2 binding physiology; to investigate hemocyanin in Hexapoda subphylum and to trace the evolutionary pathway that carried to the current complexity. Detailed structural analyses 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, 1979b; Markl, 1986; Markl and Decker, 1992).

Hemocyanins sequencing has provided a clear identification of distinct subunits in several species, allowing to work out a precise definition of subunit families and of their phylogenetic relationships (Burmester, 2001). Numerous studies have investigated the origin and the structure of the type 3 copper-binding protein domains that represent the characteristic fold of the hemocyanin subunits (Linzen et al., 1985; Salvato and Beltramini, 1990; van Holde et al., 2001; Decker and Terwilliger, 2000; Jaenicke and Decker, 2004). Quaternary arrangement and the different oligomeric assemblages have been reviewed to delineate reliable models for the different hemocyanin structures (Markl and Decker, 1992; Magnus et al., 1994; Fochetti et al., 2006; Decker et al., 2007). Other authors have evaluated the functional properties of hemocyanins with particular consideration

of the close links between physiological plasticity and environmental selection (Truchot, 1992; Terwilliger, 1998). Studies have dealt with the functionality of hemocyanins, considering their primary role as oxygen carriers as well as their non respiratory functions such as phenoloxidase and antimicrobial activity (Terwilliger, 1998; Bridges, 2001; Lee et al., 2003; Lee et al., 2004; Decker and Jaenicke, 2004; Jaenicke and Decker, 2004). Finally, subunit types have 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 hemocyanins. The wide sequence database, including sequences of crustaceans, insects, chelicerates, myriapods and onychophorans (Kusche et al., 2002) has allowed the 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). Recent studies showed that arthropod hemocyanin is not only a feature of Crustacea Malacostraca (Volbeda et al., 1989; Kusche and Burmester, 2001a), Myrapoda (Kusche and Burmester, 2001b; Kusche et al., 2003) and Chelicerata (Decker and Rimke, 1998; Martin et al., 2007) but also of some Hexapoda (Sánchez et al., 1998; Hagner-Holler et al., 2004) and the research to understand hemocyanin distribution across the subphylum is actually in progress (Burmester et al., 2007).

Beside a preliminary and uncertain clue of hemocyanin in embryonic hemolymph of grasshopper *Schistocerca americana* (Sánchez et al., 1998), the first evidence of hemocyanin in Hexapoda was reported for the stoneflies *Perla marginata* (Hanger Holler et al., 2004). Successively it was roughly assumed that hemocyanin was present across the whole order (Burmester and Hankeln, 2007).

Although hemocyanin function or multifunction in insect has to be still verified, the discovery of hemocyanin protein in the Plecoptera brought up a new and interesting insight on the evolution of respiratory proteins in the arthropods. These data seem to indicate that insect respiration may be more complex than previously thought, involving in some case both the tracheal system and oxygen carriers. The hypothesis of many authors (Beintema et al., 1994; Burmester, 2001, 2004; Terwilliger et al., 1999) is that hemocyanin is the respiratory protein of ancestral arthropods. Starting from this point, insects hemocyanins have lost the capacity to bind oxygen after divergence from Crustacea, evolving into hexamerins, which are storage proteins.

Plecoptera is a small order of hemimetabolous insects composed by about 3500 described species (Fochetti and Tierno de Figueroa, 2008a). It is a very interesting group because it retains many ancient features and is considered a key point for evolutionary inference of insects. Even if it is not yet possible to certainly assign its phylogenetic position and its relationship with the extant insect orders, many morphological features of Plecoptera are primitive and suggest that stoneflies may be

the sister-group of the extant Neoptera (Zwick, 2000; Fochetti and Tierno de Figueroa, 2008b). It has been proved that *P. marginata* (Hagner–Holler et al., 2004) and *Perla grandis* (Fochetti et al., 2006) have retained hemocyanin protein, despite they have an efficient tracheal system and abdominal gills. This confirms their ancestral position and the basal role respect to others Neoptera.

In this study we furthered the search of hemocyanin across the order of Plecoptera. We investigated 33 species globally, belonging to the European (Perlidae; Perlodidae; Chloroperlidae; Leuctridae; Taeniopterygidae; Nemouridae, Capniidae) and Oriental families (Perlidae, subfamily Perlinae, and Peltoperlidae) and some to African Notonemouridae species. We studied different steps of life cycle (nymphs and adult), and we considered various ecological conditions. Techniques of sequencing (mRNA), proteomics and use of software programs have been our work-tools.

We started to study hemocyanin genetic expression with sequencing analysis on nymphs of European fauna. We were very surprised that the hemocyanin was not uniformly present in all species. On the basis of our outcomes, we hypothesized a functional role of the hemocyanin only in observed Perloidea (*Dinocras cephalotes, Isoperla grammatica, P. marginata* and *P. grandis*). In all the investigated Nemouroidea and in *Siphonoperla torrentium* (Perloidea), this protein was probably lost. We then considered as possible explanations of these different physiological requirements of hemocyanin, the larval size range, the life-cycle length, the trophic role, the environmental induction and the phylogenetic relations. The results of this study are published in Amore et al. (2009a).

In order to verify the above mentioned hypothesis we chose sampling sites which cover Plecoptera biodiversity in species and environmental adaptations and we studied both nymphs and adults. We collected species of seasonal Mediterranean streams, tropical rivers and European mountain rivers and lakes, including wide distribution or endemic species, steno- or eurytherms species, of rhithron or potamon zones.

Firstly we compared Perlodidae (*Guadalgenus franzi* and *I. grammatica*) and Taeniopterygidae (*Brachyptera risi* and *B. vera*) species of Italian perennial rivers and Mediterranean seasonal streams of the southern Iberian Peninsula. Presumably, seasonal water species live under a major stress condition compared to the species living in perennial rivers. Seasonal streams are formed annually and expand during a short period as a consequence of melting snow and spring rains. It is presumed that life-history strategies of these species were greatly influenced by the characteristics of their environment (López-Rodríguez et al., 2009). *G. franzi* as others Perlodidae, harbours hemocyanin, but we did not find any difference between the two *Brachyptera* species. These preliminary results, let us suppose that, even if environment is an important element for local adaptations, protein expressions at molecular level cannot be understood without considering

species phylogeny. We did not detect hemocyanin in any adults, even in those species that showed it at larval stage. Nonetheless, it is to be noted that hemocyanin was recorded in adults of *P. marginata* (Hagner-Holler, 2004) and *P. grandis* (Fochetti et al., 2006). The results of this second study are published in Amore and Fochetti (2009).

Subsequently we analyzed six species of the subfamily Perlinae (Perlidae) and one species of the family Peltoperlidae, all belonging to the oriental tropical fauna, which has never been analyzed before. The reason why we decided to extend the study to Tropical Asian species is to analyze taxa that live in environments with different ecological traits respect to the previously studied ones (Palaearctic fauna). Tropical streams are characterized by seasonal and daily temperature stability of about 20 °C (Dudgeon, 1999). Here there are lower concentrations of dissolved oxygen than in European mountain and perennial rivers. In this group of species we did not find hemocyanin, but only hexamerins, which are proteins strictly related to hemocyanins. The lack of hemocyanin in Oriental Perlidae and Peltoperlidae allowed us to discard the hypothesis that the presence of hemocyanin, although in at basic amount, depends on the animal's size and life cycle. In fact European and Oriental species present, more or less, the same size and apparently display a similar life cycle. On the other hand, it suggest a multifunctionality of the protein in stoneflies nymphs. It seems unlikely that a protein involved in such a basic function as cells respiration, may not be needful for some groups, except if it is assumed that respiratory function is not the unique or main work that the hemocyanin can carry out (Amore et al., 2009b). Hemocyanin could play multiples but not exclusive functions. There is a large literature that describes phenoloxidase activity of chelicerate hemocyanins (Paul et al., 1994; Decker and Rimbke, 1998, Decker and Tuczek, 2000; Nagai and Kawabata, 2000; Nagai et al., 2001). *In vitro* experiments in Crustacea proved hemocyanin's role in the immune response and molting, (Destoumieux-Garzò et al., 2001; Lee et al., 2003; Pan et al., 2008).

Arthropod hemocyanin could be a putative multifunctional protein. Further experiments (*in vivo* and *in vitro*) are to be done for insects to verify whether others than respiration activities of the hemocyanins (Hagner-Holler, 2004) are an *in vitro* artifact or not.

In order to enrich the overall biodiversity picture of the species analyzed so far within the order, we checked stonefly nymphs and adults of European mountain rivers and lakes. Finally we inferred an evolutionary history of hemocyanin superfamily in Plecoptera and at least we enlarged the study to this complex superfamily of proteins in Arthropod Phylum (NJ and ML).

Our findings showed a discontinuous presence of hemocyanin among nymphs of different stonefly species. We considered, as possible hypothesis, that hemocyanin character may have disappeared several times during the evolution of the order. A first time might have happened in a Nemouroidea

ancestor (superfamily), where in fact we never found traces of hemocyanin. Secondarily in Perloidea (superfamily), hemocyanin might have been independently lost within families (Chloroperlidae), or genus (Perlodes). This idea is also supported by the fact that present families of Plecoptera do not seem to be very old. There are evidence of recent and repeated phenomena of speciation and extinction (Zwick, 2000), although it is a very ancient order (fossils from early Permian).

Furthermore, it is noteworthy that the hemocyanin conserved region acts like a phylogenetic molecular marker within Plecoptera. We confirmed the presence of two hemocyanin subunits (identified as hc1 and hc2) and verified that the pattern of hemocyanin evolution follows the accepted scheme of traditional phylogeny based on morphology and anatomy. On the contrary we found that hexamerins follow the same systematic relationship in a more irregular way. It indicates that a lower evolutive pressure exerted over them allowing to cumulate mutations and so a differentiation on the amino acidic sequences (Telfer and Kunkel, 1991; Burmester et al., 1998).

The sequencing and phylogenetic analysis developed in this study, is mainly focused on Arctoperlaria species, and above all on European fauna. In order to give a wider overview to the hemocyanin problematic in the Plecoptera order the study should be also extended to Antactoperlaria suborder, a goal we propose us for the future.

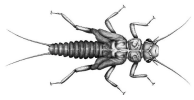
Considering the whole Phylum of Arthropods, the phylogenetic analysis carried on the hemocyanin superfamily (prophenoloxidases, pseudohemocyanins, hexamerins, dipteran hexamerin receptors and hemocyanins) supports the Pancrustacea hypothesis according to which all crustaceans and hexapods are included in a unique monophyletic taxon, unlike the Atelocerata hypothesis in which Myriapoda and Hexapoda are sister taxa, and Crustacea are only distantly related (Brusca and Brusca, 2002).

The discontinuous presence of hemocyanin observed in transcriptional material (mRNA), arose the problem of the effective presence of the protein. So far, the presence of the protein has been reported only for *P. marginata* (Hagner-Holler et al., 2004). In order to verify the expression of hemocyanin to another species a part from *P. marginata*, we analyzed SDS-PAGE bands of expected size from *D. cephalotes* and proceeded with protein identification via MS/MS. We confirmed that, as well as for *P. marginata*, hemocyanin is expressed in *D. cephalotes* hemolymph. It suggests that in Plecoptera species, whenever hemocyanin is detected as transcript mRNA sequence, this can be effectively translated into protein to be ready for physiological demand of the animals (Amore et al., 2009c).

In a subsequent study we extended the search for the protein to different stages of life cycle (nymph and adult) of a representative species' sample of the two European Plecoptera fauna subfamilies. A

first step consisted in performing western blotting analysis with a commercial antibody synthesized on *Limulus polyphemus* hemocyanin. These experiments did not produce good results probably because of the presence of multiple epitopes in the commercial antibody. Subsequently we proceeded with the study of an appropriate epitope based on a multiple alignment of known Plecoptera sequences, for the production of a specific antibody (in rabbit). Our purpose was to generate a screening tool for the detection of both hemocyanin subunit types (hc1 and hc2), to be used across the whole Plecoptera order. So far, immuno-blotting experiments have been made by using rabbit's bleeding samples on the positive control, *P. marginata*. The final purified antibody is expected on February 2010. It is interesting to add that in the last months, researches on Hexapoda hemocyanins disclosed new mRNA sequences (Pick et al., 2009a; 2009b). A comparative analysis shows that the epitope chosen for our antibody, is a very conservative amino acid sequence in Plecoptera and in all known sequences belonging to Collembola, Zygentoma, Phasmida, Blattodea, Isoptera. Starting from these bibliographic information, our anti-hemocyanin antibody has the potentiality to be a good tool for a rapid screening of hemocyanin's presence throughout species in many Hexapoda orders.

Hemocyanin is a free dissolved protein in hemolymph (Terlfer and Kunkel, 1991), and it is subject to expression regulation depending on animal needs. A rapid change in hemocyanin concentration provides an adaptive potentiality towards gradual or sudden environmental stimuli, that can be represented by special extrinsic (hypoxic exposure, ph, salinity, temperature range, microhabitat type) or intrinsic (starvation, presence of allosteric factors) conditions. The marked variation in hemocyanin synthesis and catabolism, naturally occurring in several Crustacea, probably contributes to adaptive physiological processes also involved in the successful colonization of extremely diverse habitat (Giomi and Beltramini, 2007). Experiments aimed at monitoring adaptive physiology of Plecoptera in response to environmental stimuli, at the level of protein expression modulation and subunit ratio, are in progress with quantitative real-time PCR (qRT-PCR). If oxygen affinity and cooperativity of hemocyanin, consequently oxygen-transport capacity, are tools for adaptation to the environmental conditions, owning hemocyanin represents for animals, a potential adaptative capacity in global warming. In this futuristic context, the presence of hemocyanin, and its variability in subunit types and multimeric formation, may represent a focal aspect to be analyzed also in a view of ecological selection (Schluter, 2001).



1. Introduction

The indispensable role of O_2 transport proteins is clearly remarked by the independent evolution of analogous oxygen carrier proteins. In the animal kingdom they were invented three times. Hemoglobins, hemerythrins and hemocyanins, although extremely different in structure and origin, share convergent functions across animal phyla (Mangum, 1985). Hemocyanins are the respiratory proteins of many arthropod and mollusc species (van Holde et al. 2001; Burmester, 2002). The unique protein name used for both mollusc and arthropod hemocyanins may led to misunderstandings. Mollusc and arthropod hemocyanins are very different in their quaternary architecture and subunits organizations, but they share a basic motif, typical of type 3 copper proteins. From an evolutionary point of view, a possible explanation is that, from a common ancestor, branches of these copper protein type, had segregated into proto-molluscan and proto-arthropodan hemocyanin lines. According to the more reasonable assumptions, this separation is estimated from the beginning of Cambrian, a time expected from 550 MYA (Gu, 1998) to 800-1000 MYA (Wray et al., 1996). Successively, proteins underwent to independent evolution to yield the quite different sequences found today, forming two lineages of molluscan hemocyanins and arthropodan hemocyanins (van Holde, 2001).

In this study we only refer to arthropod hemocyanins.

Several reviewers have studied arthropod hemocyanin in order to describe different characteristics such as structural features and three dimensional architecture of hemocyanin oligomers, to disclose the balance between functional constraints and adaptive modulation of the O_2 binding physiology. Detailed structural analyses 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, 1979b; Markl, 1986; Markl and Decker, 1992). The wide sequence database, about 50 sequences of crustaceans (Volbeda et al., 1989; Kusche and Burmester, 2001a), and others from insects (Sánchez et al., 1998; Hagner-Holler et al., 2004; Fochetti et al., 2006), chelicerates (Decker and Rimke, 1998; Martin et al., 2007), myriapods (Kusche and Burmester, 2001b; Kusche et al., 2003), and onychophorans (Kusche et al., 2002) permitted to trace the evolutionary scenery that have led to the current complexity and the diverse molecular evolution among the taxa (Voit et al., 2000; Burmester, 2001; Terwilliger and Ryan, 2006).

In the last few years the study of arthropod hemocyanins is at the centre of a intense research to investigate this protein across Hexapoda subphylum.

In 2004, a hexameric hemocyanin was identified in different life cycle steps of *Perla marginata* and its respiratory function was proved by kinetics methods (Hagner-Holler et al., 2004). It was the first clear evidence of a hemocyanin in insect, even if, previously, an embryonic hemolymph protein (EHL) had been detected in grasshopper embryo of *Schistocerca americana* as a putative hemocyanin, basing on phylogenetic and multivariate analysis (Sánchez et al., 1998). The finding of a respiratory metal containing protein able to bind O_2, was a novelty respect to the accepted theory on insect respiration. In fact it is commonly known that gas exchanges in insects are usually mediated through tracheae and tracheoles. These are tubular structures whose role is to connect the inner organs with the air, thus enabling diffusion of oxygen to the metabolically active tissues (Brusca and Brusca, 2002).

Plecoptera is a very interesting group because it has many ancient features and is considered a key point for evolutionary inference in insects. Even if actually it is no possible to certainly establish its phylogenetic position, and its relationship with the extant insect orders, many morphological features of Plecoptera are primitive and led to hypothesize that Plecoptera may be the sister-group of the extant Neoptera (Zwick, 2000).

In this study we wanted to enhance the search of hemocyanin within the order of Plecoptera. We verified the presence of this protein and discussed possible explanations of the resulting complex framework in an environmental and phylogenetic key. We considered ecological and autoecological parameters that can induce variations in physiological requirements of specimens and that can constitute differences in adaptative response within the Plecoptera biodiversity. We wanted to verify four principal hypotheses:

1. **larval size and life cycle**. Plecoptera size varies consistently between the two European superfamilies of Perloidea and Nemouridea. Perlidae and in a lower proportion Perlodidae nymphs (both Perloidea), have a consistent size, up to 4 cm in length, and they are very sensitive to oxygen levels in cold running waters, while Taeniopterygidae, Nemouridae, Leuctridae, Capniidae (belonging to Nemouridea) and Chloroperlidae (Perloidea), have nymphs of a smaller size, from 0.2 to 1 cm. Animal size can be correlated to the capacity of oxygen diffusion. The tracheal system may be sufficient for the smaller species to their oxygen request in water, but could be insufficient for the bigger ones. Larval size is a feature strictly related to life cycle. It

must be noted that in small size species, a complete life cycle is achieved in about one year (univoltine), while species of larger size, like Perlidae, need two or three years to develop (semivoltine). The diverse time of development and growing of nymphs, lets suppose that a different hemocyanins requirement could be necessary to comply with the life cycle, with a major energetic needs for the semivoltine species.

2. **Trophic roles**. The heterogeneity of Plecoptera adaptation to their habitat is also expressed through differences in diet. In fact, Perlidae and Perlodidae are mainly predators, while Nemouroidea are phytophagous or detritivourus. So they grow and develop on different feeding research. Predators may have major need of oxygen because of their increased activity.
3. **Environmental induction**. We hypothesized that Plecoptera larvae living in seasonal streams have to cope with heavier physiological stress than the species in perennial waters. For this reason we checked the presence of hemocyanin in European temporal water species, for Perloidea and Nemouroidea superfamily, in species of mountain lakes and rivers and we analyzed species of Oriental Fauna living at tropical conditions.
4. **Adults.** Besides larval stages we analyzed also adults. Plecoptera are hemimetaboulous insect whose ecological medium completely change when they become adults. While nymphs dwell in aquatic habitat, adult stoneflies emerge from fresh waters. They have reduced flight ability and can generally be found on the banks next to their previous habitat. The quantity of oxygen supply in air medium compared with the oxygen dissolved in water is very different and it marks a great change in physiological adaptation of the animals during its complete life cycle. In this study we have verified presence/absence in adult and the response to hemocyanin production.

We planned samplings to cover Plecoptera biodiversity in species, life stages and ecological conditions.

33 species have been studied: 25 species belong to the seven family of two European superfamily, 5 species belong to Oriental Perlidae and 1 Peltoperlidae, 2 species of African Notonemouridae. We analyzed species during different step of life cycle (nymphs and adults), and in various ecological conditions (perennial temperate rivers, European temporary streams, tropical rivers, high mountain rivers and lakes). Techniques of sequencing, proteomics and the use of software for data elaboration, were our work-tools.

Finally, we considered possible functions of hemocyanin in Plecoptera and we carry out phylogenetic inference, reconstructing molecular evolution within Plecoptera families and among Plecoptera and the others arthropod taxa.

2. A general overview

2.1 Plecoptera order

Plecoptera, commonly called stoneflies, are Pterygota, Neoptera, insects (Hexapoda). It is a small order of hemimetabolous insects, whose relationships with others orders of Exopterigota are poorly understood (Zwick, 2000). In fact, Plecoptera have retained many primitive characters, that confirm their ancestral position and the basal role respect to the rest of Neoptera; possibly they are the sister group of the remaining Exopterigota, and they could be an evolutionary link with the Ephemeroptera (Henning, 1981; Kristensen, 1991; Beutel and Gorb, 2001).

Plecoptera can be easily recognized by several morphological characters: soft body, three segmented tarsi, elongate filiform antennae, mandibulated mouthparts, two compound eyes, two or three ocelli, two usually long cerci, 10-segmented abdomen with vestiges of the eleventh segment. Adults have two pair of membranous large wings (sometimes reduced or absent), and sub equal fore and hind wings (hind wings slightly wider) that fold horizontally over and around abdomen when at rest, hence their name: plecos = folded; pteros = wings. (Fig. 2.1.1A). Nymphs are similar to adults (Fig. 2.1.1B), with a closed tracheal system with or without filamentous gills.

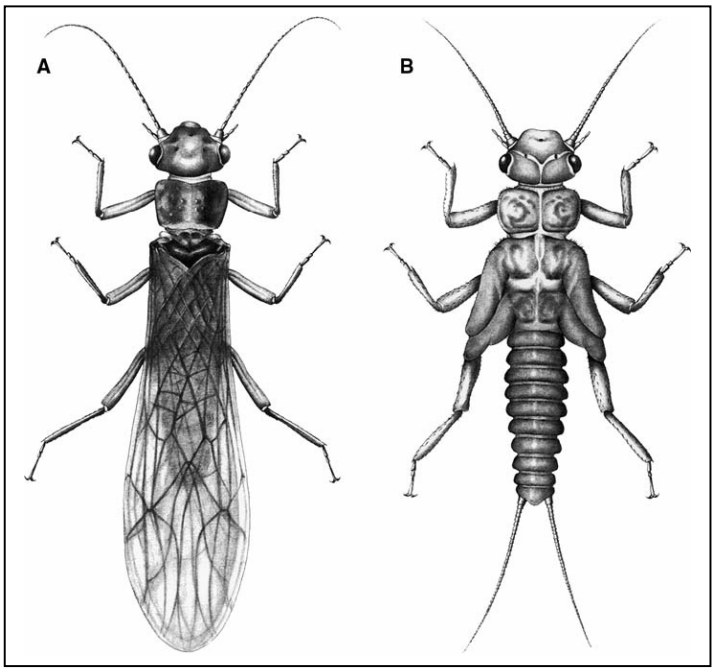


Figure 2.1.1. A-Adult of *Nemoura* and B-Nymph of *Nemoura*.

When present, gills are located on different parts of the body. Plecoptera nymphs are aquatic, and constitute a significant ecological component of running water ecosystems. They live mainly in cold, well-oxygenated running waters, although some species can also be found in mountain lakes. Few species are adapted to terrestrial life in Sub-Antarctic areas. Hynes (1976) reported the tendency of Southern hemisphere nymphs to leave the water. However, the increasing number of stoneflies described from the tropics and their high rate of endemicity, can modify the common belief that Plecoptera are cold-water specialists, and instead it may suggest that the true hot-spot for Plecoptera diversity are tropical areas (Zwick, 2000). The life cycle of stoneflies lasts for one or more years, but there are also bi- or tri-voltine species. Nymphal or egg diapause is not uncommon. The nymphs can moult up to 33 times before emerging. They feed on animal or vegetable matter as collectors, scrapers, shredders or predators. More than 3497 species has been described (Fochetti and Tierno de Figueroa, 2008). Their ecological requirements greatly limit the dispersal capacity of the nymphs and, because adults have reduced flight ability, stoneflies show a high percentage of endemism; nevertheless stoneflies are distributed over all continents except Antarctica.

The order includes 16 families whose relationships and biogeography have been studied by several authors (Henning, 1981; Kristensen, 1991; Zwick, 2000; Beutel and Gorb, 2001).

Here it is followed the more recent and widely accepted classification by Zwick (2000) and biogeography distribution resumed by Fochetti and Tierno de Figueroa (2008a) (Fig. 2.1.2 and Fig. 2.1.3). They recognize two large suborders: Antarctoperlaria, present only in the Southern Hemisphere, and Arctoperlaria, distributed mostly in the Northern Hemisphere. The first taxon includes four families; the latter includes 12 families belonging to two superfamilies (Systellognatha and Euholognatha) with six families each one.

In Europe are present 7 families (Perlidae, Perlodidae, Chloroperlidae, Taeniopterygidae, Nemouridae, Capniidae and Leuctridae), 35 genera and 426 species (Fochetti and Tierno de Figueroa, 2004).

The thirty-seven percent of the European genera are monospecific, whereas the four more diversified ones (*Leuctra*, *Protonemura*, *Nemoura* and *Isoperla*) include approximately 70% of the European stonefly species.

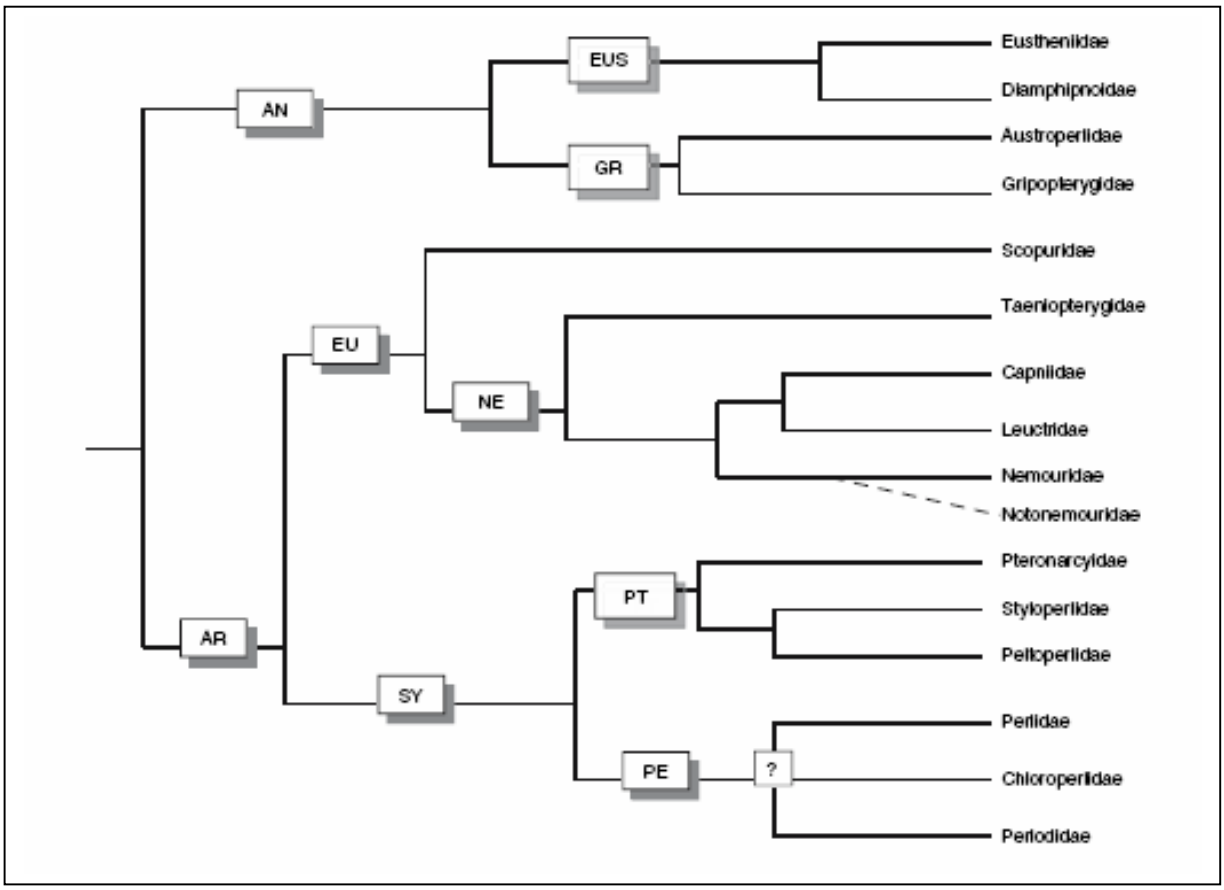


Figure 2.1.2. Plecoptera phylogeny according to Zwick (2000). AN:Antarctoperlaria; AR: Arctoperlaria; EUS: Eusthenioidea; GR: Gripopterygoidea; EU: Euholognatha; NE: Nemouroidea; SY: Systellognatha; PT: Pteronarcyoidea; PE: Perloidea (from Fochetti and Tierno de Figueroa, 2008a).

Both Zwick (1973) and Nelson (1984) list Nemouridae and Notonemouridae as sisters taxa. This is an interesting arrangement because the nemourids are uniquely northern hemisphere in distribution and the notonemourids are uniquely southern hemisphere in distribution. This is the only pattern of this sort at the family level in Plecoptera. Nemouridae and Notonemouridae are then placed as sister to Capniidae and Leuctridae (Fig. 2.1.2).

Peltoperlidae (four Palaearctic genera) have a Nearctic-Asian distribution and together with Styloperlidae and Pteronarcidae are considered sister group of Perloidea.

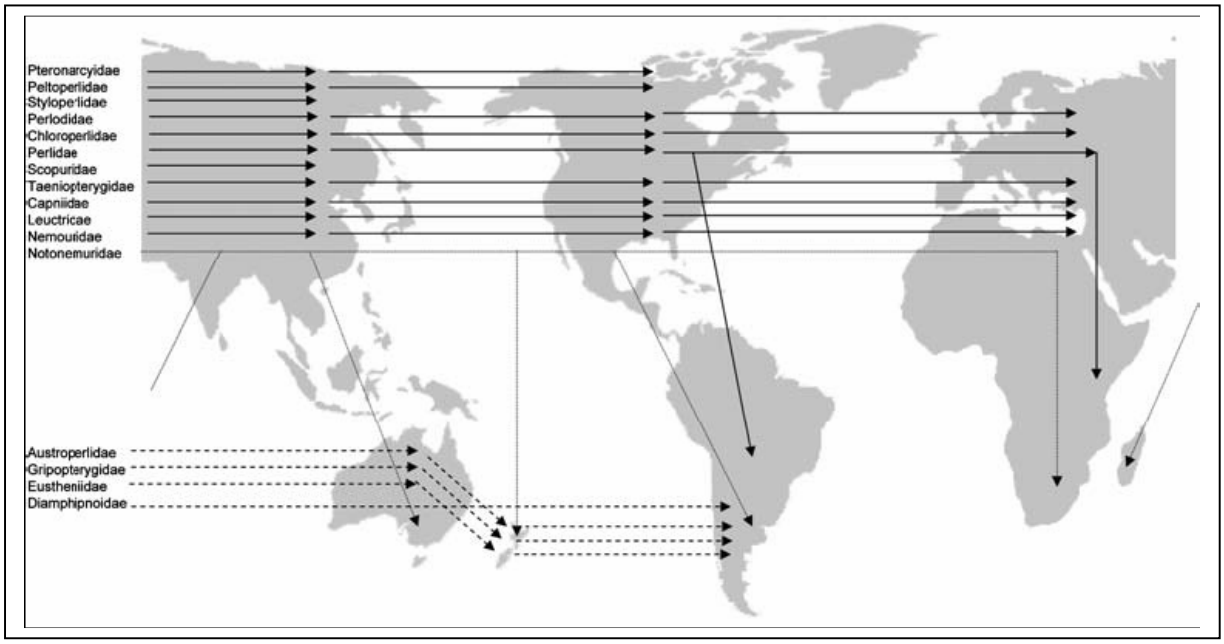


Figure 2.1.3. Plecoptera families distribution; Arctoperlaria = continuous line; Antarctoperlaria = dotted line (from Fochetti and De Figueroa, 2008a).

2.1.1 Studied species

To cover the variability of possible adaptative responses of species to ecological condition we plain to sample specimen representatives of diversity of species and habitats.

European fauna:

Species of perennial rivers:

Perlidae:

Perla marginata (Panzer, 1799)

Perla grandis (Rambur, 1841)

Dinocras cephalotes (Curtis, 1827)

Chloroperlidae

Siphoperla torrentium (Pictet, 1841)

Perlodidae:

Isoperla grammatica (Poda, 1761)

Perlodes intricatus (Pictet, 1841)

Dyctiogenus alpinum (Pictet, 1842)

Leuctridae

Leuctra fusca (Linnaeus, 1758)

Capniidae

Capnia bifrons (Newman, 1839)

Taeniopterygidae

Taeniopteryx stankovitchi Ikonomov, 1978

Brachyptera risi (Morton, 1836)

Nemouridae

Nemoura hesperiae Consiglio, 1960 ***(Italian endemic)

Nemoura cinerea (Retzius, 1783)

Protonemura tuberculata (Despax, 1929)***(Pyrenees endemic)

Protonemura ausonia (Consiglio, 1955)

Amphinemura sulcicollis (Stephens, 1836)

Species of seasonal streams:

Perlodidae:

Besdolus ravizzarum Zwick and Weinzierl, 1995

Guadalgenus franzi (Aubert, 1963)* (Iberian Peninsula endemic)

Taeniopterygidae:

Brachyptera vera Berthelemy and Gonzalez del Tanago 1983 * (Iberian Peninsula endemic)

Species of high altitude (mountain rivers and lakes).

Perlodidae:

Arcynopteryx compacta (McLachlan, 1872)

Isoperla acicularis (Despax,1936) ssp. *acicularis* **(Pyrenees endemic)

Isoperla viridinervis (Pictet, 1865) ** (Pyrenees endemic)

Leuctridae:

Pachyleuctra benllochi (Navás, 1917) **(Pyrenees endemic)

Leuctra alosi Navás, 1919 * (Iberian Peninsula endemic)

Oriental and African stoneflies.

Species of tropical rivers:

Perlidae

Neoperla sp., Needham, 1995

Caroperla sp., Kohno, 1946

Phanoperla sp., (=Dyaperla Banks, 1939)

Togoperla sp., Klapálek, 1907

Tetropina sp., Klapálek, 1909

Etrocorema sp., Klapálek, 1909

Peltoperlidae

Cryptoperla sp., Needham, 1909

Notonemouridae

Afronemura amatolae, (Balinsky, 1956)

Aphanicella bullata, Stevens and Picker, 1999

2.2 Respiration in insect

Animals that live under aerobic conditions consume large amounts of O_2, which is mainly used to sustain the production of ATP in the respiratory chain of the mitochondria. In Protozoa and small Metazoa, simple diffusion is usually considered sufficient for the supply of the inner layers of the body with O_2. Larger animals, however, require a variety of anatomical, physiological, and molecular adaptations that enhance the O_2 delivery to the cells and eventually to the mitochondria. These adaptations comprise respiratory organs, such as gills or lungs, circulatory systems, as well as respiratory proteins for transport or storage of O_2 (Willmer et al, 2000).

The largely impermeable cuticle of insect and other arthropods constrains the uptake of O_2 by diffusion across the body's surface. Terrestrial insects and myriapods (Myriapoda) acquire O_2 by the aid of tracheae. The tracheal system consists of highly branched air-filled tubes that connect the inner tissues with the atmosphere (Fig. 2.2.1) (Brusca and Brusca, 2002). Aquatic insects usually have other specialized respiratory organs such as gills, tracheal gills or a plastron (Deavis, 1988). It is largely retained that, within the tracheal system, O_2 is distributed through the body mainly by passive diffusion (Deavis, 1988; Brusca and Brusca, 2002), although some active breathing may occur, for example some big Orthoptera open selectively the fore and the back stigma with the effect of a air current, aiding the tracheal system (Deavis, 1988). O_2 uptake by the cells takes place mainly at the tips of the smallest branches, the tracheoles, which have only a thin cuticle. In highly active organs such as the insect flight muscle, the tracheoles may even enter the cells and connect with the mitochondria.

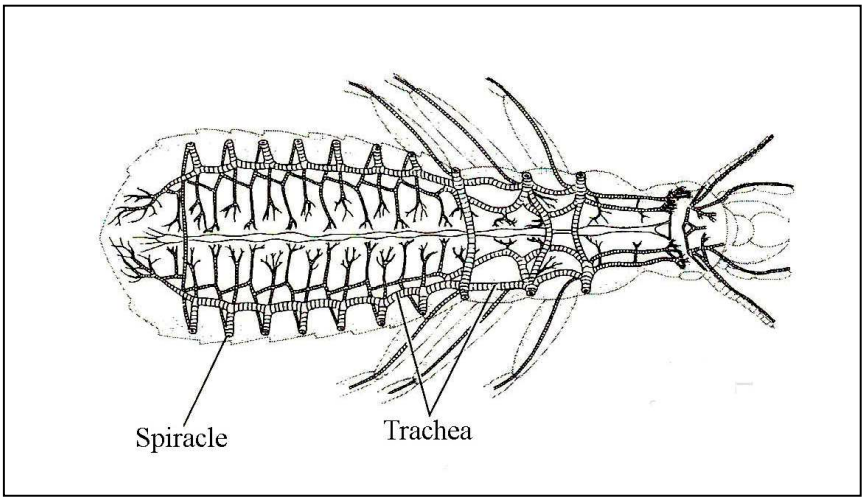


Figure 2.2.1.
General scheme of insect tracheal system
(from Brusca and Brusca, 2002).

Due to high diffusion rates and capacity coefficients, O_2 is delivered about 200,000–300,000 times more efficient in the tracheal air than in the aqueous environment of the hemolymph or blood (Kestler, 1985). These features make the air-tracheal system an extremely efficiently transport apparatus, which has been thought to comply with the O_2 requirements of even the largest insect known on earth. Therefore, until recently, the occurrence of respiratory proteins, that would enhance O_2 supply, has been largely unknown and considered unnecessary (Mangum, 1985; Law and Wells, 1989; Willmer et al., 2000). Only a few insect species that live in aquatic and hypoxic environments, as the horse botfy genus *Gasterophilus* (Weber and Vinogradov, 2001), chironomid midge larvae (Chirinomidae) (Lee et al., 2006), the aquatic backswimmers (Hemiptera: Notonectidae) (Matthews and Seymour, 2008), were known to harbor hemoglobin in their hemolymph or tissues.

However, in recent years it has become evident that O_2 transport linked to protein carries is much more widespread among insects than previously thought (Burmester and Hankeln, 1999; Hankeln et al., 2002, 2006; Hagner-Holler et al., 2004; Fochetti et al., 2006; Pick et al., 2008, 2009a).

2.3 Respiratory proteins

Respiratory proteins reversibly bind molecular O_2 for the purpose of transport or storage. They enhance the O_2 transport capacity of the body fluid, facilitate intracellular O_2 diffusion or enable O_2 storage for long or short-term periods.

In the animal kingdom, three types of metal containing respiratory proteins are known. They are: hemerythrins (He), hemoglobins (Hb), and hemocyanins (Hc).

Hemerythrins. Hemerythrins found exclusively in blood cells, occur in four marine invertebrate phyla, that are usually regarded as fairly closely related: sipunculids (Sipuncula), priapulids (Priapulida), brachiopods (Brachiopoda), and some annelid worms (Annelida) (Vinogradov, 1985; Klippenstein, 1980). The O_2 site, an iron dimer, is located in the middle of four virtually parallel α-helices. This proteins are constituted by monomers of 113-118 amino acids and molecular weight of about 13,5-13,9 kDa. The most frequent multiple is an octamer of about 110 kDa (Magnum, 1985).

Hemoglobin. Hemoglobin is a small protein, usually consisting of about 140-150 amino acids, that comprise eight α-helical segments (named A-H). It displays a characteristic 3-over-3 α-helices sandwich structure (Dickerson and Geis, 1983), which includes an iron-containing the

heme group (Fe^{2+}-protoperphyrin IX). The $Fe^{2+}$is connected to the globin chain via the proximal histidine named F (because is the 8^{th} amino acid of the helix F). The gaseous ligand, typically O_2, but also carbone monoxide (CO) or nitric oxide (NO), can bind between the Fe^{2+} ion and the distal amino acid at position E7. Hbs are phylogenetically ancient molecules, whose complex evolution is demonstrated by their widespread occurrence in bacteria, fungi, plants, invertebrate and vertebrate animals (Weber and Vinogradov, 2001). Although the various hemoglobins may display only little sequence resemblance, tertiary structures are usually conserved. Invertebrate hemoglobins may assemble to multimers of up to 144 subunits or domains. Hemoglobins are involved in many different aspects of O_2 supply. In almost all vertebrates and many invertebrates, hemoglobin is present in the circulatory system, may either be contained in specialized cells (erythrocytes) or is freely dissolved in the blood or tissues.

Hemocyanins. Hemocyanins are the respiratory proteins of many arthropod and mollusc species (van Holde et al., 2001; Burmester, 2002). In both phyla, the oxygen-binding site involves a pair of copper atoms, which are in the Cu^{+1} state in the reduce form but become Cu^{+2} upon oxygenation, binding the oxygen as O_2^{-2}. The active site, centre of the reaction, is surrounded by a strong hydrophobic environment. This change accounts for the blue color developed upon oxygenation. Arthropod hemocyanin is found as single (1x6mers) or multiples of hexamers (2x6mers, 4x6mers, 6x6mers, 8x6mers). Sequence analysis shows that an arthropod hemocyanin may contain several variants of the monomers. Each arthropod hemocyanin monomer, of about 72 kDa, folds into tree domains characterized by different folding motifs (Volbeda and Hol, 1989): domain I with five or six α-helices; domain II with four α-helices bundle and the active site containing two copper ions; and domain III with a seven stranded antiparallel β-barrel. In contrast, molluscan hemocyanin are cylindrical decamers, dodecamers or multidecamers, of about 350-400 kDa polypeptide subunit. This folds into a chain of seven or eight functional unit (FU), each one composed of two different structural domains: α-helix domain, formed by α-helix bundle carrying the copper active site and functionally correspond to domain II of arthropod; and β-sandwich domain, formed by six-stranded antiparallel β barrel functionally correspond to domain III of arthropod (Cuff et al., 1998) (Fig 2.3.1). So, arthropod and molluscan hemocyanins are very different and molecular structure at all levels, but they are similar in the coordination of copper via histidine ligands and the way in which oxygen is bound.

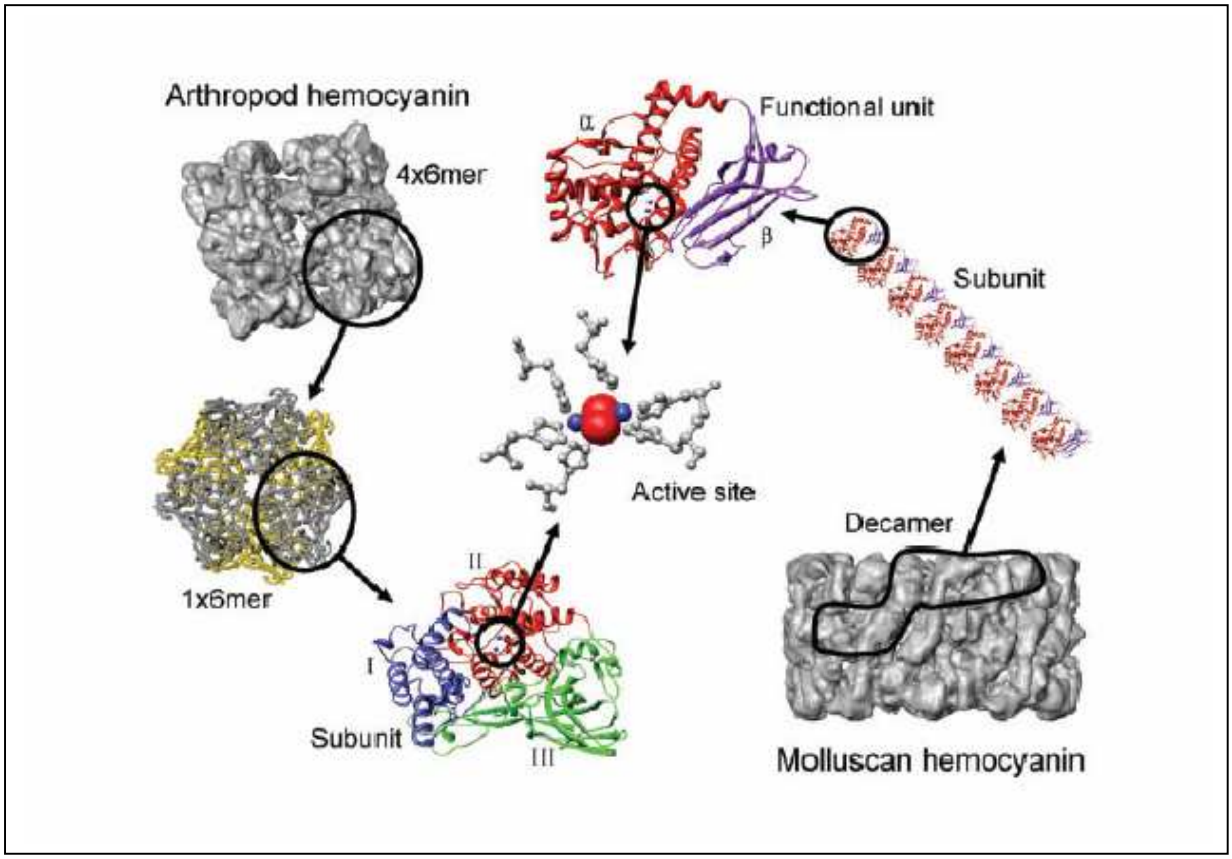


Figure 2.3.1. Structural levels of arthropod and molluscan hemocyanins. Note that in the case of arthropod hemocyanin, the subunit polypeptide carries a single active site, whereas in molluscan hemocyanin, the subunit polypeptide contains seven or eight functional units (FUs), each with an active site (from Decker et al., 2007).

Especially the 3-D arrangement of the group involved to the active site necessary to bind dioxygen is conserved. It is formed by the typical structure of type 3 copper proteins, that are protein having binuclear centre consisting of two copper atoms, named CuA and CuB, each one coordinated by three histidine residues. The adjacent amino acid residues surrounding each copper atom of the binuclear active site are generally very conserved. Among various hemocyanins, high sequence identities are found around CuB: two of the three histidines are located on one α-helix, whereas the third one is derived from another α-helix. By contrast, two different CuA sites, one for arthropods and one for molluscs, are observed: the CuA site of arthropods is similar to CuB, whereas in the CuA site of mollusc Hcs, two histidines are located in two α-helices and the third one is provided by a loop following one of the α-helices.

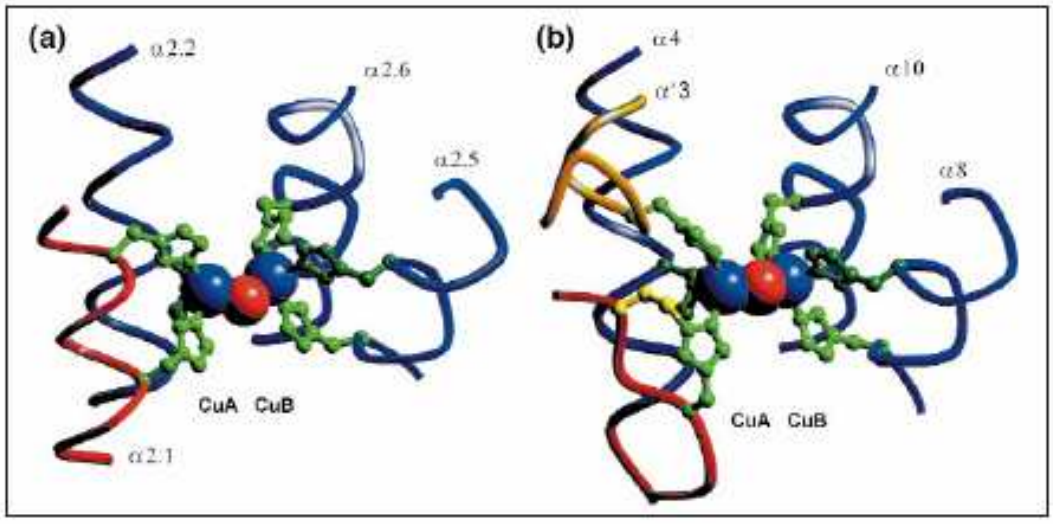


Figure 2.3.2. Comparison of the active sites of hemocyanins (Hcs) from arthropods and molluscs. The oxygen-binding sites of the Hcs of the horseshoe crab *Limulus polyphemus* (a) and the octopus *Octopus dofleini* (b) are oriented by superimposing CuA, CuB (both blue) and the a helices a2.5 and a2.6 from *Limulus* hemocyanin (Hc) and α8 and α10 from *Octopus* Hc, which provide the three histidines (green) binding to CuB. The environment of CuA is different for the two Hcs, although one of the two a helices of *Limulus* Hc (2.2) is superimposable with α4 of Octopus Hc, which provides one of the three histidines coordinating CuA. The other two histidines are provided by a helix α2.1 (Limulus Hc, red) or by loops (Octopus Hc, yellow and red). Histidine2562 is covalently stabilized by its bond with Cysteine2560 (yellow). The active site of the hemocyanins shows dioxygen (red) bound between two copper (blue). Each copper atom is ligated by three histidines. (from Decker and Tuczek, 2000).

In addition, this particular histidine forms an unusual thioether bridge with a cysteine, conserved in mollusc hemocyanin (Fig. 2.3.2). Van Holde et al. (2001) suggest that a possible explanation of the present sequence diversification is that all type 3 copper proteins evolved from a binuclear predecessor. The branches of these proteins, segregating into proto-molluscan and proto-arthropodan lines then underwent to independent evolution to yield the quite different sequences found today (Fig. 2.3.3).

The hexamers or multi-hexamers of arthropod hemocyanins are identical or similar subunits with molecular masses of around 650 amino acids and 75 kDa (Markl and Decker, 1992; van Holde et al., 2001). Each subunit folds into three structural domains and of almost similar size, but characterized by different folding motifs. The middle domain (domain II) carries the oxygen-binding site.

In contrast to hemoglobins and hemerythrins, hemocyanin sequences present an N-terminal signal peptide typical of secreted protein, so that they occur freely dissolved in the hemolymph.

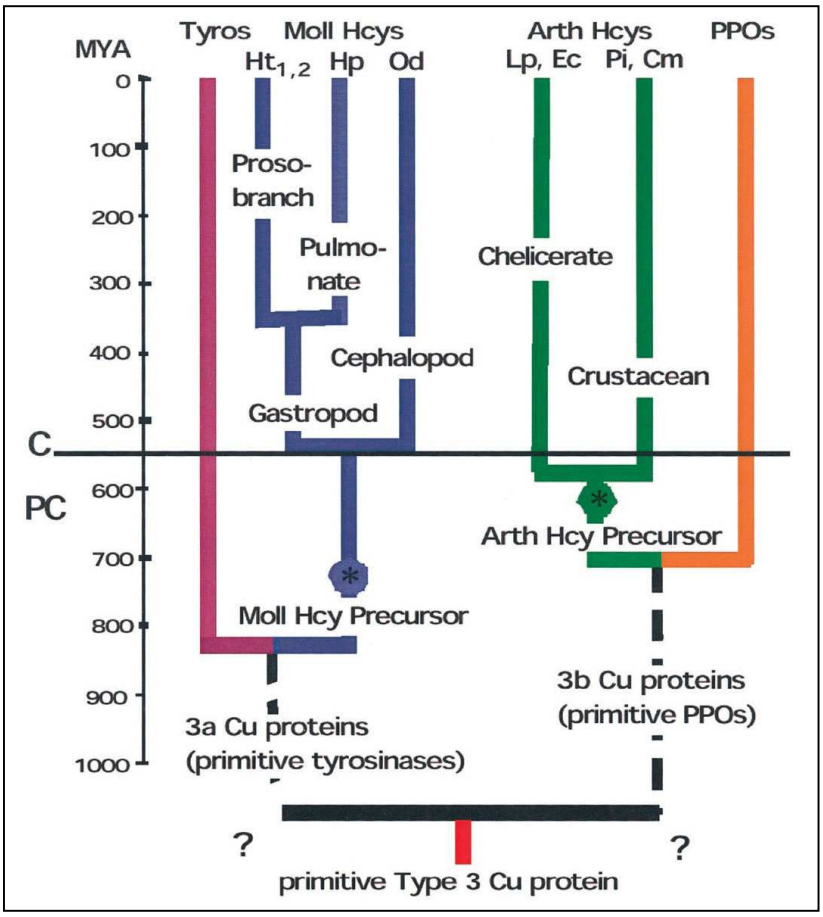


Figure 2.3.3.
A speculative map of the evolution of type 3 copper proteins. The *circled asterisks* indicate the presumed origins of the two types of hemocyanins. The horizontal line (*C*/*PC*) denotes the Cambrian/Precambrian boundary. *Tyros*, tyrosinases; *PPO*, prophenol oxidases. Symbols designatine hemocyanins: Ht1, Ht2, *Haliotis tuberculata* types 1 and 2; Hp, *Helix pomatia; Od*, *Octopus dofleini;* Lp, *Limulus polyphemus;* Ec, *Eurypelma californicum;* Pi, *Panulirus interruptus;* Cm, *Cancer magiste*r (from van Holde et al., 2001)

2.4 Hemocyanin Superfamily

Sánchez et al. (1998) suggest the name AHPH for the protein superfamily to which belong hemocyanin. The acronyms means: *a*rthropodan *h*emocyanins, *P*POs (prophenoloxidases) and *h*examerins. Often this group of proteins is mentioned as Hemocyanin Superfamily (HcSF) and includes, besides hemocyanin, four other class of exclusive arthropod protein that share significant sequence similarities but serve distinct functions (table 2.4.1) (Beintema et al., 2004; Burmester and Scheller, 1996; Burmester, 2001).

These are:

- the prophenoloxidases (PPO), activate after serine proteinase cleavage in phenoloxidases (PO: tyrosinases and catecholoxidases), that are protein involved in the initial step of the biochemical cascade of melanin biosynthesis. They play a key role in the sclerotization of

the cuticle, browning, wound healing and in immune defense (Söderhäll and Ceresius, 1998);

- the non-respiratory pseudo-hemocyanins, also called cryptocyanins, (Terwinllinget et al., 1999; Burmester 1999a) of crustacean implicated in molting process;
- the insect hexamerins, mainly of larval and nymphal stages, but also present in some adults species that serve as storage proteins (Telfer and Kunkel, 1991; Burmester, 1999b);
- the dipteran hexamerin receptors that are responsible of the uptake of the hexamerin from the hemolymph, incorporating them into storage granules of larval fat body and use up during metamorphosis (Burmester and Scheller, 1996).

Among these related proteins, only phenoloxidase and hemocyanin really bind Cu. Functionally, developmentally and evolutionary, this complex superfamily of protein permit to analyze and to infer broad consideration on Arthropoda arising. On the bases of general sequence and structural similarity, it is supposed that in the Precambrian period (600 MYA), the emergence of a unique arthropod phenoloxidases was linked to the evolution of a hard exoskeleton together with an effective immune response, and that the first member of the Hemocyanin Superfamily most likely acted like phenoloxidase.

In all this proteins, amino acids sequences and structural element have preserved an evolutionary trace with type 3 copper binding proteins, even with genetics modification substitutions or duplication and the consequent fundamental changes in function during the time.

	Main function	Occurrence	Structure	Cu^{2+}	Length (aa)[a]	Signal peptide	Evolution rate[c]
Phenoloxidase	Tyrosinase	Crustacea	1×6?	yes	~660–690	no[b]	$1.2–1.6\times10^{-9}$
		Myriapoda	1×6?	yes		?	
		Insecta	n.d.	yes		no[b]	
Hemocyanins	Oxygen transport	Chelicerata	1–8×6	yes	~630	no	$0.5–0.6\times10^{-9}$
		Crustacea	1–4×6	yes	~660	yes	$1.2–1.5\times10^{-9}$
		Myriapoda	6×6	yes	~640	yes	$0.9–1.2\times10^{-9}$
		Insecta	n.d.	yes	~650	yes	$\sim1.0\times10^{-9}$
Pseudo-hemocyanins (cryptocyanins)	Storage protein	Crustacea (Decapoda)	1×6	no	~650–660	yes	$\sim2.0\times10^{-9}$
Hexamerins	Storage protein	Insecta	1×6 or 2×6	no	~660–750	yes	$2.4–2.9\times10^{-9}$
Hexamerin receptors	Hexamerin uptake	Insecta (Diptera?)	n.d.	no	~1010–1240	yes	$\sim7.5\times10^{-9}$

Table 2.4.1. Structure and properties of arthropod phenoloxidase, hemocyanins, pseudo-hemocyanins, hexamerins and hexamerins receptors. [a]: excluding signal pepdicdes; [b]: synthetized as pro-phenoloxidases; [c]: substitucions per site and years. n.d. no determinated (from Burmaster, 2002).

2.5 Hemocyanin: a putative multifunctional protein

In the last 10 years, researches are demonstrating that additional functions can be attributed to hemocyanin.

Chelicerata lack of the specific prophenoloxidaeses. Prophenoloxidaesis mature in tyrosynases and catecholoxidases. Tyrosinase catalyzes two steps, the hydroxylation of monophenols to O-diphenols and the oxidation to O-chinons, without releasing any intermediates (Decker and Tuczek, 2000). Catecholoxidases catalyze only the second reaction.

Recent studies on the horseshoe crab *Tachypleus tridentatus* (Chelicerata) evidenced that a clotting enzyme transforms hemocyanin into a functional phenoloxidase (Nagai and Kawabata, 2000). Moreover, it was observed that hemocyanin has phenoloxidase activity after proteolytic cleavage of the N-terminal part in the tarantula *Eurypelma californicum* (Decker and Rimke, 1998), in the horseshoe crabs *Limulus polyphemus* (Decker et al., 2001) and *Tachypleus tridentatus* (Nagai and Kawabata, 2000). Chelicerata diverge early from the other arthropod taxa. It was hypothesized that the phenoloxidase activity of the chelicerate hemocyanin, could be a primordial function of hemocyanin retained, till the present, in chelicerate, or otherwise it could be an invention of the Chelicerata that recycles the phenoloxidase motif preserved in the hemocyanin to solve fundamental functions in absence of phenoloxidases.

In some crustaceans, like *Cancer magister* (Decker et al., 2001), the deep-sea crustacean *Bathynomus giganteus* (Pless et al., 2003) and *Pacifastacus leniusculus* (Lee et al. 2004), a phenoloxidase activity has been demonstrated, even if it is weaker than in Chelicerata, and it was only observed under special conditions.

The current hypothesis is that hemocyanins as well as phenoloxidases can be activated by limited proteolysis, or by inorganic as well as by organic compounds (Jaenicke and Decker 2004; Decker and Rimke, 1998). An assay on arthropodan and molluscan hemocyanins with Sodium Dodecyl Sulphate (SDS), shows a very similar movement of a flexible structural domain on hemocyanin of both arthropod and mollusc (Decker and Tuczek, 2000). It consists in opening a pocket entrance for bulky phenolic compounds to the active site (Fig. 2.5.1). This seems also to be the case with some phenoloxidases, which has been grouped as a-phenoloxidases (from *a*rthropod related) and m-phenoloxidases (from *m*olluscan related) (Jaenicke and Decker, 2004; Decker and Jaenicke, 2004). In contrast, the active site of tyrosinase becomes freely accessible after the release of the so-called caddie protein (Decker et al., 2007). In arthropods, crucial for the

activation of hemocyanins and phenoloxidases is the position of the highly conserved phenylalanine residue, the Phe49, which is located ~3–4 Å from the active site (Magnus et al., 1994). After cleavage of the N-terminal peptide, Phe49 is pulled out of a cavity of the remaining protein, leaving a pocket for potential substrates. As a result, the binuclear copper active site becomes accessible also to non gaseous bulky substrates such as phenolic derivatives (Decker and Rimke, 1998). A phenolic substrate, like tyrosine, can be modeled into this pocket exactly like Phe49. The flexibility of the substrates is constrained by hydrophobic interactions. Then, one atom of the dioxygen molecule attacks the phenol ring in the ortho position, forming a hydroxyl group which results in an O-diphenol being immediately oxidized. The other oxygen combines with protons to form a water molecule.

Anyway, differently from phenoloxidase enzyme (Ashida and Yoshida, 1998; Àspan and Söderhäll, 1991; Söderhäll and Ceresius, 1998), the phenoloxidasic activity of hemocyanin has only been demonstrated *in vitro*.

Lee et al (2003), demonstrated that, in the crayfish *Pacifastacus leniusculus* hemocyanin usually works like a respiratory protein but under acid conditions, induced *in vitro*, the C-terminal part of subunit 1 can be processed in an antibacterial peptide (asticidin-1).

This supplementary reactivity of the hemocyanin is interesting not only for the possibility of host defense proceeding from bacteria, but especially for the difference reactivity of the monomers, each occupying a specific position in the whole molecule. May be that the ratio between subunits could vary depending on environmental conditions. Different monomers activity was also observed in *L. polyphemus*, *E. californicum* and *C. magister* during hemocyanin in vitro activation versus phenoloxidase (Decker et al., 2001).

At the present, in Hexapoda, phenoloxidase activity by hemocyanin subunits was even not demonstrated, but studies suggest that hemocyanins and hexamerins could de involved in the arthropod immune system (Hayakawa, 1994; Beresford et al. 1997; Nagai and Kawabata, 2000; Decker et al. 2001).

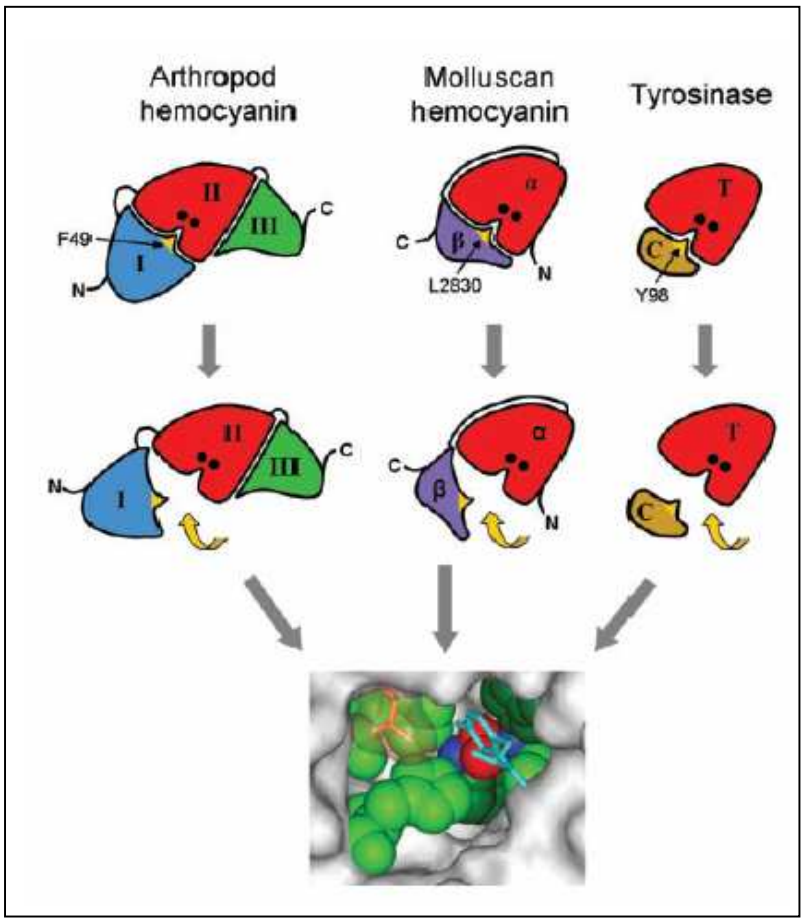


Figure 2.5.1. Hypothetical mechanism of activation of hemocyanins and tyrosinases. In all three cases, a specific amino acid acting as a sterical block (yellow) has to be removed from the entrance to the binuclear copper center (black dots) located at the active site domain (red). In the arthropod hemocyanin subunit, the removed structure is the N-terminal domain I (blue); in the molluscan functional unit, it is the C-terminal β-domain, and in tyrosinase it is the caddy protein. Topologically, domain I, the b-domain and the caddy protein are located at equivalent positions relative to the active site domain. A view into the active site reveals the open access to the copper atoms (dark blue) and the oxygen molecule (red). The phenolic substrate (light blue) directs its hydroxyl group towards CuA, that is coordinated by histidines (green) (from Decker et al., 2007).

2.6 Occurrence of Arthropod Hemocyanins

The occurrence and properties of hemocyanins have been thoroughly studied over the last 30 years in Chelicerata, in some Malacostraca and in Remipeda Crustacea (Ertas et al., 2009), but their presence in other arthropod subphyla like Onychophora (Kusche et al., 2002), Myriapoda (Kusche and Burmester, 2001b) and some Hexapoda has been discovered only recently.

A potential hemocyanin was characterized in the embryo hemolymph of the grasshopper *Schistocerca americana* (Sánchez et al., 1998). Hemocyanin was found in larvae and adults of the stonefly *Perla marginata* (Hagner-Holler et al., 2004) and this protein was proved *in vitro* to be functional and to act as an oxygen carrier. A potentially functional hemocyanin has been found also in another stonefly species, *Perla grandis*, and its 3-D structure was described and analyzed through a modeling approach (Fochetti et al., 2006).

Recently, Pick et al. (2009) have reported the presence of hemocyanin in many hexapod orders, like Collembola, Diplura, Archaeognata, Zygentoma, Orthoptera, Phasmida, Dermaptera, Isoptera and Blattodea, however, hemocyanins are apparently absent in all Eumetabola (Hemiptera and Holometabola) (Burmester and Hankeln, 2007; Pick et al., 2009) (Fig. 2.6.1).

Studies about Eumetabola revele the presence of hemoglobin; it was found in *Drosophila melanogaster* (Hankeln et al., 2002) and Hankeln et al. (2006) cloned and studied the expression of a true haemoglobin gene in the honeybee *Apis mellifera* (Hymenoptera). They also stated that hemoglobins exist in other insect orders (Hemiptera, Coleoptera, Lepidoptera), suggesting that the corresponding genes belong to the standard set of an insect genome.

All these evidence show that, in contrast with previous assumptions (Brusca and Brusca, 2002; Deavis, 1988), we have to modify the idea that consider the Hexapoda tracheal system so efficient to represent the only structure implicate in oxygen diffusion, and that the loss of hemocyanin in some taxa of insects is not correlated with the evolution of an efficient tracheal system.

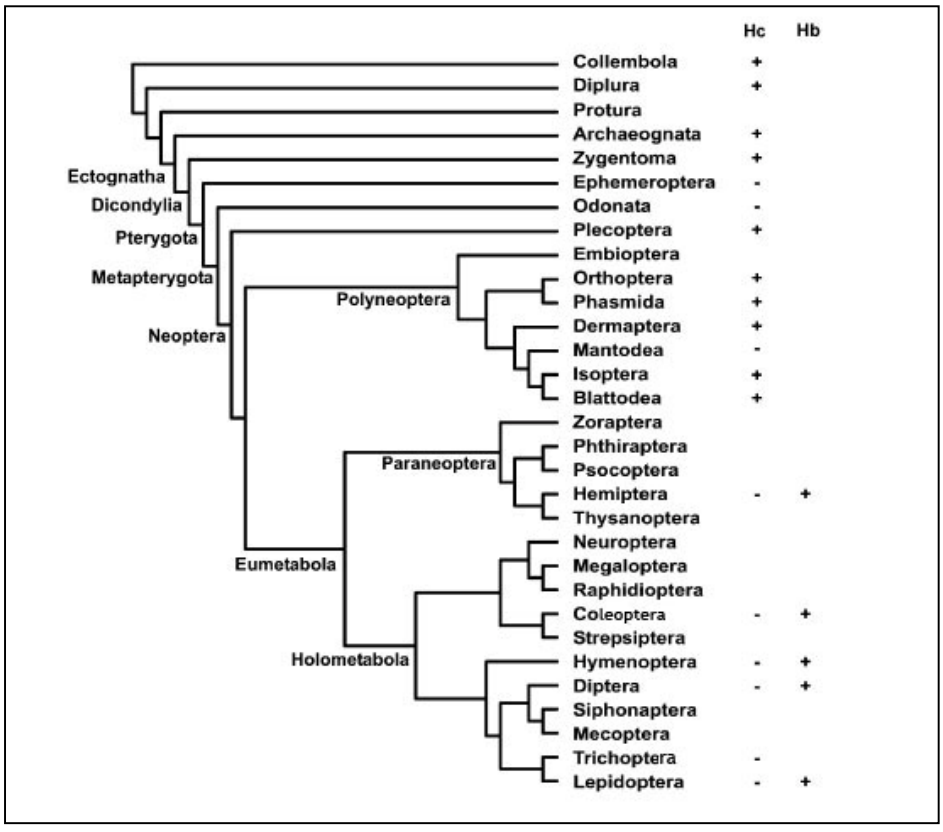


Figure 2.6.1. Distribution of respiratory proteins in insect orders. The phylogenetic tree of the insects is based on Hennig (1969), Kukalovà -Peck (1991) and Wheeler et al. (2001) (from Burmester and Hankeln, 2007).

2.7 Plecopteran hemocyanins

Hemocyanin is a protein composed by several similar or identical monomers each one occupying a particular position in the stereochemistry of the total macromolecule. In Plecoptera have been detected two hemocyanin subunits: hc1 and hc2. Studies on *Perla grandis* calculated that hc1 mature peptide should consist of 659 amino acids with a theoretical mass of 77.0 kDa and a theoretical Ip (Isoelectric point) of 5.82, and hc2 mature peptide should be of 655 amino acids with theoretical mass of 76.3 kDa and a theoretical Ip of 6.01 (Fochetti et al., 2006). Modelling 3-D structure of *P. grandis* was constructed by Fochetti et al. (2006) on the template of hemocyanin spiny lobster *Palinurus interruptus* with the results that the global folding as well as the secondary structures of hc1 and hc2 chains was maintained. In particular, the three domains characterizing each hemocyanin subunit template were conserved in most crustacean and Hexapoda species studied and consist of an amino-terminal α-helical domain (domain I), a central mostly helical copper-containing core (domain II), and a carboxyl terminal seven-stranded anti-parallel β-barrel (domain III) (Fig. 2.7.1). Like in the template chain of *P. interruptus*, the C-terminal part of domain I, in both hc1 and hc2, forms an appendix consisting of α helix and a β-strand (Fig. 2.7.1 A–C). This appendix is a very important part of the hemocyanin structure since it is involved in extensive inter-subunit contacts (Volbeda and Hol, 1989). Domain II, containing the binuclear copper site, is well maintained in both hc1 and hc2 (Fig. 2.7.1 D–F). The residues involved in the oxygen-binding site, six histidines related with the copper and three phenylalanine that stabilize the binding of O_2 in the first and second domain, are perfectly conserved. Their spatial position preserves the functional geometry and distances between faced histidines and copper ions. As for *Panulirus* hemocyanin and other type 3 copper proteins, each copper ion is ligated by three histidine side chains provided by a pair of helices. The ligands and ligand-donating helices for Cu ions are related by a pseudo-twofold axis. Besides the functional role in oxygen binding, domain II is also important from a structural point of view, because it dominates the inter-subunit contacts within the hexamer (Volbeda and Hol, 1989).

In domain III are contained most of the disordered loops (Fig .2.7.1 G-I). It is very exposed in the hexamer, and the main differences between *P. grandis* hc1 and hc2 with lobster hemocyanin chains are located in this domain and not in functional parts of the structure.

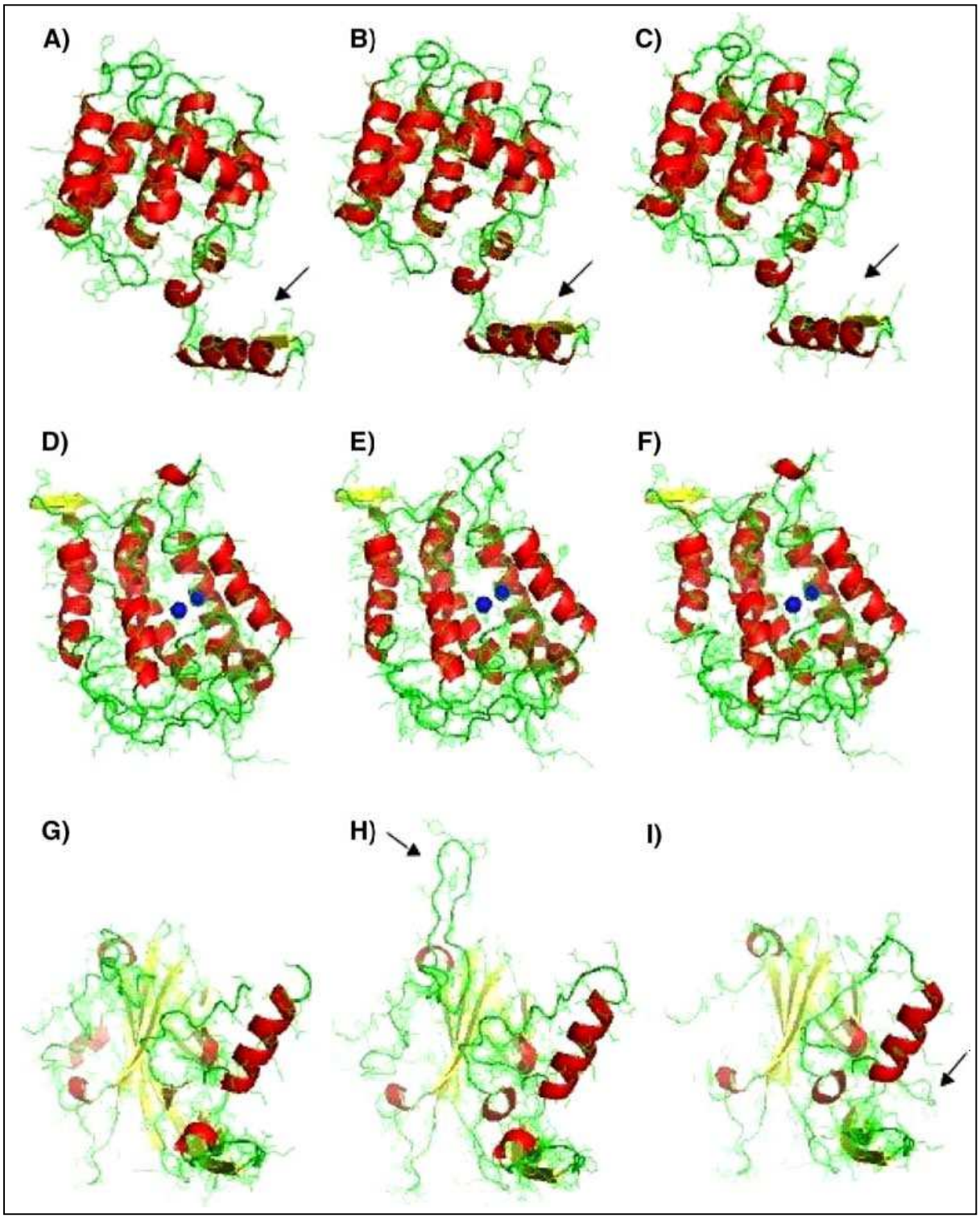


Figure 2.7.1. Domain I of 1hc5 *Panulirus interruptus* (A), hc1 (B) and hc2 (C) *Perla grandis* hemocyanin chains. The arrows indicate the conserved C-terminal part involved in subunits contacts. Domain II of 1hc5 *P. interruptus* (D), hc1 (E) and hc2 (F) *P. grandis* hemocyanin chains. Domain III of 1hc5 *P. interruptus* (G), hc1 (H) and hc2 (I) *P. grandis* hemocyanin chains. The arrows indicate the extra external loops in hc1 and hc2 chains (from Fochetti et al., 2006).

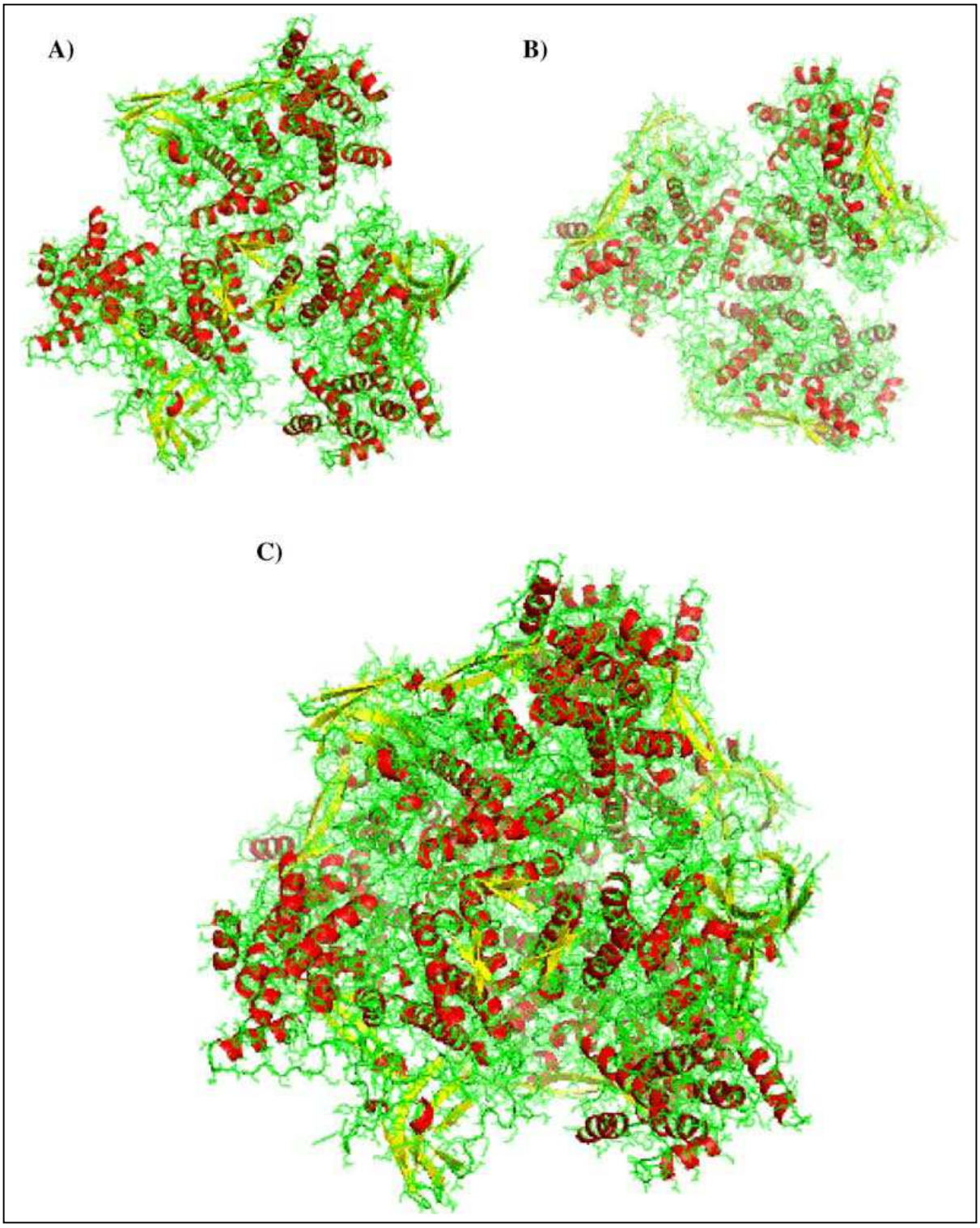
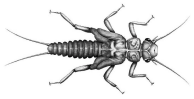


Figure 2.7.2. Frontal view of the trimers formed by hc1 *Perla grandis* subunits 1-3-5 (A), 2-4-6 (B) and the corresponding hexamer (C) (from Fochetti et al., 2006).

The high similarity between *P. interruptus* and *P. grandis* hemocyanin (about 45%) permit to infer that *P. grandis* hemocyanin, and so Plecoptera hemocyanin, can be a hexamer formed by subunits maintaining the same structure. The hexamer is best described as a dimer of trimers showing more contacts in the dimer than in the trimer (Fig. 2.7.2) even if it is unknown the hc1/hc2 ratio.



3. Scientific articles: published and in progress

* In this part, the format of the paragraphs maintains the same journal layout of the reviews where they are been published or submitted. The numbering of the pages of the paragraphs 3.1 and 3.2 follows the numbering of the published scientific journal.

Insect Molecular Biology (2009) **18**(2), 203–211 doi: 10.1111/j.1365-2583.2008.00859.x

Do all stoneflies nymphs have respiratory proteins? Further data on the presence of hemocyanin in the larval stages of plecoptera species

V. Amore*, M. Belardinelli*, L. Guerra*, F. Buonocore*, A. M. Fausto*, N. Ubero-Pascal† and R. Fochetti*

**Dipartimento di Scienze Ambientali, Università della Tuscia; and* †*Departamento de Zoologıá y Antropologıá Fısica, Universidad de Murcia*

Abstract

Contrary to what was assumed regarding the presence of respiratory proteins in insects, a functional hemocyanin was recently found in larvae and adults of the stoneflies species *Perla marginata*, whereas in the close species *Perla grandis*, hemocyanin functionality was deduced from sequence data. In order to verify if the presence of this ancient trait is widespread within the order and to investigate why stoneflies have maintained it, we have extended the search for hemocyanin to species of other Plecoptera families. In particular, we assessed the presence of hemocyanin in the larval stage of nine Plecoptera species, belonging to six of the seven families of the European stonefly-fauna, and analyzed its potential functionality as deduced by sequence data. We cloned and sequenced the corresponding cDNAs and studied their expression with RT-PCR technique. Moreover, we performed homology studies using the deduced amino acid sequences. On the basis of our analysis, we hypothesized a functional role of the hemocyanin only for two species: *Dinocras cephalotes* and *Isoperla grammatica* (Perloidea). In all the investigated Nemouroidea and in *Siphonoperla torrentium* (Perloidea), this protein may have been lost. Larval size, life-cycle length, trophic role and environmental induction are discussed as possible explanations of these different physiological requirements.

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Correspondence: Romolo Fochetti, Dipartimento di Scienze Ambientali, Università della Tuscia, Largo dell'Università snc, 01100 Viterbo, Italy. Tel.: +39 0761357178; fax.: +0761357179; e-mail: fochetti@unitus.it

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Introduction

For long time it has been paradigmatically affirmed that, due to the presence of the tracheal system, insects do not have respiratory proteins, with the exception of several chironomid midges larvae, the aquatic Hemiptera genera *Buenoa* and *Notonecta* and the parasitic horse botfly genus *Gasterophilus*. More recently, a potential hemocyanin was characterized in the embryo hemolymph of the grasshopper *Schistocerca americana* (Sánchez *et al*., 1998) and a respiratory hemoglobin was found in *Drosophila melanogaster* (Hankeln *et al*., 2002); hemocyanin was found in larvae and adults of the stonefly *Perla marginata* (Hagner-Holler *et al*., 2004) and this protein proved to be functional and to act as an oxygen carrier. A potentially functional hemocyanin has been found also in another stonefly species, *Perla grandis*, and its 3-D structure tentatively described and analyzed through a modelling approach (Fochetti *et al*., 2006). Hankeln *et al.* (2006) cloned and studied the expression of a true hemoglobin gene in the honeybee *Apis mellifera* (Hymenoptera). They also stated that hemoglobins exist in other insect orders (Hemiptera, Coleoptera, Lepidoptera), suggesting that the corresponding genes belong to the standard set of an insect genome. Besides, Burmester & Hankeln (2007) reported the presence of hemocyanin in many insect orders, from Collembola to Blattodea, without further comments. All these data indicate that insect respiration is a more complex subject than previously thought.

Plecoptera is a very interesting and enigmatic group from the systematic and phylogenetic points of view. The order is considered a key group among the Insecta: many morphological characters of the Plecoptera are so primitive that the order has been hypothesized to be the sister-group of the rest of the Neoptera (Henning 1981; Kristensen 1991; Zwick, 2000; Beutel & Gorb, 2001). We have extended the search for hemocyanin to other species of different

Plecoptera families, with the aim to verify how this ancient trait is still retained across the order and to investigate why stoneflies have retained it. In this paper, we report on the presence of hemocyanin in the larval stages of nine Plecoptera species, *Dinocras cephalotes* (Perlidae), *Isoperla grammatica* (Perlodidae), *Siphonoperla torrentium* (Chloroperlidae), *Leuctra fusca* (Leuctridae), *Taeniopteryx stankovitchi, Brachyptera risi* (Taeniopterygidae), *Nemoura hesperiae, Amphinemura sulcicollis, Protonemura ausonia* (Nemouridae), belonging to six of the seven European stonefly families, and analyze its potential functionality as deduced by sequence data.

Results

Sequences analysis

The RT-PCR reaction gave product of the expected size (586 bp) in all the investigated species; when cloning and sequencing these PCR products, we found contrasting results. The analyses of the deduced amino acid sequences showed that species belonging to the superfamily Nemouroidea and to the Choroperlidae *Si. torrentium* have substitutions in the copper-binding sites (Fig. 1), whereas in the Perloidea species we found what seems to be a true hemocyanin. A pairwise alignment of the hc2 conserved fragments of *Pe. grandis* with each plecopteran sequence, using both nucleotide and amino acid translated sequences, provided percentages of identity and similarity shown in Table 1. The highest identity (> 72%) and similarity (> 85%) was recorded between *Pe. grandis* hc2 and *Pe. marginata*, *Din. cephalotes*, and *I. grammatica* hc2 subunits. Lower similarity was shown with the hc1 subunits. The degree of similarity increased according to accepted systematic position. The percentage of identity and similarity when comparing with *Pe. grandis* hc2, was lower in the Nemouroidea superfamily: the amino acidic identity was about 33% and the similarity was about 51%. The studied sequences of species belonging to Nemouroidea showed a strong similarity with the known sequences of plecopteran hexamerins (Hagner-Holler *et al.*, 2007). This was clearly shown in the multiple alignment of aminoacidic sequences (Fig. 1), where in the *Din. cephalotes* and *I. grammatica* sequences, the binding site is conserved. In the case of *I. grammatica*, we could also distinguish two subunits, one for hc1, and one for hc2, whereas in *Din. cephalotes* we were not able to find the subunit hc1. The phenylalanine residue was present in all sequences of both hemocyanins and hexamerins. In Nemouroidea species and in *Si. torrentium*, four or five histidines of the highly-conserved region were lacking. The histidines, which are polar and weakly basic amino acids, were substituted with others that do not show similar biochemical properties, i.e. Tyrosine (Y) non-polar neutral, Threonine (T) and Asparagine (N) polar neutral, or Glutamic Acid (E) polar acid. It is noteworthy that only one histidine residue is conserved in all species, while the other four histidine residues are not

Table 1. Percentages of identity and similarity of *Perla grandis* hc2 with other Plecoptera nucleotidic and amino acidic translated sequences

			% bp identity	% aa identity	% aa similarity (P250)
Perla grandis hc2	hemocyanin	*Homarus americanus* hcA	60	53	65
		Pacifastacus leniusculus hc1	59	50	64
		Pacifastacus leniusculus hc2	59	51	66
		Perla marginata hc1	63	55	54
		Perla marginata hc2	90	90	96
		Perla grandis hc1	63	55	71
		Dinocras cephalotes hc2	86	88	95
		Isoperla grammatica hc1	62	51	67
		Isoperla grammatica hc2	79	72	85
	hexamerin	*Perla marginata* hx1	57	36	57
		Perla marginata hx2	57	37	57
		Perla marginata hx3	20	27	48
		Perla marginata hx4	54	30	54
		Diamphipnopsis samali hx1	58	39	60
		Siphonoperla torrentium hx1	34	31	55
		Leuctra fusca hx1	58	35	55
		Taeniopteryx stankovitchi hx1	56	37	55
		Taeniopteryx burksi hx2	58	38	55
		Brachyptera risi hx1	57	37	54
		Nemoura sp. hx1	58	36	56
		Nemoura sp. hx2	59	37	57
		Nemoura sp. hx3	58	36	57
		Nemoura hesperiae hx1	57	28	44
		Protonemura ausonia hx1	57	34	54
		Amphinemura sulcicollis hx1	57	28	44
		Allocapnia vivipara hx1	59	38	55

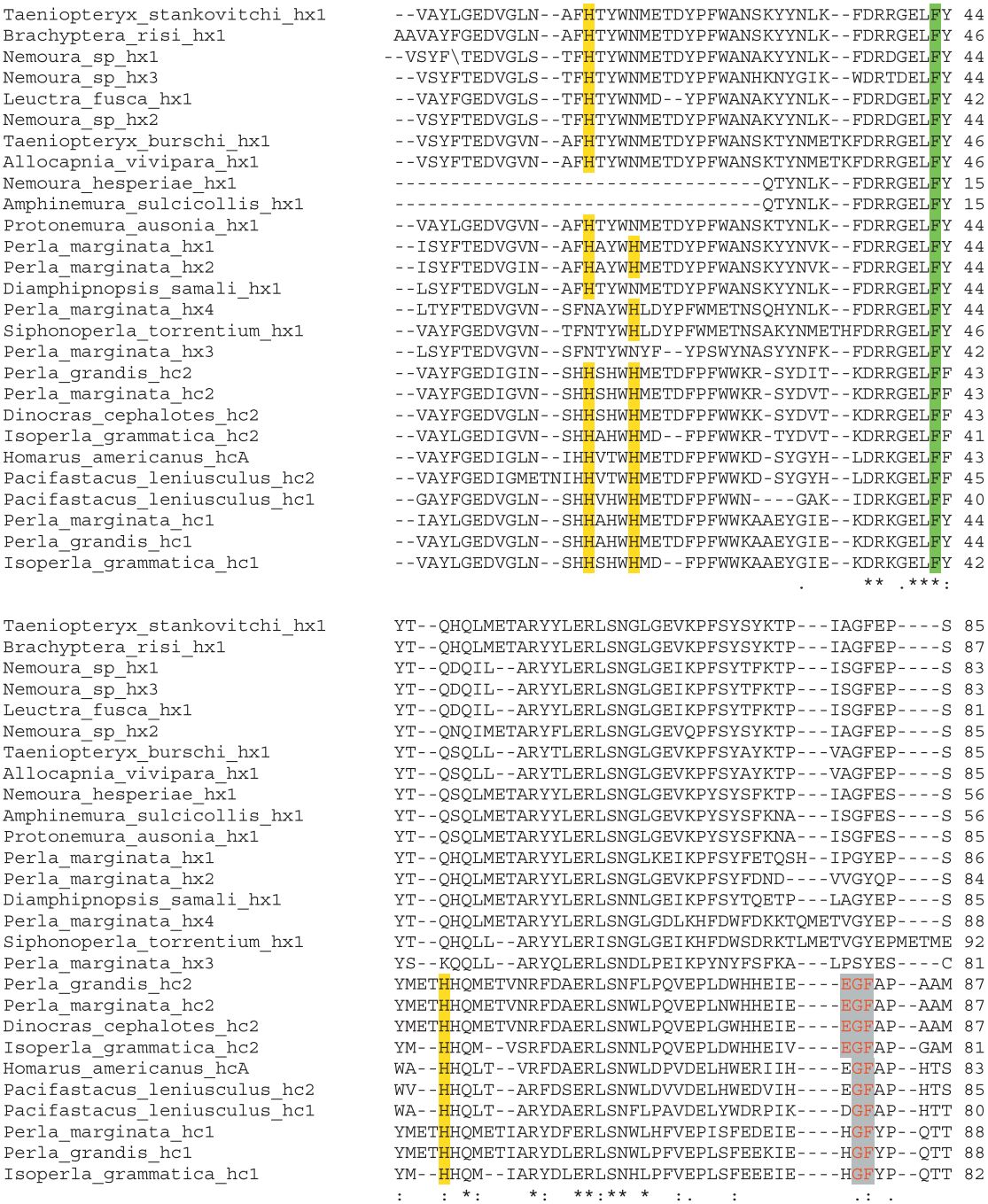

Figure 1. Multiple sequence alignment CLUSTAL W (1.83) of hemocyanins conserved aminoacid sequences (hc) and correspondent hexamerins sequences (hx). His (yellow) and Phe (green) residues involved in the oxygen-binding site are indicated. The residues involved in the trimer (blue) and dimer (red) contacts are also shown.

conserved in Nemouroidea and *Si. torrentium*, suggesting that they could lack hemocyanin or that hemocyanin in this taxa could be non-functional.

Phylogenetic analysis

The different approaches used gave similar results. Fig. 2 shows the Maximum Parsimony tree, whose consistency index is 0.729776; the retention index is 0.799745 and the composite index is 0.592892. There were a total of 113 positions in the final dataset, 99 of which were parsimony informative. The reliability of the branching pattern was tested by bootstrap analysis (Efron *et al.*, 1996) with 500 replications. In all performed analyses (NJ, MP, ME), four well-supported clades were found, namely (1) crustacean

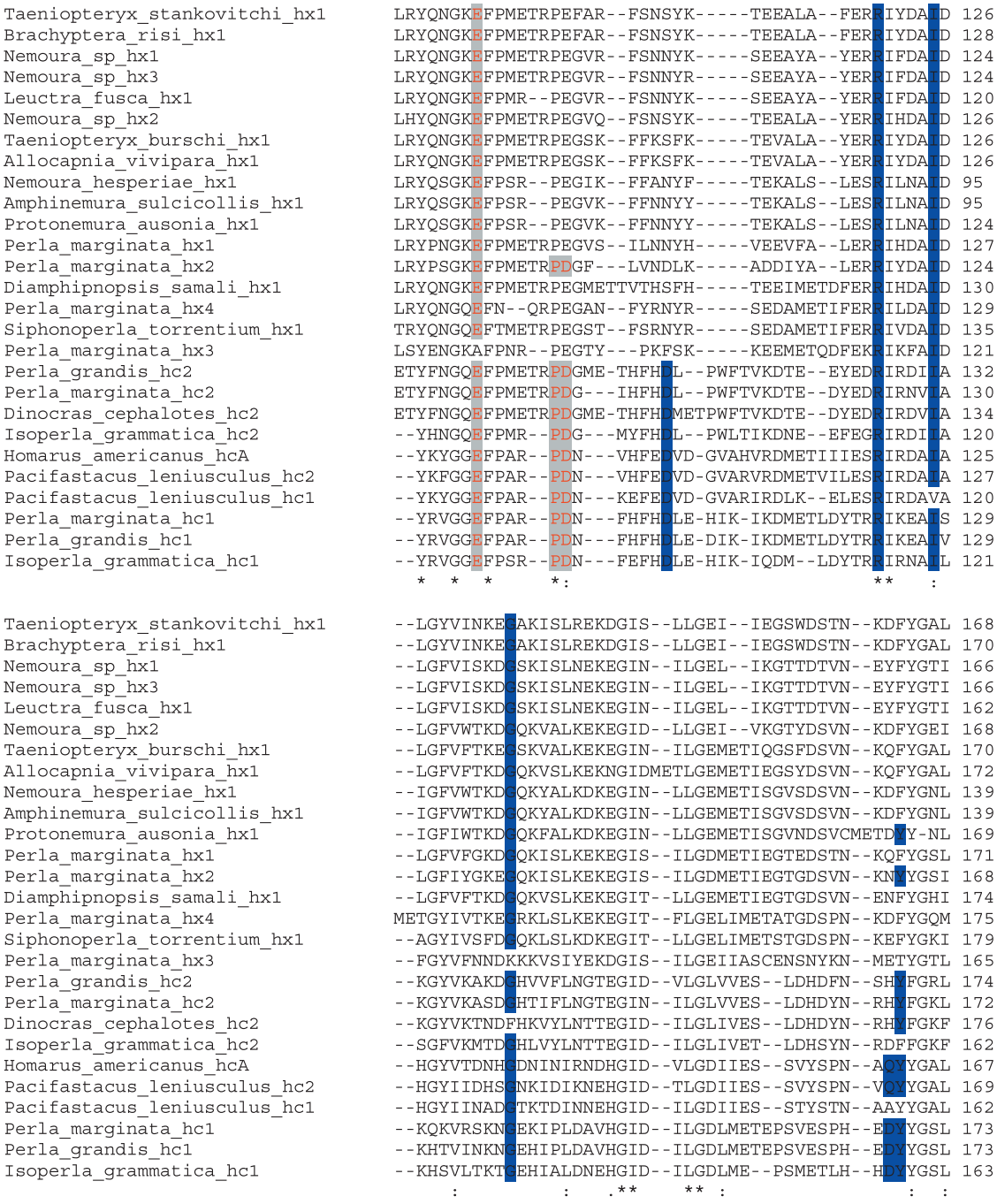

Figure 1. *Continued*

hemocyanin, (2) Plecoptera hemocyanin hc1, (3) Plecoptera hemocyanin hc2, (4) Plecoptera hexamerines.

Discussion

In previous studies regarding oxygen carrier proteins in insect orders (Hagner-Holler *et al.*, 2004; Burmester & Hankeln, 2007), only a single species was chosen to represent a whole order and define the presence of the protein within the taxon. We have performed this study with the aim of improving the knowledge on hemocyanin within Plecoptera, analyzing nine species belonging to six of the seven families of the two superfamilies (Perloidea and Nemouroidea) included in the European fauna.

```
Taeniopteryx_stankovitchi_hx1      YNIM--ETRTIFGHVTDP--TYQYGVAPGVLE--HFETA- 201
Brachyptera_risi_hx1               YNIM--ETRTIFGHVTDP--TYQYGVAPGVLE--HFETAT 204
Nemoura_sp_hx1                     YNMETMETRGIFGHVTDP--NFQYGVAPGVLE--HFET-- 200
Nemoura_sp_hx3                     YNMETMETRGIFGHVTDP--NFQYGVAPGVLE--HFET-- 200
Leuctra_fusca_hx1                  YNMM----RGIFGHVTDP--NFQYGVAPGVLE--HFETA- 193
Nemoura_sp_hx2                     YNMETMETRTIFGHVTDP--TYKYGTAPGVTE--HFET-- 202
Taeniopteryx_burschi_hx1           YNIM--ETRTIFGHVTDP--TFQYGVAPGVLE--HFETA- 203
Allocapnia_vivipara_hx1            YNIM--ETRTIFGHVTDP--AFQYGVAPGVLE--HFETA- 205
Nemoura_hesperiae_hx1              YNLM--ETRTIFGHVTDP--NFKYGVAPGVMETEHFET-- 173
Amphinemura_sulcicollis_hx1        YNLM--ETRSIFGHVTDP--NFKYGVAPGVMETEHFE--- 172
Protonemura_ausonia_hx1            YNL----TRKISDHLTDP--NVRGGVTHGLMETEPFETA- 202
Perla_marginata_hx1                YNME--TLRTVYGHYADPMETYQYEVAPSVLE--HFTTA- 206
Perla_marginata_hx2                YN----TIRTVFGHHADPMETYQYLVAPGVLE--HFQTA- 201
Diamphipnopsis_samali_hx1          YSLM--ETRTVFGHATDP--KYQYDVAPGVLE--HFETA- 207
Perla_marginata_hx4                ETYS--ILCTVFGHVMETDPTFKYDTVPSVLE--HYETA- 210
Siphonoperla_torrentium_hx1        YTN----LCTIFGHVMETDHTFAFDTVPSVLE--HSKPP- 212
Perla_marginata_hx3                YYS----ILKTFGHEVDP--QYQYKFAPPALEQIHTT--- 196
Perla_grandis_hc2                  HLNAH----VLLSKITDPEQKFG--TPPGVMETEHFETA- 207
Perla_marginata_hc2                HSNAH----VLLSKVTDPEQKFG--TPPGVMETEHFETA- 205
Dinocras_cephalotes_hc2            HSNAH----VLLSKITDPEQKFGMETPPGVMETEHFETA- 211
Isoperla_grammatica_hc2            HANSH----VMLGRITDPMLKFG--MPPGVME--HFETAT 194
Homarus_americanus_hcA             HNTAHI--METLGRQGDPHGKFNMETPPGVMETEHFETA- 204
Pacifastacus_leniusculus_hc2       HNTAHI--METLGRQGDPHGKFDMETPPGVMETEHFETA- 206
Pacifastacus_leniusculus_hc1       HNQAH----RVLGAQSDPKHKFNMETPPGVMETEHFETA- 197
Perla_marginata_hc1                HNDAH----VLLGQITDPLGKFDL--PPGVMETEHFETA- 206
Perla_grandis_hc1                  HNYAH----ILLGKVTDPLGKFDL--PPGVMETEHFETAT 207
Isoperla_grammatica_hc1            HNYAH----ILLGQITDPLGKFNM--PPGVME--HFETP- 194
                                              .   :.          *
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Figure 1. *Continued*

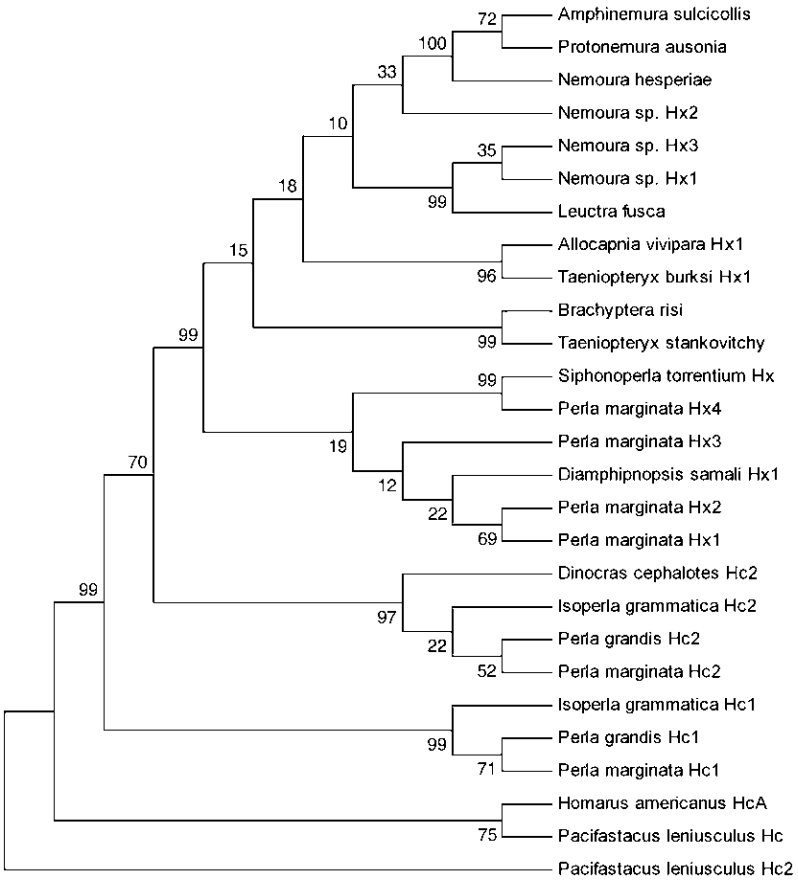

Figure 2. Phylogeny of stonefly hemocyanins and hexamerins. The numbers at the nodes represent bootstrap support of MP analyses.

Sequence analysis

The presence of hemocyanin transcripts in aquatic gilled nymphs, corresponding to a respiratory protein, is unexpected. From a phylogenetic point of view, the hemocyanin is an ancestral and retained character that supports, together with the other known aspects of this order, the primitivity of Plecoptera (Henning 1981; Kristensen, 1991; Zwick, 2000; Beutel & Gorbh, 2001). The sequence analyses of the fragments we studied in Plecoptera allowed us detecting important modifications of the protein conserved region among the different species. In fact, according to the sequence data, we could assume that all the analyzed Nemouroidea and *Si. torrentium* do not have a functional hemocyanin. The amplified fragment probably belong to hexamerins. This agrees with the hypothesis of the molecular evolution of these subunits from a common ancestor, i.e. recent insects and crustacean hemocyanins could derive from an ancestral arthropod (probably a primitive crustacean according to the Pancrustacea hypothesis). Thus, insect hexamerins might have lost their copper-binding capability (Burmester, 2002; Burmester & Hankeln, 2007). In fact, in the sequences of crustacean hemocyanins which have been characterized so far, the oxygen-binding site is always conserved (it is formed by six histidines and four phenylalanines; Volbeda & Hol, 1989). Furthermore, the analysis of the multiple alignment between a Plecoptera species (*Pe. grandis*) and a Crustacean (*Palinurus interruptus*) showed that the conserved residues involved in three-dimensional structure of the hemocyanin molecules were constant in hemocyanin fragments (Fochetti *et al*., 2006). In *Pe. marginata*, the oxygen-binding property of the purified hemocyanin has been supported by a functional test (Hagner-Holler *et al*., 2004), whereas in *Pe. grandis*, the respiratory function for hemocyanin has been only hypothesized on the basis of its three-dimensional structure (Fochetti *et al*., 2006). In the present study, by comparing the sequences, we can hypothesize a functional role for the hemocyanins of *Din. cephalotes* and *I. grammatica.* On the contrary, in all the studied Nemouroidea nymphs (*T. stankovitchi, B. risi, N. hesperiae, Am. sulcicollis, Pr. ausonia* and *L. fusca)* and in *Si. torrentium* nymphs, this respiratory protein might have been lost.

Amino acid analysis

From literature data (Hagner-Holler *et al*., 2007) we know that the average content of aryl-groups (Phe and Tyr) of known insect hemocyanins (Plecoptera and *Schistocerca americana*) is about 10.8%, which is significantly lower than that of plecopteran hexamerins, which is about 16.5%, while the content of Met is not significantly different. We calculated the proportions of aryl and Met groups, deduced from cDNA sequences, for each sequence of the 9 studied plecoptera species (looking for another evidence allowing us to distinguish among hemocyanins and hexamerins). According to the studied sequences, Nemouroidea species and *Si. torrentium* displayed the amino acid values expected for hexamerins (mean = 15.67%), whereas *Din. cephalotes* and *I. grammatica* values of aryl-group were similar to those of *Pe. marginata* and *Pe. grandis* hemocyanins and to those of *Sc. americana* (mean = 10.83%). These data support the idea that only some species of Plecoptera have functional hemocyanins.

Phylogenetic analysis

Our results suggest possible differences in the presence of hemocyanins among nymphs of different stonefly species, even though they share the same aquatic habitat and show the same ecological requirements. One possible explanation is that nymphs belonging to the family Perlidae and, to a minor extent, Perlodidae, are large-sized and, at least the studied Perlidae species, are very sensitive to oxygen levels in cold running waters (Hynes, 1976; Zwick, 2000). On the other hand, in small-size nymphs such as those of Taeniopterygidae, Nemouridae, Leuctridae and Chloroperlidae, the tracheal system and gills (if present) may be sufficient for their oxygen request in water. Furthermore, the complete life cycle of small-size species is achieved in about one year, whereas large-size species (Perlidae) need two or three years to develop, thus requiring an oxygen supply for the periods of stress. Nevertheless, *I. grammatica* (a medium-size species, 1.2–1.5 cm length), shows an univoltine life cycle, despite possessing both hemocyanin subunits. Another possible difference between these two groups of Plecoptera is related to their different trophic roles. In fact, most Perlidae and Perlodidae are predators, therefore requiring more oxygen for their increased activity, whereas Nemouroidea are phytophagous or detritivorous. This hypothesis works except for *Si. torrentium* – where we did not find hemocyanin evidence- which is a predator, although the juvenile stages feed on vegetal matter (Hynes, 1976; Tierno de Figueroa *et al*., 2003).

However, in recent years it has become evident that the combination of an efficient respiratory system, composed by gills and tracheae, with a respiratory protein is a peculiar feature not only of Plecoptera, because O_2 carrier-proteins are much more widespread among insects than previously thought (Burmester & Hankeln, 1999; Hankeln *et al*., 2002, 2006; Hagner-Holler *et al*., 2004; Burmester *et al*., 2006; Burmester & Hankeln, 2007).

It is remarkable that the studied conserved region acts like a phylogenetic molecular marker within plecoptera: the pattern of hemocyanin and hexamerin evolution follows the accepted scheme of the phylogeny of the Plecoptera based on morphology and anatomy, with the exception of *Allocapnia vivipara* (Capnidae) which appears to be closer to the Taeniopterygidae rather than to Leuctridae as in the traditional approaches. Our results add evidence supporting

the theory that hexamerins evolved from hemocyanins in the early steps of insect evolution. Our data would indicate they evolved from hc2 subunit, even though the analysis of a more complete dataset led Burmester & Hankeln (2007) to hypothesize hc1 as the probable closest subunit.

Finally, alternative physiological functions for insect hemocyanins may also be considered. This hypothesis arises by an interesting feature of the hemocyanin molecules of Chelicerates, suggesting that evolution has developed a double function for this molecule. In fact, recent studies about chelicerate hemocyanins, suggested that hemocyanin aquires a phenoloxidase activity after proteolitic cleavage at the amino-terminal part (Decker & Rimbke, 1998, Decker & Tuczek, 2000). Under normal conditions the hemocyanin functions as an oxygen carrier protein, but it may convert to pheniloxidases after microbial infections. In Crustaceans, a study on *Pacifastacus leniusculus* showed that, under acidic conditions, some hemocyanins can be processed by a cysteine-like protein to generate an antimicrobial peptide (Lee *et al.*, 2002). In relation to this topic, studies on Plecoptera are needed to verify if the RT-PCR fragments detected in all the studied species belong to a molecule with an immunological role.

Experimental procedures

Insects

The nymphs of nine Plecoptera species were collected by kick method in the following sites:

Perloidea

Dinocras cephalotes (Curtis, 1827): Nera River (Arrone, Terni; Central Italy), 7-VI-2007;
Isoperla grammatica (Poda, 1761), Leja River (Viterbo, Central Italy); 13-II-2007;
Siphonoperla torrentium (Pictet, 1841), tributaries of Siele River (Piancastagnaio, Siena, Central Italy), 21-III-2007;

Nemouroidea

Taeniopteryx stankovitchi, Ikonomov, 1978: Mignone River (Canale Monterano, Roma; Central Italy), 16-XI-2007;
Brachyptera risi (Morton, 1836), tributaries of Siele River, (Piancastagnaio, Siena, Central Italy), 21-III-2007;
Leuctra fusca (Linnaeus, 1758): Mignone River, (Oriolo Romano and Canale Monterano, Roma; Central Italy), 16-XI-2007.
Amphinemura sulcicollis (Stephens, 1836), Nera River (Arrone, Terni; Central Italy), 21-V-2007;
Nemoura hesperiae Consiglio, 1960, tributaries of Siele River (Piancastagnaio, Siena, Central Italy), 21-III-2007;
Protonemura ausonia (Consiglio, 1955), Arlena River (Viterbo, Central Italy), 13-II-2007;

Total RNA isolation and cDNA synthesis

Total RNA was extracted from larvae of the analyzed species using the phenol-guanidine thiocyanate method (Chomczynski & Sacchi, 1987) or the mini or micro Qiagen Kit (Qiagen, Hilden, Germany), following to the manufacturer's instructions, according on the size and weight of the samples. The first cDNA strand was subsequently synthesized using 1 μg of total RNA, 0.2 μg of random hexamers, 100 mM dNTP mix (25 mM each) and 50 U/μl of BioScript Rnase H Minus BIOLINE (Duotech, Bioline, London, UK). The mixture (20 μl of total volume) was pre-incubated at 25 °C for 10 min, successively at 37 °C for one hour and then at 70 °C for 10 min to inactivate the enzyme.

Cloning and partial sequencing of stonefly hemocyanin

The multiple alignment of crustacean amino acid sequences (*Pacifastacus leniusculus, Palinurus interruptus, Homarus americanus*) and insect hemocyanins (*Perla grandis*, *Perla marginata* and an embryonic hemocyanin-like protein of *Schistocerca americana*), was performed with ClustalW program (Thompson *et al.*, 1994). BLAST searches (Altschul *et al.*, 1997) show that crustacean hemocyanin sequences are most homologous, to *Pe. grandis*, *Pe. marginata* hemocyanins and to embryonic hemocyanin-like protein of *Sc. americana*. The multiple alignment of these sequences allowed detecting a couple of degenerated primers against the most conserved region, the binding site, about 600 bp. This region codes for 5 of 6 histidine residues directly linked to a copper ion, and 1 of 4 phenylalanine residues that stabilizes the oxygen-copper bond. The forward primer was: Lfor: 5′-GT(GC) GC(CT) TA(CT) (CT)T (GCT) GG(AGCT) GA(AG) G-3′, and the reverse primer was Lrev: 5′-GT(AGT) GC(AGT) GT(CT) TC(AG) AA(AG) TG(CT) TCC-3′. We purchased them from MWG-Biotech (MWG Operon, Ebersberg, Germany). The primers Lfor and Lrev were used in RT-PCR with 1 μg of total RNA of nymphs of each species, using the Eppendorf MiniCycler™ model PTC-150-16 (MJ Research, Waltham, MA, USA). In the same reactions the amplification of a 18S rRNA fragment (343 bp) was used as internal control. RT-PCR products were visualized on 1% (w/v) agarose gels containing GelRed™ Biotium (10 ng/μl), using 1 kb DNA Ladder GeneRuler™ (Geneco-*LifeScience*, Dorset, UK) as size marker. PCR-fragments of expected size were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and cloned into pGEM-T Easy cloning vector (Promega) and transfected into competent *Escherichia coli* cells (JM 109). Plasmid DNA of independent clones was purified using GFXTM Micro Plasmid Prep Kit (Amersham Biosciences, GE Healthcare, Fairfield, UK) and sequenced by a commercial service (MWG-Biotech).

Sequence analyses

The obtained sequences (see accession numbers) were analysed for similarity in BLAST (Altschul *et al.*, 1997). Amino acid composition was deduced from translated cDNA sequences. The tools provided by the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (http://www.expasy.org) were used for the analysis of DNA and amino acid sequences. The obtained sequences of Plecoptera were included in a multiple alignment of hemocyanins and hexamerins sequences of Plecoptera and selected hemocyanins of Crustacea, from GenBank databases (Table 2). The nucleotide sequences were manually adjusted with MEGA version 4 (Tamura *et al.*, 2007), and the respective conserved region aligned with *Pe. grandis* hc2. We used *Pe. grandis* as internal control of the systematic group because of previous knowledge of its hemocyanin sequence. The final alignment includes 27 sequences and 17 species: three crustacean hemocyanins of two species, all presently known hemocyanin subunits of Plecoptera (hc1 and hc2 of *Pe. grandis* and *Pe. marginata*), ten fragments of

Table 2. List of species used for phylogenetic analysis

Species	Accesion number	Protein
Perla marginata	AJ555403	Hemocyanin Hc1
Perla marginata	AJ555404	Hemocyanin Hc2
Perla marginata	AM690365	Hexamerin Hx1
Perla marginata	AM690366	Hexamerin Hx2
Perla marginata	AM690367	Hexamerin Hx3
Perla marginata	AM690368	Hexamerin Hx4
Perla grandis	DQ118369	Hemocyanin Hc1
Perla grandis	DQ118370	Hemocyanin Hc2
Dinocras cephalothes	EF218621	Hemocyanin Hc2
Diamphipnopsis samali	EF620538	Hexamerin Hx1
Isoperla grammatica	EU672885	Hemocyanin Hc1
Isoperla grammatica	EU672886	Hemocyanin Hc2
Siphonoperla torrentium	EU672887	Hexamerin Hx1
Taeniopteryx stankovitchi	EF218622	Hexamerin Hx1
Taeniopteryx burksi	EF617598	Hexamerin Hx1
Brachyptera risi	EU672888	Hexamerin Hx1
Leuctra fusca	EU218620	Hexamerin Hx1
Nemoura sp.	AM690369	Hexamerin Hx1
Nemoura sp.	AM690370	Hexamerin Hx2
Nemoura sp.	AM690371	Hexamerin Hx3
Nemoura hesperiae	EU672889	Hexamerin Hx4
Protonemura ausonia	EU672890	Hexamerin Hx1
Amphinemura sulcicollis	EU715327	Hexamerin Hx1
Allocapnia vivipara	EF617597	Hexamerin Hx1
Homarus americanus	AJ272095	Hemocyanin HcA
Pacifastacus leniusculus	AF522504	Hemocyanin Hc1
Pacifastacus leniusculus	AY193781	Hemocyanin Hc2

the nine studied species of Perloidea and Nemouroidea, and ten hexamerins sequences of Perloidea, Nemouroidea and Eusthenoidea (another stoneflies superfamily). Alignment of the arthropod hemocyanins was performed and manually adjusted to the studied conserved region with CLUSTALW (Thompson *et al.*, 1994).

Molecular phylogeny

The same 27 aminoacidic sequences used in multiple alignment were run for phylogenetic analysis with MEGA version 4 (Tamura *et al.*, 2007). Phylogenetic trees were calculated employing Neighbour Joining, Minimum Evolution and Maximum Parsimony methods. The reliability of trees was tested with bootstrap analysis (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The MP tree was obtained using the Close-Neighbour-Interchange algorithm, with initial trees obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option).

References

Altschul, S.F., Madden,T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Beutel, R.G. and Gorb, S.N. (2001) Ultrastucture of attachmentof exapods: evolutionary patterns inferred from a revised ordinal phylogeny. *J Zool Syst Evol Research* **39**: 177–207.

Burmester, T. (2002) Origin and evolution of arthropod hemocyanins and related proteins. *J Comp Physiol* **172**: 95–107.

Burmester, T. and Hankeln, T. (1999) A globin gene of *Drosophila melanogaster*. *Mol Bio Evol* **16**: 1809–1811.

Burmester, T. and Hankeln, T. (2007) The respiratory proteins of insects. Review. *J Insect Physiol* **53**: 285–294.

Burmester, T., Storf, J., Hasenjäger, A., Klawitter, S. and Hankeln, T. (2006) The hemoglobin genes of *Drosophila*. *FEBS J* **273**: 468–480.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159.

Decker, H. and Rimke, T. (1998) Tarantula hemocyanin shows phenoloxidase activity. *J Biol Chem* **273**: 25889–25892.

Decker, H. and Tuczek, F. (2000) Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. *Trends Biochem Sci* **25**: 392–397.

Efron, B., Halloran, E. and Holmes, S. (1996) Bootstrap confidence levels for phylogenetic trees. *Proc Natl Acad Sci USA* **93**: 7085–7090.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fochetti, R., Belardinelli, M., Guerra, L., Buonocore, F., Fausto A.M. and Caporale, C. (2006) Cloning and Structural Analysis of a Haemocyanin from the Stonefly *Perla grandis*. *Protein J* **25**: 443–454.

Hagner-Holler, S., Schoen, A., Erker, W., Marden, J.H., Rupprecht, R., Decker, H., *et al.* (2004) A respiratory hemocyanin from an insect. *PNAS* **101**: 871–874.

Hagner-Holler, S., Pick, C., Girgenrath, S., Marden, J.H. and Burmester, T. (2007) Diversity of stonefly hexamerins and implication for the evolution of insect storage proteins. *Insect Biochem Mol Biol* **37**: 1064–1074.

Hankeln, T., Jaenicke, V., Kiger, L., Dewilde, S., Ungerechts, G., Schmidt, M., *et al.* (2002) Characterization of *Drosophila* hemoglobin. Evidence for hemoglobin-mediated respiration in insects. *J. Biol Chem* **277**: 29012–29017.

Hankeln, T., Klawitter, S., Krämer, M. and Burmester, T. (2006) Molecular characterization of hemoglobin from the honeybee *Apis mellifera*. *J Insect Physiol* **52**: 701–710.

Henning, W. (1981) Insect phylogeny. *Chichester: Wiley and Sons*, New York.

Hynes, H.B.N. (1976) Biology of Plecoptera. *Annu Rev Entomol* **21**: 135–153.

Kristensen, N.P. (1991) Phylogeny of extant hexapods. In *Insects of Australia: A Textbook for Students and Research Workers*. pp. 125–140. Volume I and II. Second Edn.

Lee, S.Y., Lee, B.L. and Söderhäll, S. (2002) Processing of an Antibacterial Peptide from Hemocyanin of the Freshwater Crayfish *Pacifastacus leniusculus*. *J Biol Chem* **7**: 7927–7933.

Sánchez, D., Ganfornina, M.D., Gutiérrez, G. and Bastiani, M.J. (1998) Molecular characterization and phylogenetic relationships of a protein with potential oxygen-binding capabilities in the grasshopper embryo. A hemocyanin in insects? *Mol Biol Evol* **15**: 415–426.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**:1596–1599.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap

penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.

Tierno de Figueroa, J.M., Sánchez-Ortega, A., Membiela Iglesia, P., Luzón-Ortega, J.M. (2003) Plecoptera. In *Fauna Ibérica* (Ramos, M.A. *et al*. eds) **22**: pp. 404. Museo Nacional de Ciencias Naturales, CSIC, Madrid.

Volbeda, A. and Hol, W.G. (1989) Crystal structure of hexameric haemocyanin from *Panulirus interruptus* refined at 3.2 A resolution. *J Mol Biol* **209**: 249–279.

Zwick, P. (2000) Phylogenetic system and zoogeography of the plecoptera. *Annu Rev Entomol* **45**: 709–746.

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Present knowledge on the presence of hemocyanin in stoneflies (Insecta: Plecoptera)

Valentina Amore [a]; Romolo Fochetti [a]
[a] Dipartimento di Scienze Ambiental, Università della Tuscia, Viterbo, Italia

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Present knowledge on the presence of hemocyanin in stoneflies (Insecta: Plecoptera)

Valentina Amore* and Romolo Fochetti

Dipartimento di Scienze Ambiental, Università della Tuscia, Viterbo, Italia

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Hemocyanin is a respiratory protein that occurs in the main lineages of Arthropoda. In insects hemocyanin is presently known in many orders. Recently, a functional hemocyanin has also been found in the Plecoptera. Further studies have revealed that hemocyanin seems to be not uniformly distributed within this order. In this paper we report additional data, obtained with RT-PCR sequencing, on the presence of hemocyanin in different stonefly species. In addition, we summarise the present knowledge about the distribution of hemocyanin in the Plecoptera. Biological aspects such as larval size, life cycle length, trophic roles and environmental induction are discussed as possible factors that may be correlated with the presence or absence of hemocyanin in the studied species.

Keywords: respiratory proteins; oxygen; arthropod; larva; adult

Introduction

Insects have evolved a very peculiar yet simple apparatus to transport atmospheric oxygen directly to the cells of different body parts for respiration and subsequent production of ATP in the mitochondria. The tracheal system consists of a network of small tubes and provides a thin moist interface for the exchange of gases between atmospheric air and living cells. Many aquatic insects are equipped with a variety of morphological adaptations, like cuticular respiration, gills, breathing tubes, air bubbles and plastron, which allow them to supply oxygen under water, or to acquire it directly from the environment. For a long time the tracheal system has been thought to comply with the requirements of insect respiration. Therefore, the occurrence of respiratory proteins in insects has never been taken into consideration. Only several chironomid midges larvae (Diptera: Nematocera), the aquatic Hemiptera genera *Buenoa* and *Notonecta* (Hungerford 1922) and the parasitic horse botfly genus *Gasterophilus* (Diptera: Brachycera) (Keilin and Wang 1946) have been known to harbour extracellular and intracellular hemoglobin that works as a short-term oxygen storing device. In the last decade further studies have suggested a more complex situation. A potential hemocyanin has been characterised in the embryonal hemolymph of the grasshopper *Schistocerca americana* (Sánchez, Ganfornina,

*Corresponding author. Email: valamore@unitus.it

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Gutiérrez and Bastiani 1998); hemocyanin was found in *Thermobia domestica* and *Lepisma saccarina* (Zygentoma) (Pick, Hagner-Holler and Burmester 2008), and in larvae and adults of the stonefly *Perla marginata* (Hagner-Holler et al. 2004), *Perla grandis* (Fochetti et al. 2006) and other species of Perloidea (Amore et al. 2009). Recently, Pick, Schneuer and Burmester (2009) have reported the presence of hemocyanin in many hexapod orders, like Collembola, Diplura, Archaeognata, Zygentoma, Orthoptera, Phasmida, Dermaptera, Isoptera and Blattodea.

In this paper we report further new data regarding the presence of hemocyanin in larvae and adults of stonefly species and summarise the present state of knowledge on hemocyanin in the Plecoptera. On basis of the available data, we discuss the occurrence of hemocyanin in the different species and suggest a possible explanation involving larval size, life cycle length, trophic roles, and autoecology.

Materials and methods

Studied species

17 species of Plecoptera belonging to the two European superfamilies Perloidea and Nemouroidea were analysed by RT-PCR. The results regarding most of them have been already published (Amore et al. 2009); new data here presented concern *Capnia bifrons* (Newman, 1839), *Guadalgenus franzi* (Aubert, 1963), *Afronemoura amatolae* (Balinsky, 1956); and *Aphanicercella bullata* Stevens and Picker, 1999.

Larvae

Perloidea

Perlidae: *Perla grandis* Rambur, 1840; *Perla marginata* (Panzer, 1799); *Dinocras cephalotes* (Curtis, 1827); Perlodidae: *Isoperla grammatica* (Poda, 1761), *Guadalgenus franzi* (Aubert, 1963); Chloroperlidae: *Siphonoperla torrentium* (Pictet, 1841);

Nemouroidea

Taeniopterygidae: *Taeniopteryx stankovitchi* Ikonomov, 1978; *Brachyptera risi* (Morton, 1896); *Brachyptera vera* Berthélemy and González del Tanago, 1983; Leuctridae: *Leuctra fusca* (Linnaeus, 1758); Nemouridae: *Amphinemura sulcicollis* (Stephens, 1836); *Nemoura hesperiae* Consiglio, 1960; *Protonemura ausonia* (Consiglio, 1955); Notonemouridae: *Afronemoura amatolae* (Balinsky, 1956).

Adults

Perloidea

Perlidae: *Perla grandis* Rambur, 1840; *Perla marginata* (Panzer, 1799); *Dinocras cephalotes* (Curtis, 1827); Perlodidae: *Isoperla rivolorum* (Pictet, 1841).

Nemouroidea

Capnidae: *Capnia bifrons* (Newman, 1839); Notonemouridae; *Afronemoura amatolae* (Balinsky, 1956); *Aphanicercella bullata* Stevens and Picker, 1999.

RT-PCR cloning of stonefly hemocyanin

Total RNA was extracted from larvae and adults, and degenerate oligonucleotide primers, targeting the hemocyanin conserved region (about 600 nucleotides), were used in an RT-PCR reaction. The amplification of a 18S rRNA fragment (343 bp) was used as internal control. PCR fragments of expected size were cloned into

a pGEM-T easy vector, and sequenced by a commercial service, as described elsewhere (Amore et al. 2009).

Results

Cloning and sequencing hemocyanin

Based on cloning and sequencing RT-PCR products of expected size [see Genebank accession numbers DQ118369, DQ118370 (Fochetti et al. 2006), EF218621, EU672885, EU672886, EU672887, EF218622, EU672888, EF218620, EU672889, EU672890, EU715327 (Amore et al. 2009), FJ384672; FJ393060, FJ415315], we can conclude that hemocyanin is not homogeneously widespread across the order Plecoptera and that its presence can vary during the life cycle (Table 1).

All studied larvae of Perlidae and Perlodidae species, except of *Siphonoperla torrentium*, harbour hemocyanin; this hemocyanin consists of two distinct subunit types (Hc1 and Hc2). In particular, *Perla* shows hemocyanin in larval stage of the 1st, the 2nd and the 3rd years, and in the adults. On the contrary, RT-PCR reaction in adults of *Dinocras cephalotes* and *Isoperla rivulorum* did not result in PCR products or gave aspecific products. *Siphonoperla torrentium* (Chloroperlidae) and all the studied species of Nemouroidea (see Table 1 under Euholognatha) lack a functional hemocyanin because the conserved region sequence shows substitution of key amino acids involved in Cu^{+} bond and in stabilisation of the Cu^{+}–O_2 complex (see also Amore et al. 2009). Moreover, these sequences have a certain similarity to insect hexamerins (hx). Hexamerins, which are nonrespiratory proteins, evolved independently from the same superfamily proteins and acquired a storage function (Hagner-Holler, Pick, Girgenrath, Marden and Burmester 2007). PCR products of the expected size were obtained from adult *Capnia bifrons* but sequences were very similar to those of the other Nemouroidea.

Discussion

Sequence analysis

Respiratory proteins reversibly bind molecular O_2 for the purpose of transport or storage. Hemocyanins are copper-containing (Cu^{+}), oxygen carrying proteins that only occur in molluscs and arthropods. Although similar in function, mollusc and arthropod-derived hemocyanins are not phylogenetically related (Burmester 2004; Burmester and Hankeln 2007).

The data presented here and those reported in Amore et al. (2009) show that hemocyanin is not homogenously distributed across the Plecoptera (see Table 1), event though only species of Arctoperlaria have been analysed so far. We cannot exclude the possibility that hemocyanin is present in Nemouroidea species, but we have the evidence that our primers failed to amplify hemocyanin in Nemouroidea but not in the Perloidea (with the exception of *S. torrentium*). Besides, analysis on South African Notonemouridae species (sister group of Nemouridae) gave the same result as Nemouridae species.

In order to understand this complex picture we considered a series of ecological factors that could be involved and responsible for different physiological requirements, i.e. autoecology, larval size, life cycle length, trophic roles and environmental induction (Amore et al. 2009). We analysed species living in different ecological conditions, from perennial rivers to seasonal streams. Seasonal streams

Table 1. Summary of known data on the presence of hemocyanin in stoneflies.

	Family	Species	Life stage (years)	Size (mm) animal	diet	Life cycle	Stream type	Hc	Non functional hc or hx
Systellognatha: Perloidea	Perlidae	*Perla marginata*	1st, 2nd, 3rd		carnivorous	semivoltine	permanent	hc1; hc2	–
			adult	12.5–25	–			hc1; hc2	
		Perla grandis	1st, 2nd, 3rd		carnivorous	semivoltine	permanent	hc1; hc2	–
			adult	20–33	–			hc1; hc2	
		Dinocras cephalotes	larva		carnivorous	semivoltine	permanent	hc1; hc2	–
			adult	11–30	–			no	
	Perlodidae	*Isoperla grammatica*	larva	8–12	carnivorous	univoltine	permanent	hc1; hc2	–
		Isoperla rivulorum	adult	10–15	carnivorous	univoltine		no	
		Guadalgenus franzi	larva	12–18	carnivorous	univoltine	temporal	hc1; hc2?	–
	Chloroperlidae	*Siphonoperla torrentium*	larva	9–11	carnivorous, phytophagous	univoltine	permanent	–	yes
Euholognatha: Nemouroidea	Taeniopterygidae	*Taeniopteryx stanckovitchi*	larva	8–12.5	phytophagous	univoltine	permanent	–	yes
		Brachyptera risi	larva	8–13.5	phytophagous	univoltine	permanent	–	yes
		Brachyptera vera	larva	8.5–10.5	phytophagous	univoltine	temporal	–	yes
	Leuctridae	*Leuctra fusca*	larva	5.5–10	phytophagous	univoltine	permanent	–	yes
	Nemouridae	*Nemoura hesperiae*	larva	6–9	phytophagous	univoltine	permanent	–	yes
		Protonemura ausonia	larva	7–11	phytophagous	univoltine	permanent	–	yes
		Amphinemura sulcicollis	larva	4.5–8.8	phytophagous	univoltine	permanent	–	yes
	Capnidae	*Capnia bifrons*	adult	5.3–11	phytophagous	univoltine	permanent	–	yes
	Notonemouridae	*Afronemoura amatolae*	larva		phytophagous	univoltine	permanent	–	–
			adult	6–7.5	phytophagous			–	–
		Aphanicercella bullata	adult	5–7	phytophagous	univoltine	permanent	–	–

are formed annually and expand during a short period as a consequence of melting snow and spring rains. Firstly, we hypothesised that Plecoptera larvae living in seasonal streams have to cope with heavier physiological stress than species in perennial water. For this reason we checked for the presence of hemocyanin in two temporal water species, one for each stonefly superfamily, *Guadalgenus franzi* and *Brachyptera vera*, and we compared the results with other studied species belonging to the same families living in perennial rivers. As shown in Figure 1, habitat differences do not seem to affect hemocyanin mRNA production. In fact, the analysed *Guadalgenus franzi* harbours hemocyanin while *Brachyptera vera* does not.

We then considered the differences in larval size, and therefore, the life cycle lengths, since larval size largely overlaps with life cycle modality. There is a relevant interspecific range in size among species: Perlidae, and in lower proportion, Perlodidae have often a consistent size (up to 4 cm in length), and they are generally very sensitive to oxygen levels in cold running waters. They usually achieve the imaginal stage in 2–4 years. Taeniopterygidae, Nemouroidae, Leuctridae, and Chloroperlidae have larvae of smaller size (0.5–1.5 cm approximately) and a complete life cycle is achieved in about one year. For these small stoneflies, the tracheal system may be sufficient for their oxygen demand in water. Our data are moderately in agreement with the hypothesis that bigger species could need a carrier protein to meet increased oxygen requirements. Nevertheless, the medium sized *I. grammatica* (1.2–1.5 cm) with a univoltine life cycle has the gene for hemocyanin. To prove a correlation between species size and presence of hemocyanin, a larger number of species needs to be analysed and hemocyanin should be quantified at different points in the larval lifetime.

Trophic roles can also determine oxygen requirements. Perlidae and Perlodidae are mainly carnivorous, while Nemouroidea are phytophagous or detritivorous. Active predators may have a major need of oxygen because of their increased activity, while the tracheal system may be sufficient to meet the metabolic demands of small, less active detritivorous insects. This hypothesis is congruent with our data except for the carnivorous *S. torrentium* that possesses a nonfunctional hemocyanin-like protein. However, it must be said that *S. torrentium* ingests vegetal matter in the

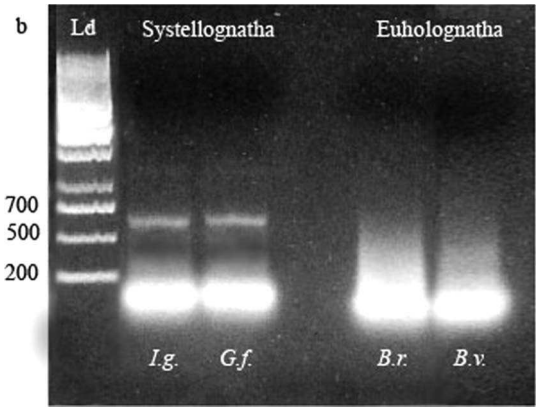

Figure 1. RT-PCR identification of the conserved fragment of 586 nucleotides in species of perennial (1st and 3rd) and seasonal (2nd and 4th) freshwaters. (*I.g.*) *Isoperla grammatica*; (*G.f.*) *Guadalgenus franzi*; (*B.r.*) *Brachyptera risi*; (*B.v.*) *Brachyptera vera*.

juvenile stages. Our preliminary data on Notonemouridae, a family usually related to Nemouridae (but see for instance Zwick 2000), confirm the absence of hemocyanin in both larvae and adults (see Table 1).

While the discovery of respiratory protein in larvae can be explained by an enhanced oxygen demand in an aquatic environment, the presence of a respiratory pigment in adults remains unexplained. Nevertheless, we know that hemocyanin also may occur in adult stoneflies, though it has been proven with certainty only in the imago of *Perla marginata* (Hagner-Holler et al. 2004) and *Perla grandis* (Fochetti et al. 2006) so far.

Further considerations

The role of respiratory proteins in insects may be more significant than what we believed in the past. Recently, a hemoglobin gene has been detected in *Drosophila* (Burmester and Hankeln 1999) and in *Apis* (Hankeln, Klawitter, Krämer and Burmester 2006), terrestrial insects that have no difficulty with oxygen supply or energy (ATP) shortages for flight activity. The physiological role of Plecoptera hemocyanin, which is present at low concentrations, is not well understood. Hagner-Holler et al. (2004) tested the functionality of hemocyanin with polarographic fluorometric methods and in vitro demonstrated the oxygen-binding property of *Perla marginata*. It could be also hypothesised that, under particular conditions, the N- and C-terminal part of the protein subunits are processed and function as an antibacterial peptide and as phenoloxidase, respectively, as is the case in *Pacifastacus leniusculus* (Lee, Lee and Söderhäll 2003, 2004). Finally, the role of environmental induction in activating a respiratory response mediated by a carrier protein must be studied.

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References

Amore, V., Belardinelli, M., Guerra, L., Buonocore, F., Fausto, A.M., Ubero-Pascal, N., and Fochetti, R. (2009), 'Do really all stonefly nymphs have hemocyanin? Further data on the presence of hemocyanin in the larval stages of Plecoptera species', *Insect Molecular Biology*, 18, 2003–2011.

Burmester, T. (2004), 'Evolutionary history and diversity of arthropod hemocyanins', *Micron*, 35, 121–122.

Burmester, T., and Hankeln, T. (1999), 'A globin gene of *Drosophila melanogaster*', *Molecular Biology and Evolution*, 16, 1809–1811.

Burmester, T., and Hankeln, T. (2007), 'The respiratory proteins of insects', *Journal of Insect Physiology*, 53, 285–294.

Fochetti, R., Belardinelli, M., Guerra, L., Buonocore, F., Fausto, A.M., and Caporale, C. (2006), 'Cloning and Structural Analysis of a haemocyanin from the Stonefly *Perla grandis*', *Protein Journal*, 25, 443–454.

Hagner-Holler, S., Pick, C., Girgenrath, S., Marden, J.H., and Burmester, T. (2007), 'Diversity of stonefly hexamerins and implication for the evolution of insect storage proteins', *Insect Biochemestry and Molecular Biology*, 37, 1064–1074.

Hagner-Holler, S., Schoen, A., Erker, W., Marden, J.H., Rupprecht, R., Decker, H., and Burmester, T. (2004), 'A respiratory hemocyanin from an insect', *Proceedings of the National Academy of Sciences of the United States of America*, 101, 871–874.

Hankeln, T., Klawitter, S., Krämer, M., and Burmester, T. (2006), 'Molecular characterization of hemoglobin from the honeybee *Apis mellifera*', *Journal of Insect Physiology*, 2, 701–710.

Hungerford, H.B. (1922), 'Oxyhaemoglobin present in the backswimmer, *Buenoa margaritacea*, Bueno (Hemiptera)', *The Canadian Entomologist*, 54, 262–263.

Keilin, D., and Wang, Y.L. (1946), 'Haemoglobin of *Gasterophilus* larvae. Purification and properties', *Biochemical Journal*, 40, 855–866.

Lee, S.Y., Lee, B.L., and Söderhäll, S. (2003), 'Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus*', *Journal of Biological Chemistry*, 278, 7927–7933.

Lee, S.Y., Lee, B.L., and Söderhäll, S. (2004), 'Processing of crayfish hemocyanin subunits into phenoloxidase', *Biochemical and Biophysical Research Communications*, 322, 490–496.

Pick, C., Hagner-Holler, S., and Burmester, T. (2008), 'Molecular characterization of hemocyanin and hexamerin from the firebrat *Thermobia domestica* (Zygentoma)', *Insect Biochemistry and Molecular Biology*, 38, 977–983.

Pick, C., Schneuer, M., and Burmester, T. (2009), 'The occurrence of hemocyanin in Hexapoda', *FEBS Journal*, 276, 1930–1941.

Sánchez, D., Ganfornina, M.D., Gutiérrez, G., and Bastiani, M.J. (1998), 'Molecular characterization and phylogenetic relationships of a protein with potential oxygen-binding capabilities in the grasshopper embryo. A hemocyanin in insects?', *Molecular Biology and Evolution*, 15, 415–426.

Zwick, P. (2000), 'Phylogenetic System and Zoogeography of the Plecoptera', *Annual Review of Entomology*, 45, 709–746.

Lack of hemocyanin in Oriental Perlidae and Peltoperlidae (Plecoptera) suggests multifunctionality of the protein in stoneflies larvae.

V. Amore*, B. Gaetani and R. Fochetti

[1] Dipartimento di Scienze Ambientali, Università della Tuscia, Viterbo, Italia

*Corresponding author. Email: valamore@unitus.it

Valentina Amore, Dipartimento di Scienze Ambientali, Università della Tuscia, snc, Largo dell'Università 01100, Viterbo, Italy. Tel: + 39 0761357178; Fax: + 0761357179;

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ABSTRACT: Data on the presence/absence of hemocyanin in Plecoptera species belonging to the Oriental fauna are reported, being previously known data on the presence/absence of this protein based only on European species. We studied six species of the family Perlidae and one species of the family Peltoperlidae from Thailand but we did not obtain mRNA hemocyanin sequences from these Oriental stoneflies. All species show hexamerins similar to the hexamerins previously found in species of the same families. The above results suggest us that the presence of hemocyanins, at least to basic quantitative, does not depend on sizes or life cycle, nor on trophic role, as hypothesized in the past. We suggest that in the Plecoptera the specific role of this protein has not still completely understood. The respiratory function hypothesis can not be rejected, but the hemocyanin expression pattern we obtained across the whole Plecoptera order cannot be explained unless hypothesizing other functions.

Key words: stonefly, respiratory protein, immune defense

INTRODUCTION

Hemocyanins are copper-containing and multi-subunit proteins that are freely dissolved in the hemolymph of many molluscs and arthropods (Linzen 1989; Markl & Decker 1992; van Holde *et al.* 2001). Hemocyanins are considered, together with hemerithryns

and hemoglobins, animal metal-containing respiratory proteins with the function to reversibly bind O_2 for the purpose of transport or storage. They are composed by six identical or similar subunits with molecular mass of around 75 kDa each (van Holde *et al.* 1995; Salvato & Beltramini 1990). The active site, in domain II of each subunit, is a highly conserved region able to bind an O_2 molecule by the means of two Cu^+, coordinated by three histidines in two distinct binding sites. These proteins have been studied thoroughly in Crustacea Malacostraca and Chelicerata (Mangum 1980; 1983a, 1983b, 1985; Volbeda & Hol 1989), but only in the last years, hemocyanins have been reported in many Hexapoda order like Collembola, Diplura, Archaeognata, Zygentoma, Plecoptera, Orthoptera, Phasmida, Dermaptera, Isoptera and Blattodea (Burmester & Hankeln 2007; Pick *et al.* 2009).

The recent finding of hemocyanin in many insect orders raises doubts about the paradigm on tracheal system and respiratory proteins. The notion that is widely accepted is that the tracheal system is sufficient for insect respiration (Brusca & Brusca 2002).

In addition, in all insects investigated so far, copper-less proteins, which resemble hemocyanins in structure and sequence, have been identified. These are referred to as hexamerins (Telfer & Kunkel, 1991; Beintema *et al.* 1994).

In the last years, our study has been focused on the presence of hemocyanin in Plecoptera. Starting from the first report of hemocyanin in the stonefly *Perla marginata* (Panzer, 1999) (Hagner-Holler *et al.* 2004), we extended our research to better understand presence, functional significance and role of this protein in the Plecoptera. We analyzed many species of both European superfamilies (Eulognatha and Systellognatha) and the mRNA expression of hemocyanin in different stages of their life cycle (Fochetti *et al.* 2006; Amore *et al.* 2009; Amore & Fochetti 2009). We reported mRNA hemocyanin sequences in various species, and the absence of this product in many others. We hypothesized three causes that could be correlated to the presence of hemocyanin in Plecoptera considering a series of ecological and autoecological parameters. These hypotheses refer to: (1) environmental induction, (2) larval size, a feature that is strictly related to life cycle (univoltine or semivoltine) and (3) trophic roles.

In this paper we report new data about species belonging to the stonefly fauna of Thailandi. We analyzed six species of the family Perlidae and one species of the family Peltoperlidae, never analyzed before. Enlarging the study to Tropical Asian taxa has the

meaning to analyze species that live in environments with different ecological traits respect to the ones previously studied, belonging to the Palaearctic fauna. This would allow us to text the relationship between presence of hemocyanin and environment conditions.

MATERIALS AND METHODS

Insects

Larvae of seven genera of Plecoptera (species not further identified) belonging to the Oriental fauna were collected in the following sites, conserved in RNA later and analyzed in RT-PCR reaction. They are:

Perloidea

Peltoperlidae

Cryptoperla sp. Huai Sai Leung – April 2009. 1030 mt.

Perlidae – Perlinae

Togoperla sp., Tetropina sp. Phanoperla sp., Neoperla sp. Mae Chaen - April 2009, 1025 mt.

Etrocorema sp. Huai Sai Leung – Abril 2009. 1030 mt

Caroperla sp. Kung Klang Siriphum waterfall- April 2009. 1300 mt

Total RNA isolation, identification and molecular cloning of sequences

Total RNA was extracted from larvae of the analyzed species using the phenol-guanidine thiocyanate method (Chomczynski & Sacchi, 1987) or the Mini or Micro Qiagen Kit (Hilden, Germany), following to the manufacturer's instructions, according on the size and weight of the samples. First strand cDNA syntheses and subsequent PCR were carried out by using BioScript Rnase H Minus and BIOTAQ^{TM} DNA Polymerase (Bioline). For control of the efficiency of cDNA synthesis, β-actin was amplified using the following degenerated oligonucleotide primers: 5'-TGGCAYCAYACNTTYTAYAA-3' and 5'-GCDATNCCNGGRTACATNGT-3'. Degenerated oligonucleotide primers were designed on the conserved amino acid sequence of hemocyanins to amplify a fragment of domain II, containing key amino acid for copper-binding, aligning known arthropodan hemocyanins of *Pacifastacus leniusculus* (Dana, 1852), *Palinurus interruptus* (Randall, 1840), *Homarus americanus* (H. Milne-Edwards, 1837), *Schistocerca americana (*Drury, 1773), and Plecoptera

species. The primers were: 5'-GT(GC) GC(CT) TA(CT) (CT)T (GCT) GG(AGCT) GA(AG) G-3', and 5'- GT(AGT) GC(AGT) GT(CT) TC(AG) AA(AG) TG(CT) TCC-3'. We purchased them from MWG-BIOTECH (Germany). PCR-fragments of expected size were purified using Wizard SV Gel and PCR Clean-Up System (Promega), and were cloned into pGem-T Easy/JM109 system (Promega). Plasmid DNA of independent clones was purified using GFXTM Micro Plasmid Prep Kit (Amersham Biosciences) and sequenced by a commercial service (Eurofins MWG Operon- Germany).

Sequence and phylogenetic analysis

Resulting cloned sequences were translated with the tools provided with the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (http://www.expasy.org). Statistical analysis was carried out with VassarStart web site for statistical computation (http://faculty.vassar.edu/lowry/VassarStats.html). A multiple alignment of cloned sequences of Perloidea hexamerins [hx: *Siphonoperla torrentium* (Pictet, 1841) EU672887, and *P. marginata* AM690365, AM690366, AM690367, AM690368)] and hemocyanins [(hc: *Isoperla grammatica* (Poda, 1761) EU672885, EU672886; *Guadalgenus franzi* (Aubert, 1963) FJ393060; *P. marginata* AJ555403; AJ555404; *Perla grandis* (Rambur, 1842), DQ118369, DQ118370; *Dinocras cephalotes* (Curtis, 1827) FJ415315, EF218621)], and of Myriapoda hemocyanins [(hc: *Scutigera coleoptrata* (Linnaeus, 1758) AJ344359, AJ344360, AJ431378, AJ431379, AJ512793 and *Spirostreptus* (Brandt, 1833) *sp.* AJ297738)] was performed using MAFFT online version (Kazutaka 2002) (http://align.genome.jp/mafft), matrix BLOSUM62. BioEdit (Hall 1999) was used for multiple sequence alignment analysis. In table 1 are listed analyzed species.

The final alignment includes 27 sequences and 15 species: 9 Perlidae, 2 Perlodidae, 1 Chloroperlidae, 1 Peltoperlidae. 6 Myriapoda species belonging to two different families are chosen as outgroup (Tab. 1) (Kusche & Burmester 2001). After the exclusion of N- and C-terminal extensions, the final alignment contains 209 positions. Neighbor Joining (NJ) tree was inferred with MEGA 4.1 (Tamura *et al.* 2007) and the evolutionary distances were computed using the JTT matrix-based method (Jones *et al.* 1992). The reliability of the branching pattern was tested by bootstrap (Felsenstein 1985), with 1000 replications.

Maximum Likelihood analysis was run with PhyML online version (Guindon & Gascuel 2003), the appropriate model of amino acid sequence evolution (LG + G; Le &

Gascuel 2008) was selected by ProtTest (Abascal *et al.* 2005) using the Acaik Information Criterion (AIC), and bootstrap of 100 replicates.

RESULTS

Molecular cloning, sequence analyses and comparisons

The designed primers were applied to cDNA from studied species and produced an amplified sequence of about 570 bp length, the translated amino acid sequences are composed by about 188 amino acids. The obtained sequences of Plecoptera (*Cryptoperla sp*. hx: GU121387; *Tetropina sp.* hx: GU121388; *Togoperla sp*.: hx GU121389; *Neoperla sp*. hx: GU121390; *Etrocorema sp.* hx: GU121391; *Phanoperla sp*. hx: GU121392; *Caroperla sp.* hx: GU121400) (Tab.1) were run in the BLAST, Blastn and Blastp (Altschul *et al.* 1997).

In order to give a detailed description of these new Plecoptera sequences, we compared them with an identity matrix, built with known Plecoptera sequences of Perlidae (*P. marginata*) and Perlodidae (*I. grammatica*) hemocyanins and hexamerins (Tab. 2). Nucleotidic and amino acids translated sequences showed high degree of identity among all Thai hexamerins (>0.87 at nucleotide level, and >0.82 at amino acid level). The higher value at nucleotidic than at amino acidic level shows that, in the multiple alignment, the major part of nucleotidic changes is at the 1st or 2nd nucleotide of the ORF. Within these species, hexamerins of *Neoperla sp.*, *Phanoperla sp.*, *Togoperla sp.*, on the one hand, and *Etrocorema sp.* and *Caroperla sp.* on the other hand, were found closely related. Thai species display a low identity with *P. marginata* hexamerins, a European Perlidae of wide distribution. The values are between 0.59 and 0.69 for amino acids and between 0.70 and 0.76 for nucleotides with the exception of *P. marginata* hx3, whose value are lower and differ also from other *P. marginata* known hexamerins.

All hexamerins have a similar identity value when compare with hemocyanins (hc1 and hc2). The identity range is from 0.30 to 0.40 for amino acids, and from 0.49 to 0.54 for nucleotide level.

For further comparisons, the amino acid composition of Plecoptera hexamerins mentioned above, and known Plecoptera hemocyanins are listed in table 3 and table 4. Amino acid proportions were deduced from the cDNA translated sequences for each of the insect hexamerins and hemocyanins (Tab.3 and Tab. 4).

In hemocyanins and hexamerins, Tryptofane (Try) and Cystidine (Cys) values are next to zero. The content of aryl groups, Phenylalanine and Tyrosine (Phe+Tyr

mean=15.9%) is not significantly different to the typical value of 16.5% (P=0.009 t-test) reported in literature (Telfer and Kunkel, 1991). The average of Methionine (1,8%), and it is significantly different from the average value of hexamerins of 4% (P<0,0001 t-test). Histidine content (His) is lower in hexamerins (2,65%) than in hemocyanin (8,24%). The multiple sequence alignment (Fig.1) shows that some conserved residues, 4 of the 5 histidines (H) for this fragment, required for reversible oxygen binding, have been lost, while the Phenylalanine (F), necessary to hemocyanin to stabilize O_2 binding, is present in all sequences. Some key groups for the three dimensional fold of the molecules (Fig. 1) are conserved too.

Phylogenetic analysis. Both analyses, NJ and ML point out four principal groups; oriental stonefly hexamerins form a monophyletic and well supported clade with others Perloidea hexamerins.

In the Neighbors Joining analysis (Fig. 2), four principal clusters are evident: one for Myriapoda hemocyanin at ancestral position, one for Plecoptera hemocyanin subunit 1 (hc1), then a derived hemocyanin subunit 2 (hc2) and finally all hexamerins.

Tropical Plecoptera hexamerins join the cluster with others Perloidea hexamerins. *Caroperla sp.*, whose systematic position is still uncertain, clustered together with *Etrocorema sp*. and they are sister group of all others sequence of Thai species, included *Cryptoperla sp*., a Peltoperlidae belonging to Pteronarcyoidea superfamily. Inside each group traditional systematic positions are respected. *D. cephalotes* joins the cluster with *P. marginata* and *P. grandis*, also belonging to the Perlidae. The same is true for *I. grammatica* and *G. franzi*, both belonging to Perlodidae.

In the Maximum Likelihood analysis (not shown), hexamerins are found as sister group of both hemocyanin subunits.

Discussion

Amino acid composition. For most proteins, a simple percentage composition of amino acids is no longer an interesting topic since they closely conform to the average composition described by King and Jukes (1969). In our case this analysis could be a tool to quantitatively evaluate similarity between hexamerins and hemocyanins, two classes of proteins closely related. Hexamerins are described like arylphorin and methinine rich proteins, whose value totally exceed more than twice the average determined for typical polypeptides (Telfer & Kunkel 1991). The aromatic amino acids,

generally about 16.5%, could be related to the demands of these constituents during cuticle sclerotization. The comparison of hemocyanins domain II with hexamerins shows that aryl groups have a low percentage in hemocyanin (10%); the quantity of Tyrosine is similar, and the different percentage is due to Phenylalanine. Tryptofane and Cystidine values are very low in both hexamerins and hemocyanins, like in any other hemolymph proteins, because both inter- and intrachain disulfide bridges are highly unusual (Telfer & Kunkel 1991). Relevant amino acids difference regards aspartic acid (Asp) and Histidine, important to bind Cu^{+} in hemocyanin. Consistently with Hagner-Holler *et al.* (2007), in Plecoptera the content of Methionine is lower than the typical high value for hexamerins.

Phylogenetic analysis. The hexamerin sequences of studied Plecoptera were included in an alignment of Perloidea hemocyanins and hexamerins. We selected Myriapoda hemocyanin sequences as outgroup on the base of phylogenetic analysis of Kusche and Burmester (2001), where Myriapoda hemocyanin is in ancestral position. All Thai species cluster with others Perloidea hexamerins. The analysis of the conserved region of Plecoptera hemocyanins agrees with traditional systematic position (Fochetti & Tierno de Figueroa 2008). All hexamerins join the same clade (100% Bootstrap value, Fig. 2), but within the clade, these proteins follow more loosely the systematic relationship than hemocyanin sequences. The lower degree of adherence to systematic relationship indicates that hexamerins are subjected to a lower evolutive pressure that permitted them to cumulate mutations and a successively considerable amino acids differentiation (Telfer & Kunkel 1991; Burmester *et al.* 1998, 2002).

Further consideration: hemocyanin as a putative multifunctional protein. Little is known about the ecology of this tropical fauna (Zwick 1982; 1984; Stark & Sheldon 2008; Stark & Sivec 2008). The specimens have been collected in tropical rivers, characterized by a great seasonal and daily temperature stability of about 20 °C (Dudgeon 1999). At these conditions, the quantity of oxygen dissolved in water is less if compare with ecological conditions of European mountain and perennial rivers.

Togoperla sp., Tetropina sp., Etrocorema sp., Phanoperla sp., Neoperla sp., Caroperla sp., and *Cryptoperla sp.,* are species of large or medium size. Contrarily to what has been observed till now for the analyzed European Perloidea (*I. grammatica, G. franzi,*

D. cephalotes, P. marginata and Perla grandis), the Asiatic Perlidae do not have hemocyanins in their mRNA, but they have hexamerins similar to those of the species of the same family.

The above discussed results suggest that the presence of hemocyanins, at least to basic quantitative, does not depend on sizes and biological cycle because European and Oriental species are, more or less, of the same size, and presumable display a similar life cycle. The great stability of tropical water courses compared to European streams, especially regarding to water temperature range of variation, and the incidence on dissolved oxygen concentrations (Dudgeon 1999), can account for the lack of hemocyanins in Thai species. In a possible evolutionary framework, different physiological needs, correlated with water range temperature and pH, might led to secondary loss of hemocyanin in an ancient oriental Perloidea with a secondary divergent evolution.

Hemocyanin capability to bind oxygen has only been demonstrated by kinetic methods in *P. marginata* (Hagner Holler *et al.* 2004), but there is no clue that the respiratory role were its exclusive function. As it has been reported by Decker *et al.* (2007) for Chelicerata and Crustacea, hemocyanins can play a role as functional phenoloxidase, immune or storage proteins in non feeding periods.

Moreover, we have to take into account that Decapoda hemocyanin concentration is associated with molting cycle, suggesting a specific utilization during starvation (Depledge & Bjeregaard 1989). Under some circumstances, hemocyanin is metabolically recycled and employed as source of energy and amino acids (Zuckerkandl 1960; Hagerman 1983). Recent studies on Chelicerata and Crustacea reported that hemocyanin can manifest, *in vitro,* phenoloxidase activity after a proteolytic cleavage of the N-terminal part (Nagai & Kawabata 2000; Decker & Rimke 1998; Decker & Tuczek 2000; Decker *et al.* 2001; Pless *et al.* 2003; Jaenicke & Decker 2004). Phenoloxidases (tyrosinases and catecholoxidases) are proteins involved in the initial step of the biochemical cascade of melanin biosynthesis, that play a key role in the sclerotization of the cuticle after molting, browning, wound healing and in immune defense (Söderhäll & Ceresius 1998). Studies on similar function were never carried out in Hexapoda. Anyway, it is to verify if phenoloxidase activity of hemocyanin is an *in vitro* experimental artifact.

While for a long time hemocyanin has been considered mainly a respiratory protein, we suggest that in the Plecoptera the specific role of this protein has not still completely

understood. The respiratory function hypothesis can not be rejected, but the hemocyanin expression pattern we obtained across the whole Plecoptera order cannot be explained unless hypothesizing other functions. We can suppose that hemocyanin function is in a fine balancing between oxygen binding and amino acid storage.

REFERENCES

Abascal F, Zardoya R, Posada D (2005) ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21, 2104-2105.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

Amore V, Belardinelli M, Guerra L, Buonocore F, Fausto AM, Ubero-Pascal N, Fochetti R (2009) Do really all stonefly nymphs have hemocyanin? Further data on the presence of hemocyanin in the larval stages of Plecoptera species *Insect Molecular Biology* 18, 203-11.

Amore V, Fochetti R (2009) Present knowledge on the presence of hemocyanin in stoneflies (Insecta: Plecoptera) *Aquatic Insect* 31 (4): 577-583..

Beintema JJ, Stam WT, Hazes B, Smidt MP (1994) Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). *Mololecular Biology and Evolution* 11, 493–503.

Brusca RC, Brusca GJ (2002) *Invertebrates.* Second ed. Sinauer Associates Sunderland, MA.

Burmester T (2001) Molecular evolution of the arthropod hemocyanin superfamily, *Molecular Biology and Evolution* 18, 184-195.

Burmester T (2002) Origin and evolution of arthropod hemocyanins and related proteins. *Journal of Comparative Physiology, B: Biochemical, Systematic, and Environmental Physiology* 172, 95–117.

Burmester T, Massey HC Jr, Zakharkin SO, Beneš H (1998) The evolution of hexamerins and the phylogeny of insects. *Journal of Molecular Evolution.* 47, 93-108.

Burmester T, Hankeln T (2007) The respiratory proteins of insects. Review. *Journal of Insect Physiology* 53, 285-94.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analitical Biochemestry* 162, 156–159.

Decker H, Rimke T (1998) *Tarantula* hemocyanin shows phenoloxidase activity, *Journal of Biological Chemistry* 273, 25889–25892.

Decker H, Tuczek F (2000) Tyrosinase/catecholoxidasse activity of hemocyains: structural basis and molecular mechanism. *Trends in Biochemical Sciences* 25, 392-397.

Decker H, Ryan M, Jaenicke E, Terwinlliger N (2001) SDS induced pheneloxidase activity of hemocyanin fron *Limulus polyphemus*, *Eurypelma californicum*, Cancer *magister*. *Journal of Biological Chemistry* 276, 17796- 17799.

Decker H, Hellmann H, Jaenicke E, Lieb B, Meissner U, Markl J (2007) Recent progress in hemocyanin research. *Integrative and Comparative Biology* 1-14.

Depledge MN, Bjerregaard P (1989) Haemolymph protein composition and copper levels in decapod crustaceans. *Helgol Meeresunters* 43, 207–223.

Dudgeon D (1999) Tropical *Asian streams: zoobenthos, ecology and conservation*. Hong Kong University Press.

Felsenstein J (1985) Confidence limits of phylogenies: an approach using the bootstrap *Evolution* 39, 783-791.

Fochetti R, Belardinelli M, Guerra L, Buonocore F, Fausto, AM, Caporale C (2006), Cloning and Structural Analysis of a haemocyanin from the Stonefly *Perla grandis Protein Journal* 25, 443-454.

Fochetti R, Tierno de Figueroa JM (2008) Global diversity of stoneflies (Plecoptera; Insecta) *Hydrobiologia* 594, 365-377.

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52,696-704.

Hagner-Holler S, Schoen A, Erker W, Marden JH, Rupprecht R, Decker H, Burmester T (2004) A respiratory hemocyanin from an insect *Proceedings of the National Academy of Sciences USA* 101, 871–874.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95-98.

Hagerman L (1986) Haemocyanin concentration in the shrimp *Crangon crangon* (L.) after exposure to moderate hypoxia *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 85, 721–724.

Jaenicke E, Decker H (2004) Functional changes in the family of type 3 copper proteins during evolution. *Chembiochem* 5, 163-9.

Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences *Computer Applications in the Biosciences* 8, 275-282.

Kazutaka K, Kazuharu M, Kei-ichi K, Takashi M (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* 30, 3059-3066.

King T, Jukes J (1969) Non-darwinian evolution *Science* 164, 788-98.

Kusche K, Burmester T (2001) Diplopod Hemocyanin Sequence and the Phylogenetic Position of the Myriapoda *Molecular Biology and Evolution* 18, 1566-73.

Le SQ, Gascuel O (2008) LG: An improved general amino acid replacement matrix. *Molecular Biology and Evolution* 25, 1307-1320.

Linzen B (1989) Blue blood: structure and evolution of hemocyanin *Naturwissenschaften* 76:206-11 (in German).

Mangum CP (1980) Respiratory function of the hemocyanins *American Zoology* 20, 19-38.

Mangum CP (1983a) Adaptability and anadaptability among HcO_2 transport system: an apparent paradox. *Life Chemistry Reports Supplement 1*: 335-352

Mangum CP (1983b) The distribution of lactate sensitivity among the hemocyanins. *Marine Biology Letters* 4, 139-149.

Mangum CP (1985) Oxygen transport in invertebrates *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 248, 505–514.

Markl J, Decker H (1992) Molecular structure of the arthropod hemocyanins *Advances in Comparative and Environmental Physiology* 13, 325–376.

Nagai T, Kawabata S (2000) A link between blood coagulation and prophenoloxidase by horseshoe crab antimicrobial peptides. *The Journal of Biological Chemistry* 276, 29264-29267.

Pick C, Schneuer M, Burmester T (2009) The occurrence of hemocyanin in Hexapoda *FEBS Journal*, 276, 1930-1941.

Pless DD, Aguilar MB, Falcón A, Lozano-Alvarez E, Heimer de la Cotera EP (2003) Latent phenoloxidase activity and N-terminal amino acid sequence of hemocyanin from *Bathynomus giganteus*, a primitive crustacean *Archives of Biochemistry and Biophysics* 409, 402–410.

Salvato B, Beltramini M (1990) Hemocyanin molecular architecture: structure and reactivity of the binuclear copper-active site *Life Chemistry Reports* 8:1-47.

Söderhäll K, Cerenius L (1998) Role of the prophenilosidase-activating system in invertebrate immunity. *Current Opinion in Immunology.* 10, 23-28.

Stark BP, Sheldon AL (2008) Records of Neoperlini (Plecoptera: Perlidae) from Brunei darussalam and Sarawak, with descriptions of new *Phanoperla* bancks and *Neoperla* Needham species *Illiesia* 5,11-19.

Stark BP, Sivec I (2008) The genus *Togoperla* Klapálek (Plecoptera: Perlidae). *Illiesia* 4, 208-225.

Tamura K, Dudley J, Nei M, Kumar S (2007) *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24,1596-1599.

Telfer, H, Kunkel JG (1991) The function and evolution of insect storage hexamers. *Annual* Review of *Entomology* 36, 205-28.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673-80.

van Holde KE, Miller KI (1995) Hemocyanins *Advances in Protein Chemistry* 47:1-81.

van Holde KE, Miller KI, Decker H. (2001) Hemocyanins and invertebrate evolution. J. *Journal of Biological Chemistry* 276, 15563–15566.

Volbeda A, Hol WG (1989) Crystal structure of hexameric hemocyanin from *Palinurus interruptus* refined at 3.2 Å resolution. Journal of Molecular Biology 209, 249–279.

Zwick P (1982) A revision of the Oriental stonefly genus *Phanoperla* (Plecoptera: Perlidae) *Systematic Etomology* 7, 87-126.

Zwick P (1984) The genera *Tetropina* and *Neoperlops* (Plecoptera: Perlidae) *Aquatic Insect* 6, 169-176.

Zuckerkandl E (1960) Hémocyanine et cuivre chez un Crustacé Décapode dans leurs rapports avec le cycle d'intermue. Annuaire.d'Institute.d'Océanographique 38, 1–122 (in Frances).

Order	**Family**	***Specie***	**Type**	**Id**	**GeneBnk Accesion number**
Plecoptera	Perlidae	*Neoperla sp.*	hx	Neo_sp.hx	GU121390
		Phanoperla. sp.	hx	Pha_sp.hx	GU121392
		Togoperla sp.	hx	Tog_so.hx	GU121389
		Etrocorema sp.	hx	Etr_sp.hx	GU121391
		Tetrotina sp.	hx	Tet_sp.hx	GU121388
		Caroperla sp.	hx	Car_sp.hx	GU121400
		Perla grandis	hc1	Per_gr.hc1	DQ118369
			hc2	Per_gr.hc2	DQ118370
		Perla marginata	hc1	Per_ma.hc1	FJ393060
			hc2	Per_ma.hc2	AJ555404
			hx1	Per_ma.hx1	AM690365
			hx2	Per_ma.hx2	AM690366
			hx3	Per_ma.hx3	AM690367
			hx4	Per_ma.hx4	AM690368
		Dinocras cephalotes	hc1	Dic_ce.hc1	FJ415315
			hc2	Dic_ce.hc2	EF218621
	Peltoperlidae	*Cryptoperla sp.*	hx	Cry_sp.hx	GU121387
	Chloroperlidae	*Siphonoperla torrentium*	hx	Sip_to.hx	EU672887
	Perlodidae	*Isoperla grammatica*	hc1	Iso.gr.hc1	EU672885
			hc2	Iso.gr.hc2	EU672886
		Guadalgenus franzi	hc2	Gua_fr.hc2	FJ393060
Myriapoda	Scutigeridae	*Scutigera coleoptrarta*	hcA	Scu_co.hcA	AJ344359
			hcB	Scu_co.hcB	AJ512793
			hcC	Scu_co.hcC	AJ431379
			hcD	Scu_co.hcD	AJ34436’
			hcX	Scu_co.hcX	AJ431378
	Spirostreptidae	*Spirostreptus sp.*	hc	Spi_sp.hc	AJ297738

Table 1: List of species analyzed in Phylogenetic analysis. Molecule type, acronyms and GenBank accession numbers are shown.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Seq.	***Iso_gr*.hc1**	**Iso_gr.hc2**	***Per_ma*.hc1**	***Per_ma*.hc2**	***Neo_sp*.hx**	***Pha_sp*.hx**	***Tog_sp*.hx**	***Tet_sp*.hx**	***Etr_sp*.hx**	***Car_sp*.hx**	***Per_ma*.hx1**	***Per_ma*.hx2**	***Per_ma*.hx3**	***Per_ma*.hx4**	***Cry_sp*.hx**
1	***Iso_gr.* hc1**	**ID**	0,62	0,78	0,61	0,53	0,53	0,54	0,53	0,55	0,54	0,52	0,55	0,49	0,53	0,53
2	***Iso_gr*.hc2**	0,53	**ID**	0,59	0,79	0,52	0,52	0,53	0,53	0,53	0,51	0,54	0,55	0,49	0,55	0,52
3	***Per_ma*.hc1**	0,81	0,52	**ID**	0,62	0,54	0,53	0,53	0,54	0,52	0,51	0,53	0,53	0,49	0,52	0,54
4	***Per_ma*.hc2**	0,54	0,75	0,54	**ID**	0,51	0,50	0,50	0,52	0,50	0,50	0,52	0,53	0,5	0,54	0,51
5	***Neo_sp*.h**	0,36	0,36	0,39	0,35	**ID**	0,98	0,96	0,98	0,87	0,86	0,75	0,75	0,64	0,71	0,98
6	***Pha_sp*.hx**	0,37	0,37	0,38	0,35	0,96	**ID**	0,96	0,98	0,87	0,86	0,75	0,75	0,65	0,70	0,99
7	***Tog_sp*.h**	0,37	0,39	0,39	0,35	0,93	0,95	**ID**	0,97	0,89	0,87	0,76	0,74	0,64	0,71	0,97
8	***Tet_sp*.hx**	0,37	0,38	0,40	0,36	0,95	0,96	0,96	**ID**	0,87	0,86	0,76	0,75	0,64	0,70	0,98
9	***Etr_sp*.hx**	0,38	0,39	0,39	0,35	0,85	0,86	0,89	0,86	**ID**	0,97	0,75	0,73	0,62	0,72	0,87
10	***Car_sp*.hx**	0,37	0,35	0,38	0,34	0,81	0,83	0,83	0,82	0,93	**ID**	0,74	0,73	0,61	0,70	0,87
11	***Per_ma*.hx1**	0,37	0,38	0,39	0,37	0,68	0,69	0,68	0,69	0,68	0,67	**ID**	0,84	0,65	0,73	0,76
12	***Per_ma*.hx2**	0,40	0,39	0,43	0,37	0,68	0,68	0,67	0,68	0,66	0,65	0,78	**ID**	0,65	0,71	0,75
13	***Per_ma*.hx3**	0,31	0,30	0,32	0,31	0,48	0,49	0,48	0,48	0,47	0,46	0,52	0,49	**ID**	0,62	0,65
14	***Per_ma*.hx4**	0,35	0,39	0,38	0,35	0,61	0,60	0,61	0,61	0,63	0,59	0,61	0,61	0,46	**ID**	0,71
15	***Cry_sp*.hx**	0,37	0,37	0,39	0,35	0,96	0,98	0,96	0,98	0,87	0,84	0,69	0,68	0,49	0,61	**ID**

Table 2: Nucleotidic (up) and amino acidic (down) identity. Species acronyms are the same used in phylogenetic analysis.

Sp		**Ala**	**Cys**	**Asp**	**Glu**	**Phe**	**Gly**	**His**	**Ile**	**Lys**	**Leu**	**Met**	**Asn**	**Pro**	**Gln**	**Arg**	**Ser**	**Thr**	**Val**	**Trp**	**Tyr**	**Tyr+Phe**
Caroperla sp.	hx	3,61	1,03	4,12	8,76	6,7	8,25	4,12	5,15	6,19	10,31	1,03	4,12	4,64	3,09	5,15	5,15	6,7	4,12	1,03	6,7	13,4
Neoperla sp.	hx	4,26	0,53	3,72	9,04	9,04	9,04	3,72	4,79	7,45	7,98	1,6	3,72	3,19	2,66	4,26	6,38	5,32	5,32	1,06	6,91	15,95
Phanoperla sp.	hx	4,26	0	4,26	9,57	9,04	8,51	3,72	4,79	6,91	7,98	2,13	3,72	3,19	2,66	4,26	6,38	5,85	4,79	1,06	6,91	15,95
Togoperla sp.	hx	3,72	1,06	4,26	7,98	8,51	9,57	4,26	4,79	6,91	7,98	1,6	4,79	3,19	3,19	4,79	5,32	5,32	5,32	1,06	6,38	14,89
Etrocorema sp.	hx	3,72	0,53	4,26	9,04	7,98	8,51	3,72	5,85	6,38	8,51	1,06	4,26	3,72	2,66	5,32	4,79	7,45	4,26	1,06	6,91	14,89
Tetropina sp.	hx	4,26	0	4,26	9,04	7,98	8,51	3,72	4,79	7,45	8,51	1,6	3,72	3,19	2,66	4,26	6,91	5,85	5,32	1,06	6,91	14,89
Cryptoperla sp.	hx	4,26	0	4,26	9,04	9,04	9,04	3,72	4,79	7,45	7,98	1,6	3,72	3,19	2,66	4,26	5,85	5,85	5,32	1,06	6,91	15,95
Perla marginata	hx1	4,62	0	4,62	8,72	6,67	7,18	4,1	5,13	5,13	7,69	3,08	4,62	4,62	3,08	4,62	6,15	4,1	5,13	1,03	9,74	16,41
Perla marginata	hx2	5,21	0	7,29	5,73	6,25	8,85	3,13	6,25	5,21	7,81	2,6	4,69	4,17	3,13	4,69	5,21	3,13	5,21	1,04	10,42	16,67
Perla marginata	hx3	4,15	1,04	4,15	7,77	8,29	4,66	1,04	5,18	8,81	7,25	1,04	7,25	5,18	3,63	3,11	8,29	3,63	2,59	1,04	11,92	20,21
Perla marginata	hx4	3,59	0,51	6,67	7,18	7,18	7,18	3,08	3,59	5,13	9,23	4,1	5,13	3,08	4,1	5,64	4,62	5,64	4,1	1,54	8,72	15,9
Siphonoperla torrentium	hx	3,61	0,52	6,7	6,7	7,22	7,73	3,61	5,15	4,64	7,22	4,64	4,64	3,61	2,58	5,67	6,19	6,19	4,64	1,55	7,22	14,44
Avg		**4,11**	**0,44**	**4,88**	**8,21**	**7,83**	**8,09**	**3,50**	**5,02**	**6,47**	**8,20**	**2,17**	**4,53**	**3,75**	**3,01**	**4,67**	**5,94**	**5,42**	**4,68**	**1,13**	**7,97**	**15,80**

Table 3: Mole percent of amino acids in Plecoptera hexamerins (hx). Amino acid acronyms follow standard abbreviations. (Sp.: specie; Avg: average).

Sp		**Ala**	**Cys**	**Asp**	**Glu**	**Phe**	**Gly**	**His**	**Ile**	**Lys**	**Leu**	**Met**	**Asn**	**Pro**	**Gln**	**Arg**	**Ser**	**Thr**	**Val**	**Trp**	**Tyr**	**Tyr+Phe**
Guadalgenus franzi	hc1	4,08	0	7,14	8,67	6,12	7,14	8,67	6,12	5,1	7,65	4,59	3,57	5,1	2,04	4,59	4,08	4,08	4,59	1,53	5,1	11,22
Isoperla grammatica	hc1	3,61	0	6,7	9,79	5,67	7,22	9,28	6,19	3,61	9,79	4,12	3,61	5,67	2,06	4,12	4,12	4,12	3,09	1,55	5,67	11,34
Isoperla grammatica	hc2	4,12	0	7,73	7,22	7,73	8,25	7,73	5,15	3,09	7,22	5,67	4,12	5,15	1,55	5,15	3,09	5,15	5,67	2,58	3,61	11,34
Dinocras cephalotes	hc1	5,67	0	8,25	8,25	6,7	7,73	7,73	6,19	5,67	7,73	3,09	2,58	5,15	1,55	4,12	3,09	3,09	5,15	2,06	6,19	12,89
Dinocras cephalotes	hc2	4,15	0	8,29	7,77	8,29	6,74	8,29	3,63	5,18	5,7	4,66	4,15	5,18	2,07	4,15	3,63	4,15	6,22	3,11	4,66	12,95
Perla grandis	hc1	5,13	0	7,69	9,23	5,64	7,18	8,21	6,15	5,64	8,72	3,08	3,08	6,15	1,03	3,59	3,08	3,59	5,13	2,05	5,64	11,28
Perla grandis	hc2	4,64	0	8,25	8,25	8,76	7,22	8,25	4,64	4,12	6,7	3,61	3,61	5,15	2,06	4,64	3,61	4,12	6,19	2,58	3,61	12,37
Perla marginata	hc1	5,15	0	8,25	9,28	6,19	7,22	8,25	6,7	5,67	7,73	3,09	2,58	5,67	2,06	4,12	4,12	2,58	4,12	2,06	5,15	11,34
Perla marginata	hc2	4,66	0	7,25	7,77	7,77	7,25	8,29	4,15	4,15	6,22	3,11	5,18	5,18	2,07	4,66	4,15	4,15	6,74	3,11	4,15	11,92
Avg		**4,58**	**0,00**	**7,73**	**8,47**	**6,99**	**7,33**	**8,30**	**5,44**	**4,69**	**7,50**	**3,89**	**3,61**	**5,38**	**1,83**	**4,35**	**3,66**	**3,89**	**5,21**	**2,29**	**4,86**	**11,85**

Table 4: Mole percent of amino acids in Plecoptera (hemocyanin) hc. Amino acid acronyms follow standard abbreviations. (Sp.: specie; Avg: average).

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Brachyptera_risi.hx              VAYFGEDVGLNAFHTYWNMDYPFWANSKYYNLKFDRRGELFYYTQHQLMA 50
Taeniopterix_stankovitchi.hx     VAYLGEDVGLNAFHTYWNMDYPFWANSKYYNLKFDRRGELFYYTQHQLMA 50
Leuctra_fusca.hx                 VAYFGEDVGLSTFHTYWNMDYPFWANAKYYNLKFDRDGELFYYTQDQILA 50
Nemoura_sp.hx1                   VSYFTEDVGLSTFHTYWNMDYPFWANAKYYNLKFDRDGELFYYTQDQILA 50
Nemoura_sp.hx3                   VSYFTEDVGLSTFHTYWNMDYPFWANHKNYGIKWDRTDELFYYTQDQILA 50
Nemoura_sp.hx2                   VSYFTEDVGLSTFHTYWNMDYPFWANAKYYNLKFDRDGELFYYTQNQIMA 50
Capnia_bifrons.hx                ----------------------------YNMKFDRRGELFYYTQSQLLA 21
Taeniopteryx_burksi.hx           -SYFTEDVGVNAFHTYWNMDYPFWANSKTYNMKFDRRGELFYYTQSQLLA 49
Allocapnia_vivipare.hx           VSYFTEDVGVNAFHTYWNMDYPFWANSKTYNMKFDRRGELFYYTQSQLLA 50
Protonemura_ausonia.hx           --------------------------------------------------
Amphinemura_sulcicollis.hx       ---------------------------QTYNLKFDRRGELFYYTQSQLMA 23
Nemoura_hesperiae.hx             ---------------------------QTYNLKFDRRGELFYYTQSQLMA 23
Perla_marginata.hx1              ISYFTEDVGVNAFHAYWHMDYPFWANSKYYNVKFDRRGELFYYTQHQLMA 50
Perla_marginata.hx2              ISYFTEDVGINAFHAYWHMDYPFWANSKYYNVKFDRRGELFYYTQHQLMA 50
Perlodes_intricatus.hx           ISYFTEDVGLNAFHTYWNLDYPFWANSKNYNLKFDRRGELFYYTQHQLMA 50
Caroperla_sp.hx                  HPSKRQCGGLEVFHTYWNFDYPFWAESKHYNLKFDRRGALFYYTQHQLMA 50
Etrocorema_sp.hx                 -------GGFKVFHTYWNFDYPFWAESKHYNLKFDRRGELFYYTQHQLMA 43
Neoperla_sp.hx                   ------CGSFKMFHTYWNFDYPFWAESKHYNLKFDRRGELFYYTQHQLMA 44
Phanoperla_sp.hx                 -------GSFEMFHTYWNFDYPFWAESKHYNLKFDRRGELFYYTQHQLMA 43
Tetropina_sp.hx                  -------SGFKVLHTYWNFDYPFWAESKHYNLKFDRRGELFYYTQHQLMA 43
Cryptoperla_sp.hx                -------GGFKVFHTYWNFDYPFWAESKHYNLKFDRRGELFYYTQHQLMA 43
Togoperla_sp.hx                  -------GGFKVLHTYWNFDYPFWAESKHYNLKFDRRGELFYYTQHQLMA 43
Diamphipnopsis_samali.hx         LSYFTEDVGLNAFHTYWNMDYPFWANSKYYNLKFDRRGELFYYTQHQLMA 50
Perla_marginata.hx4              LTYFTEDVGVNSFNAYWHLDYPFWMNSQHYNLKFDRRGELFYYTQHQLMA 50
Siphonoperla_torrentium.hx       VAYFGEDVGVNTFNTYWHLDYPFWMNSAKYNMHFDRRGELFYYTQHQLLA 50
Perla_marginata.hx3              LSYFTEDVGVNSFNTYWNYFYPSWYNASYYNFKFDRRGELFYYSKQQLLA 50
Guadalgenus_franzi.hc1           VAYLGEDVGLNSHHAHWHMDFPFWWKAAEYGVEKFRKGELFYYMHHQMIA 50
Isoperla_grammatica.hc1          VAYLGEDVGLNSHHSHWHMDFPFWWKAAEYGIEKDRKGELFYYMHHQMIA 50
Perla_grandis.hc1                VAYLGEDVGLNSHHAHWHMDFPFWWKAAEYGIEKDRKGELFYYMHHQMIA 50
Perla_marginata.hc1              IAYLGEDVGLNSHHAHWHMDFPFWWKAAEYGIEKDRKGELFYYMHHQMIA 50
Dinocras_cephalotes.hc1          VAYFGEDVGLNSHHAHWHMDFPFWWKAAEYGIEKDRKGELFYYMHHQMIA 50
Isoperla_grammatica.hc2          VAYLGEDIGVNSHHAHWHMDFPFWWKR-TYDVTKDRRGELFFYMHHQMVS 49
Isoperla_acicularis.hc2          VAYLGEDIGVNSHHAHWHMDFPFWWKR-TYDVTKDRRGELFFYMHHQMVN 49
Perla_grandis.hc2                VAYFGEDIGINSHHSHWHMDFPFWWKR-SYDITKDRRGELFFYMHHQMVN 49
Perla_marginata.hc2              VAYFGEDIGVNSHHSHWHMDFPFWWKR-SYDVTKDRRGELFFYMHHQMVN 49
Dinocras_cephalotes.hc2          VAYFGEDVGLNSHHSHWHMDFPFWWKK-SYDVTKDRRGELFFYMHHQMVN 49

Brachyptera_risi.hx              --RYYLERLSNGLGEVKPFSY-SYKTPIAGFEPSLRYQNGKEFPMRPEFA 97
Taeniopterix_stankovitchi.hx     --RYYLERLSNGLGEVKPFSY-SYKTPIAGFEPSLRYQNGKEFPMRPEFA 97
Leuctra_fusca.hx                 --RYYLERLSNGLGEIKPFSY-TFKTPISGFEPSLRYQNGKEFPMRPEGV 97
Nemoura_sp.hx1                   --RYYLERLSNGLGEIKPFSY-TFKTPISGFEPSLRYQNGKEFPMRPEGV 97
Nemoura_sp.hx3                   --RYYLERLSNGLGEIKPFSY-TFKTPISGFEPSLRYQNGKEFPMRPEGV 97
Nemoura_sp.hx2                   --RYFLERLSNGLGEVQPFSY-SYKTPIAGFEPSLHYQNGKEFPMRPEGV 97
Capnia_bifrons.hx                --RYTLERLSNGLGEVKPFSY-AYKTPVAGFEPSLRYQNGKEFPMRPEGS 68
Taeniopteryx_burksi.hx           --RYTLERLSNGLGEVKPFSY-AYKTPVAGFEPSLRYQNGKEFPMRPEGS 96
Allocapnia_vivipare.hx           --RYTLERLSNGLGEVKPFSY-AYKTPVAGFEPSLRYQNGKEFPMRPEGS 97
Protonemura_ausonia.hx           --------------------------------------------------
Amphinemura_sulcicollis.hx       --RYYLERLSNGLGEVKPYSY-SFKNAISGFESSLRYQSGKEFPSRPEGV 70
Nemoura_hesperiae.hx             --RYYLERLSNGLGEVKPYSY-SFKTPIAGFESSLRYQSGKEFPSRPEGI 70
Perla_marginata.hx1              --RYYLERLSNGLKEIKPFSYFETQSHIPGYEPSLRYPNGKEFPMRPEGV 98
Perla_marginata.hx2              --RYYLERLSNGLGEVKPFSYFDND--VVGYQPSLRYPSGKEFPMRPDGF 96
Perlodes_intricatus.hx           --RYYLERLSNGLGEVKAFSYFDSE--IVGYQPSLRYQNGKEFPMRPEGM 96
Caroperla_sp.hx                  --RYYLERLSNGLGEVKPFSYFTHETNIEGFEPSLRYQNGKEFPMRPEGL 98
Etrocorema_sp.hx                 --RYYLERLSNGLGEVKPFSYFTHKTNIEGFEPSLRYQNGKEFPMRPEGL 91
Neoperla_sp.hx                   --RYYLERLSNGLGEVKPFSYFTYKSKIEGFEPSLRYQNGKEFPVRPEGA 92
Phanoperla_sp.hx                 --RYYLERLSNGLGEVKPFSYFTYKSKIEGFEPSLRYQNGKEFPMRPEGA 91
Tetropina_sp.hx                  --RYYLERLSNGLGEVKPFSYFTYKSKIEGFEPSLRYQNGKEFPMRPEGA 91
Cryptoperla_sp.hx                --RYYLERLSNGLGEVKPFSYFTYKSKIEGFEPSLRYQNGKEFPMRPEGA 91
Togoperla_sp.hx                  --RYYLERLSNGLGEVKPFSYFTHKTNIEGFEPSLRYQNGKEFPMRPEGA 91
Diamphipnopsis_samali.hx         --RYYLERLSNNLGEIKPFSY-TQETPLAGYEPSLRYQNGKEFPMRPEGM 97
Perla_marginata.hx4              --RYYLERLSNGLGDLKHFDWFDKKTQMVGYEPSLRYQNGQEFNQRPEGA 98
Siphonoperla_torrentium.hx       --RYYLERISNGLGEIKHFDWSDRKTLMVGYEPMMRYQNGQEFTMRPEGS 98
Perla_marginata.hx3              --RYQLERLSNDLPEIKPYNYFSFK-ALPSYESCLSYENGKAFPNRPEGT 97
Guadalgenus_franzi.hc1           --RYDLERLSNHLPVVKPLSF--EEEIEHGFYPQTTYRVGGEFPSRPDNF 96
Isoperla_grammatica.hc1          --RYDLERLSNHLPFVEPLSF--EEEIEHGFYPQTTYRVGGEFPSRPDNF 96
Perla_grandis.hc1                --RYDLERLSNWLPFVEPLSF--EEKIEHGFYPQTTYRVGGEFPARPDNF 96
Perla_marginata.hc1              --RYDFERLSNWLHFVEPISF--EDEIEHGFYPQTTYRVGGEFPARPDNF 96
Dinocras_cephalotes.hc1          --RYDLERLSAWLHFVEPLSF--EDKIEHGFYPQTTYRVGGEFPARPDNF 96
Isoperla_grammatica.hc2          --RFDAERLSNNLPQVEPLDW--HHEIVEGFAPGAMYHNGQEFPMRPDGM 95
Isoperla_acicularis.hc2          --RFDAERLSNDLPQVEPLDW--HHEIVEGFAPGAIYHNGQEFPMRPDGM 95
Perla_grandis.hc2                --RFDAERLSNFLPQVEPLDW--HHEIEEGFAPAAMYFNGQEFPMRPDGM 95
Perla_marginata.hc2              --RFDAERLSNWLPQVEPLNW--HHEIEEGFAPAAMYFNGQEFPMRPDGI 95
Dinocras_cephalotes.hc2          --RFDAERLSNWLPQVEPLGW--HHEIEEGFAPAAMYFNGQEFPMRPDGM 95
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Figure 1: Multiple alignment (Blosum 62) of plecoptera hemocyanins (hc) and hexamerins (hx). Continued.

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Brachyptera_risi.hx              RFSNSY--KTEEALAFERRIYDAIDLGYVINKEGAKISLREKDGISLLGE 145
Taeniopterix_stankovitchi.hx     RFSNSY--KTEEALAFERRIYDAIDLGYVINKEGAKISLREKDGISLLGE 145
Leuctra_fusca.hx                 RFSNNY--KSEEAYAYERRIFDAIDLGFVISKDGSKISLNEKEGINILGE 145
Nemoura_sp.hx1                   RFSNNY--KSEEAYAYERRIFDAIDLGFVISKDGSKISLNEKEGINILGE 145
Nemoura_sp.hx3                   RFSNNY--RSEEAYAYERRIFDAIDLGFVISKDGSKISLNEKEGINILGE 145
Nemoura_sp.hx2                   QFSNSY--KTEEALAYERRIHDAIDLGFVWTKDGQKVALKEKEGIDLLGE 145
Capnia_bifrons.hx                KFFKSF--KTEVALAYERRIYDAIDLGFVWTKEGQKVALKEKEGINILGE 116
Taeniopteryx_burksi.hx           KFFKSF--KTEVALAYERRIYDAIDLGFVFTKEGSKVALKEKEGINILGE 144
Allocapnia_vivipare.hx           KFFKSF--KTEVALAYERRIYDAIDLGFVFTKDGQKVSLKEKNGIDMLGE 145
Protonemura_ausonia.hx           --FNNY--YTEKALSLESRILNAIDIGFVWTKDGQKFAIKDKEGINLLGE  46
Amphinemura_sulcicollis.hx       KFFNNY--YTEKALSLESRILNAIDIGFVWTKDGQKYALKDKEGINLLGE 118
Nemoura_hesperiae.hx             KFFANY--FTEKALSLESRILNAIDIGFVWTKDGQKYALKDKEGINLLGE 118
Perla_marginata.hx1              SILNNY--HVEEVFALERRIHDAIDLGFVFGKDGQKISLKEKEGISILGD 146
Perla_marginata.hx2              -LVNDL--KADDIYALERRIYDAIDLGFIYGKEGQKISLKEKEGISILGD 143
Perlodes_intricatus.hx           SVVYNR--QTEELYTLERRIQDAIDLGFVFGKEGQKVSLKEKEGISILGE 144
Caroperla_sp.hx                  SFVDSY--KTEEVIVLERLIRDATDLGFVVGKEGQKISLKDKEGITLLGE 146
Etrocorema_sp.hx                 SFVDSY--KTEEVIVLERRIRDAIDLGFVVGKEGQKISLKDKEGITLLGE 139
Neoperla_sp.hx                   SFVGSL--KTEDLIVFERRIHDAIDLGFVFGKEGQKISLKEKEGIAILGE 140
Phanoperla_sp.hx                 SFVDSL--KTEDLIVFERRIHDAIDLGFVFGKEGQKISLKEKEGIAILGE 139
Tetropina_sp.hx                  SFVDSL--KTEDLIVFERRIHDAIDLGFVSGKEGQKISLKEKEGIAILGE 139
Cryptoperla_sp.hx                SFVDSL--KTEDLIVFERRIHDAIDLGFVFGKEGQKISLKEKEGIAILGE 139
Togoperla_sp.hx                  SFVDSL--KTEDLIVFERRIHDAIDLGFVFGKEGQKISLKEKEGIAILGE 139
Diamphipnopsis_samali.hx         TVTHSF--HTEEIMDFERRIHDAIDLGFVFTKDGQKVSLKEKEGITLLGE 145
Perla_marginata.hx4              NFYRNY--RSEDAMIFERRILDAIDMGYIVTKEGRKLSLKEKEGITFLGE 146
Siphonoperla_torrentium.hx       TFSRNY--RSEDAMIFERRIVDAIDAGYIVSFDGQKLSLKDKEGITLLGE 146
Perla_marginata.hx3              -YPKFS--KKEEMQDFEKRIKFAIDFGYVFNNDKKKVSIYEKDGISILGE 144
Guadalgenus_franzi.hc1           EFHDLDHIKIKDMIDYTRRIREAIFKKSVLTKNGDHISLDNMHGIDILGD 146
Isoperla_grammatica.hc1          EFHDLEHIKIQDMLDYTRRIRNAILKHSVLTKTGEHIALDNEHGIDILGD 146
Perla_grandis.hc1                HFHDLEDIKIKDMLDYTRRIKEAIVKHTVINKNGEHIPLDAVHGIDILGD 146
Perla_marginata.hc1              HFHDLEHIKIKDMLDYTRRIKEAISKQKVRSKNGEKIPLDAVHGIDILGD 146
Dinocras_cephalotes.hc1          YFHDLEDIKIKDMLDYTKRIRNAIYKQGVLTKDGERVPLDAVHGIDILGD 146
Isoperla_grammatica.hc2          YFHDLPWLTIKDNEEFEGRIRDIIASGFVKMTDGHLVYLNTTEGIDILGL 145
Isoperla_acicularis.hc2          YFHDLPWLTTKDNEEFEGRIRDIIASGFVKMTDGHLVYLNTTEGIDILGL 145
Perla_grandis.hc2                HFHDLPWFTVKDTEEYEDRIRDIIAKGYVKAKDGHVVFLNGTEGIDVLGL 145
Perla_marginata.hc2              HFHDLPWFTVKDTEDYEDRIRNVIAKGYVKASDGHTIFLNGTEGINILGL 145
Dinocras_cephalotes.hc2          HFHDMPWFTVKDTEDYEDRIRDVIAKGYVKTNDFHKVYLNTTEGIDILGL 145
                                           .       *       :        :   .** .**

Brachyptera_risi.hx              I--IEGSWDSTNKDFYGALYNIMRT--IFGHVTDPTYQYGV--APGVLE- 188
Taeniopterix_stankovitchi.hx     I--IEGSWDSTNKDFYGALYNIMRT--IFGHVTDPTYQYGV--APGVLE- 188
Leuctra_fusca.hx                 L--IKGTTDTVNEYFYGTIYNMMRG--IFGHVTDPNFQYGV--APGVLE- 188
Nemoura_sp.hx1                   L--IKGTTDTVNEYFYGTIYNMMRG--IFGHVTDPNFQYGV--APGVLE- 188
Nemoura_sp.hx3                   L--IKGTTDTVNEYFYGTIYNMMRG--IFGHVTDPNFQYGV--APGVLE- 188
Nemoura_sp.hx2                   I--VKGTYDSVNKDFYGEIYNMMRT--IFGHVTDPTYKYGT--APGVTE- 188
Capnia_bifrons.hx                M--IEGSYDSVNKQFYGTLYNIMRT--IFGHVTDPTFQYGV--APGVLE- 159
Taeniopteryx_burksi.hx           M--IQGSFDSVNKQFYGALYNIMRT--IFGHVTDPTFQYGV--APGVLE- 187
Allocapnia_vivipare.hx           M--IEGSYDSVNKQFYGALYNIMRT--IFGHVTDPAFQYGV--APGVLE- 188
Protonemura_ausonia.hx           M--ISGVSDSVNKDFYGNLYNLMRS--IFGHVTDPNFKYGV--APGVME-  89
Amphinemura_sulcicollis.hx       M--ISGVSDSVNKDFYGNLYNLMRS--IFGHVTDPNFKYGV--APGVME- 161
Nemoura_hesperiae.hx             M--ISGVSDSVNKDFYGNLYNLMRT--IFGHVTDPNFKYGV--APGVME- 161
Perla_marginata.hx1              M--IEGTEDSTNKQFYGSLYNMLRT--VYGHYADPMYQYEV--APSVLE- 189
Perla_marginata.hx2              M--IEGTGDSVNKNYYGSIYNTIRT--VFGHHADPMYQYLV--APGVLE- 186
Perlodes_intricatus.hx           M--IEGTADSANKNFYGSVYNMMKT--VFGHVTDPTFQFQV--APSALE- 187
Caroperla_sp.hx                  L--IEGTGDSANKNFYCSLYNIIRT--VFGHITDPTYQHTI--APTALE- 189
Etrocorema_sp.hx                 L--IEGTGDSANKNFYCSLYNIIRT--VFGHITDPTYQHTI--APTALE- 182
Neoperla_sp.hx                   M--IEGTGDSVNKNFYGSLYNLIKT--VFGHVTDVTYQHTV--APSALE- 183
Phanoperla_sp.hx                 M--IEGTGDSVNKNFYGSLYNLIKT--VFGHVTDVTYQHTV--APSALE- 182
Tetropina_sp.hx                  M--IEGTGDSVNKNFYGSLYNLIKT--VFGHVTDVTYQHTV--APSALE- 182
Cryptoperla_sp.hx                M--IEGTGDSVNKNFYGSLYNLIKT--VFGHVTDVTYQHTV--APSALE- 182
Togoperla_sp.hx                  M--IEGTGDSVNKNFYGSLYNLIKT--VFGHVTDVTYQHTV--APQCSG- 182
Diamphipnopsis_samali.hx         M--IEGTGDSVNENFYGHIYSLMRT--VFGHATDPKYQYDV--APGVLE- 188
Perla_marginata.hx4              L--IMATGDSPNKDFYGQMYSILCT--VFGHVMDPTFKYDT--VPSVLE- 189
Siphonoperla_torrentium.hx       L--IMSTGDSPNKEFYGKIYTNLCT--IFGHVMDHTFAFDT--VPSVLE- 189
Perla_marginata.hx3              I--IASCENSNYKNMYGTLYYSILK--TFGHEVDPQYQYKF--APPALE- 187
Guadalgenus_franzi.hc1           L--MEPSMETVHQDYYGSLHNHAHI--LLGQITDPKGRFNMETPPGVME- 191
Isoperla_grammatica.hc1          L--MEPSMETLHHDYYGSLHNYAHI--LLGQITDPLGKFNM--PPGVME- 189
Perla_grandis.hc1                L--MEPSVESPHEDYYGSLHNYAHI--LLGKVTDPLGKFDL--PPGVME- 189
Perla_marginata.hc1              L--MEPSVESPHEDYYGSLHNDAHV--LLGQITDPLGKFDL--PPGVME- 189
Dinocras_cephalotes.hc1          L--IEPSVESVHENFYGSLHNYAHI--MLGKITDPHGKFDL--PPGVME- 189
Isoperla_grammatica.hc2          I--VETLDHSYNRDFFGKFHANSHV--MLGRITDPMLKFGM--PPGVME- 188
Isoperla_acicularis.hc2          I--VETLDHGYNRDFFGKFHANSHV--VLSRITDPMLKFGM--PPGVME- 188
Perla_grandis.hc2                V--VESLDHDFNSHYFGRLHLNAHV--LLSKITDPEQKFGT--PPGVME- 188
Perla_marginata.hc2              V--VESLDHDYNRHYFGKLHSNAHV--LLSKVTDPEQKFGT--PPGVME- 188
Dinocras_cephalotes.hc2          I--VESLDHDYNRHYFGKFHSNAHV--LLSKITDPEQKFGM--PPGVME- 188
                                 :  :    .      :  .:         .:  *    .     *
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Figure 1: Continued.

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Brachyptera_risi.hx               -HFETAT 194
Taeniopterix_stankovitchi.hx      -HFETA- 193
Leuctra_fusca.hx                  -HFETA- 193
Nemoura_sp.hx1                    -HFETAL 194
Nemoura_sp.hx3                    -HFETAL 194
Nemoura_sp.hx2                    -HFETAL 194
Capnia_bifrons.hx                 -HFET-- 163
Taeniopteryx_burksi.hx            -HFETAL 193
Allocapnia_vivipare.hx            -HFETAT 194
Protonemura_ausonia.hx            -HFETA-  94
Amphinemura_sulcicollis.hx        -HFE--- 164
Nemoura_hesperiae.hx              -HFET-- 165
Perla_marginata.hx1               -HFTTAL 195
Perla_marginata.hx2               -HFQTAL 192
Perlodes_intricatus.hx            -HFETA- 192
Caroperla_sp.hx                   -HLKPL- 194
Etrocorema_sp.hx                  -HFETAT 188
Neoperla_sp.hx                    -HFETA- 188
Phanoperla_sp.hx                  -HFETAT 188
Tetropina_sp.hx                   -HFETAT 188
Cryptoperla_sp.hx                 -HFETAT 188
Togoperla_sp.hx                   -AFRNCH 188
Diamphipnopsis_samali.hx          -HFETAT 194
Perla_marginata.hx4               -HYETAL 195
Siphonoperla_torrentium.hx        -HSKPP- 194
Perla_marginata.hx3               -QIHTTL 193
Guadalgenus_franzi.hc1            -HFETA- 196
Isoperla_grammatica.hc1           -HFETP- 194
Perla_grandis.hc1                 -HFETAT 195
Perla_marginata.hc1               -HFETA- 194
Dinocras_cephalotes.hc1           -HFETA- 194
Isoperla_grammatica.hc2           -HFETAT 194
Isoperla_acicularis.hc2           -HFETAT 194
Perla_grandis.hc2                 -HFETAT 194
Perla_marginata.hc2               -HFETA- 193
Dinocras_cephalotes.hc2           -HFETA- 193
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Figure 1: Continued.

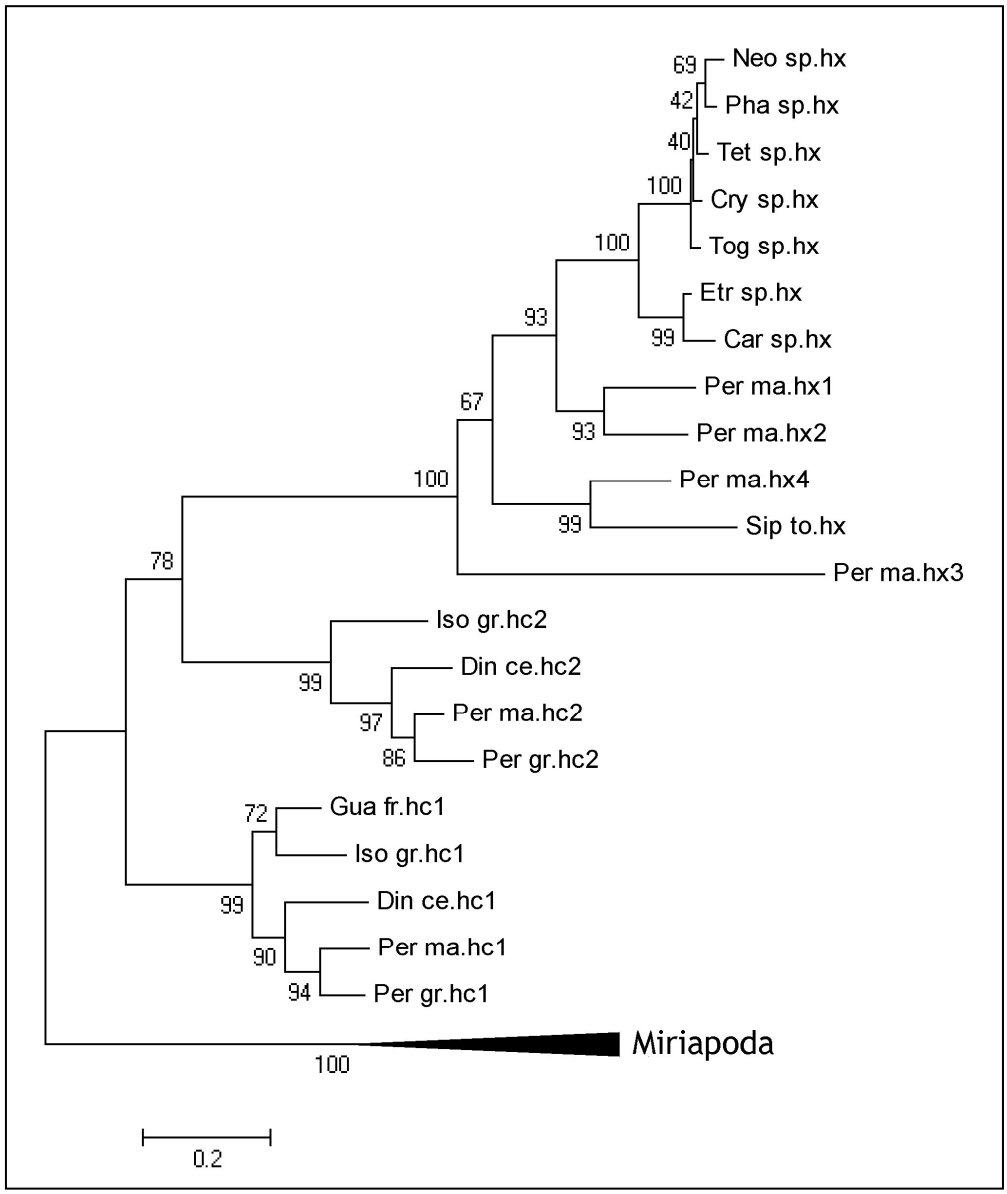

Figure 2: NJ analysis of Perloidea hemocyanins (hc) and hexamerins (hx). The numbers represent the bootstrap support. The bar equals 0.2 substitutions per site.

Amore et al.: Proteonomic analysis of hemocyanin in stoneflies

Environmental Entomology

Romolo Fochetti,
Dipartimento di Scienze Ambientali, Università della Tuscia,
snc Largo dell'Università 01100, Viterbo, Italy.
Tel: + 39 0761357178;
Fax: + 0761357179;
Email: fochetti@unitus.it

Preliminary data on comparative proteomic analysis of hemocyanins in the stoneflies *Dinocras cephalotes* and *Perla marginata* (Plecoptera)

V. Amore[1,2], M.A. Puig García[2], A.M. Timperio[1], G. Egidi[1], N. Ubero-Pascal[3], R. Fochetti[1].

[1] Dipartimento di Scienze Ambientali, Università della Tuscia, Viterbo, Italia

[2] Center of Advanced Studies of Blanes (CEAB-CSIC), Blanes, Girona, Spain

[3] Departamento de Zoología y Antropologá Fısica, Universidad de Murcia, Campus de Espinardo

Abstract: Hemocyanins are large oligomeric respiratory proteins found in many arthropods and mollusks. The overall pattern of presence distribution of hemocyanin mRNA, revealed by studies on plecoptera hemocyanin sequencing, has raised the question whether the protein was expressed or not. The presence of expressed hemocyanin, in fact, was reported in literature only for one species *Perla marginata*. In this work we report the presence of hemocyanin (hc) and hexamerin (hx) in *Dinocras cephalotes*, a species closely related to *P. marginata*. By inference we can assume that regardless to its putative functions (respiratory, immune defense, storage protein), in species where hemocyanin sequence is present, it is actually expressed. To assess hemocyanin presence, a reproducible and highly sensitive method, based on liquid chromatography tandem mass spectrometry, was used.

Keyword: Copper binding proteins; nano-RP-HPLC–ESI–MS; insects.

Hemocyanin, together with hemoglobin and hemerytrin, is known as the unique respiratory proteins of the animal kingdom. (Mangum 1985). Under the name of hemocyanin are usually grouped mollusks and arthropod proteins. They were originally given the same name because both are oxygen transport proteins and share the basic motif of type 3 copper proteins, that suggest a very far common ancestor (van Holde et al. 2001). However, mollusk and arthropod hemocyanins are profoundly different in molecular structure (Decker et al. 2007).

Arthropod hemocyanins are large multimetric (nx6), copper-containing proteins, composed by subunit types of about 75 kDa (Mangum 1985, van Holde et al. 2001; Decker et al. 2007). Sequence analysis showed that these hemocyanin macromolecules can contain several variants of monomers (Markl 1986, Voit et al. 2000, Hagner-Holler et al. 2004). The presence of the N-terminal signal peptide is typical of arthropod hemocyanins, presumably for the modality of their secretion in the hemolymph by hepatopancreas cells or fatty bodies cells (Fochetti et al. 2006, Kusche and Burmester, 2001a, 2001b, Sánchez et al. 1998, Pick et al. 2008), so hemocyanins are freely dissolved in the hemolymph. Despite the O_2-binding capacity earned to hemocyanin the common name of "respiratory protein", there is a large literature that states an *in vitro* multifuntionality in Chelicerata and Crustacea. Hemocyanin can potentially work as phenoloxidase after proteolytic cleavage (Decker and Rimke 1998, Nagai and Kawabata 2001, Decker et al. 2001, Pless et al. 2003, Lee et al. 2004), as anti-bacterial or anti-myotic (Destoumieux-Garzón et al. 2001, Lee et al. 2002, Pan et al. 2008) and so participate to the immune defense system, or take part to molting process and work as an hexamerin (Jaenicke et al. 1999).

The presence of hemocyanin in insects is an ongoing scientific debate. The first insect hemocyanin was reported for the stonefly *Perla marginata* (Hagner-Holler et al. 2004). Two different subunits were identified: subunit 1 (hc1) of 77 kDa, and subunit 2 (hc2) of 76.3 kDa (Fochetti et al. 2006, Hagner-Holler 2004), while 3-D structure studies were carried on a close species, *P. grandis* (Fochetti et al. 2006). Successively was assumed that hemocyanin was present across the whole order (Burmester and Hankeln 2007).

Plecoptera is a very interesting order because it has retained many ancient features and is considered a key point for the understanding of insect phylogeny. During the last years we focused our studies to investigate the presence of this protein across Plecoptera. To this regard we analyzed 32 species of Plecoptera belonging to different European, African and Oriental families in RT-PCR from total mRNA. We considered ecological and autoecological parameters that can induce variations in physiological requirements of specimens and can constitute difference in adaptative response inside the stonefly biodiversity (Fochetti et al. 2006, Amore et al. 2009, Amore and Fochetti, 2009, our unpublished data). We noted that, while hemocyanin lacks in Nemouroidea, one of the two European superfamilies, and in *Cryptoperla*, a Peltoperlidae belonging to Pteronarcyoidea, hemocyanin mRNA is expressed in many, even though not in all, essayed Perloidea. These data led us to make hypotheses on the probable multifunction of hemocyanin in the Plecoptera (Amore and Fochetti 2009). Moreover, the discontinuous presence of hemocyanin observed in transcriptional material, arose the problem of the actual existence of the proteins. In order to verify the expression of hemocyanin to another species other than *P. marginata*, we analyzed samples from *Dinocras cephalotes* by using SDS-PAGE electrophoresis as a first dimension and nano-reversed-phase high-performance liquid chromatography–electrospray ionization–

mass spectrometry (nano-RP-HPLC–ESI–MS) in order to identify proteins after triptic digestion. This method resulted rapid, highly sensitive and reproducible and by this approach we was able to identify specific proteins belonging to *D. cephalotes* and this is the novelty of the present paper.

Materials and methods

Insects. Nymphs of *Perla marginata* (Panzer, 1799) and *Dinocras cephalotes* (Curtis, 1827) were collected by the kick method, stored in liquid nitrogen in the field, and then at -80°C in the laboratory.

SDS PAGE. Total proteins were extracted by homogenizing two specimens for each species, using an antiproteases cocktail (Radio-Immunoprecipitation Assay Buffer, and Protease inhibitor Cocktail SIGMA) and 10% SDS. Cell debris were removed by 30min centrifugation at 13,000x r.p.m. at 4º C. The total protein concentration was determined according to the method of Bradford (1976) with the Quanti Pro BCA Assay Kit, SIGMA. Denaturing SDS/PAGE was performed on polyacrylammide discontinuous gel (16-5%) following standard procedures, to separate proteins according to their electrophoretic mobility. Proteins were dissolved 5 minutes at 98 °C, and 5 minutes in ice. After electrophoresis, the gel was fixed in methanol/acetic acid and stained with 0,1% Comassie Brilliant Blue G-250.

In-Gel Digestion. Protein bands were carefully excised from Blue silver stained gels and subjected to in-gel trypsin digestion according to Shevchenko and collaborators (1996), with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM of ammonium bicarbonate (NH_4HCO_3) and 12.5 ng/µL of trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in an ice bath. After 30 minutes the supernatant was removed and discarded, 20 µL of 50 mM

NH_4HCO_3 were added to the gel pieces and digestion allowed to proceed at 37 °C overnight. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to mass spectrometric analysis, the peptide mixtures were redissolved in 10 µL of 5% of Formic Acid (FA).

Peptide Sequencing by Nano-RP-HPLC-ESI-MS/MS. Peptide mixtures were separated using a nanoflow-HPLC system (Ultimate; Switchos; Famos; LC Packings, Amsterdam, The Netherlands). A sample volume of 10 µL was loaded by the autosampler onto a homemade 2 cm fused silica precolumn (75 µm I.D.; 375 µm O.D.; Reprosil C18-AQ, 3 µm - Ammerbuch-Entringen, DE) at a flow rate of 2 µL/min. Sequential elution of peptides was accomplished using a flow rate of 200 nL/min and a linear gradient from Solution A (2% acetonitrile; 0.1% formic acid) to 50% of Solution B (98% acetonitrile; 0.1% formic acid) in 40 minutes over the precolumn in-line with a homemade 10-15 cm resolving column (75 µm I.D.; 375 µm O.D.; Reprosil C18-AQ, 3 µm - Ammerbuch-Entringen, Germany). Peptides were eluted directly into a High Capacity ion Trap (model HCTplus, Bruker-Daltonik, Germany). Capillary voltage was 1.5-2 kV and a dry gas flow rate of 10 L/min was used with a temperature of 230 °C. The scan range used was from 300 to 1800 m/z. Protein identification was performed by searching the National Center for Biotechnology Information non-redundant database (NCBInr, www.ncbi.nlm.nih.gov) using the Mascot program (in-house version 2.2, Matrix Science, London, UK). The following parameters were adopted for database searches: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide Mass Tolerance ± 1.2 Da, Fragment Mass Tolerance ± 0.9 Da, missed cleavages 2. For positive identification, the score of the result of (- 10 x Log (P) had to be over the significance threshold level ($P < 0.05$). We have taken into account identification with at least 5 peptide fragments recognized.

Results

Total proteins samples of larval specimens of *P. marginata* and *D. cephalotes* were analysed in SDS-PAGE. In both species we observed the same pattern of bands between 97-66 kDa (fig. 1), the predicted range for hemocyanin and hexamerin molecular weight (75-80 kDa) (Markl and Decker 1992, Burmaster 2002, Telfer and Kunkel 1991). We analyzed three bands for *P. marginata* (P1, P2, P3 in fig. 1), and three bands for *D. cephalotes* (D1, D2, D3 in fig. 1) in the size range of expected bands. Proteins were successfully identified according to homology status with proteins that already existed in the taxa databases at the NCBI (www.ncbi.nlm.nih.gov).

Overall, four different expressed proteins were defined and all of the identified proteins can be clustered in the hemocyanin superfamily, corresponding to hexamerin 1 (hx1), 2 (hx2), and 4 (hx4) of *P. marginata,* and hemocyanin subunits 1 (hc1) and 2 (hc2) (Tab. 1). The identification mainly concerns polypeptide at N- and C-terminal part, probably due to intensity of fragmentation. In P1 and D1, the band of highest molecular weight, only includes hexamerins, while D2, P2, D3, P3 are a mixture of hexamerins and hemocyanins. In this study *P. marginata* has been used as positive control, to check goodness of total protein extraction and spectrometry procedures. Here we report for the first time *D. cephalotes* hexamerins, and we confirm the presence of both hemocyanin subunits (hc1 and hc2) in its total protein repertory.

Some peptide fragments of *P. marginata* and *D. cephalotes* identified hc1 and hc2, are coincident. These are, in hc1 from 24 to 35 amino acid position, (GSVPADQDFLTR), from 542 to 557 (KSSESSVTIPDRETTK) and from 556 to 666 (EDFFTDNMYTK) (tab. 2); in hc2 from 393 to 398 (DPAFFR), from 420 to 433 (EELDFPGVTVDAVK), and from 532 to 541 (LTAGENVITR) (Tab. 2).

Discussion

In a continuous SDS-PAGE electrophoresis, protein bands match more than a unique protein. Hemocyanin belongs to a protein superfamily that includes other classes of proteins that share significant sequence similarities but perform distinct functions (Burmester 2002). In Hexapoda hemocyanins and hexamerins are paralogous proteins (Burmester 2001). Hexamerin are proteins of the insect repertory and have been sequenced in all insect taxa investigated so far. Even if experimental data is scarce for some taxa, these proteins are likely to be ubiquitous in insects, but, presently, only Zygentoma and Plecoptera possess both type of hexameric proteins in their hemolymph (Hagner-Holler et al. 2007, Pick et al. 2009). As for pseudo-hemocyanin and crustacean hemocyanins (Burmaster 2007), hexamerins and insect hemocyanins are strictly related and it is supposed that hexamerins derived from a modified subunit type of hemocyanin who lost copper-binding capacity. Probably their ancestor was a protein similar to a copper-free hemocyanin-like protein (Burmester 2004). Hemocyanins and hexamerins are multisubunit proteins organized in hexamers whose typical molecular weight is of about 75 and 80 kDa. (Markl and Decker 1992, Burmaster 2002, Telfer and Kunkel 1991). Hemocyanins, differently from hexamerins, are proteins subjected to allosteric regulation (Decker et al., 2007). The three dimensional folding of the protein is strictly related to the bind with an effector and so to the plastic modulation to environmental conditions (Mangum 1983, Decker and Tuczek 2000, Hellman et al. 2004, Decker et al. 2007).

From the present mass spectrometry study, as well as from sequence analysis (Hagner-Holler et al. 2004, Fochetti et al. 2006, Amore et al. 2009, Amore and Fochetti 2009),

two defined subunits of hemocyanin were found, with the typical 3 copper protein active site (van Holde et al. 2001).

The restrictions of amino acid change in hexamerins are lesser than in hemocyanins. Due to the lost of oxygen binding property, these proteins are subject to a less strong selective pressure. Hexamerins serve mainly as storage proteins during non feeding periods, in larval molting or adult development (Telfer and Kunkel 1991, Haunerland 1996, Beintema et al. 1994), but can also fulfill other function as carrier proteins for small organic compound, like steroid hormone, riboflavin and juvenile hormone (Enderle et al. 1983, Magee et al. 1994, Braun and Wyatt 1996), or may be involved in immune response (Hayakawa 1994, Beresford et al. 1997). Burmester and Scheller (1996), hypothesized that the only limitation in the evolution of hexamerins was the conservation of the hexameric structure, probably associated with the maintenance of osmotic pressure in the hemolymph, with the results of a variety of hexamerin subunits that differ little to each other. Four distinct hexamerins are known for *P. marginata* (Hagner-Holler 2007), and three type have been identified in the spectrometry analysis, both in *P. marginata* and in *D. cephalotes.* The analysis of more than 30 Plecoptera species belonging to the seven European families, two Oriental families and one south African family, allowed us to detect a differential presence of hemocyanin across the order (Amore et al. 2009, Amore and Fochetti 2009). From sequence data comparison we hypothesized functionality of this protein, even though we could not safely state that with certainty. The question then if the protein is really expressed in the studied species.

P. marginata and *D. cephalotes* are rheophilous species widely distributed in Europe. They live in river where the water is typically fast-moving, shallow, relatively cold and well oxygenated (Fochetti and Tierno de Figueroa 2008, Tierno de Figueroa et al. 2003). The present study on protein expression shows that *D. cephalotes*, as well as *P.*

marginata, really translates hemocyanin mRNA into proteins, therefore confirming that, as well as for *P. marginata*, hemocyanin is expressed in *D. cephalotes* hemolymph too, and suggesting that in Plecoptera species, where hemocyanin is detected as transcript mRNA sequence, this is probably really translated into protein to be ready for physiological demand of the animal.

Aknowlegment

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References Cited

Amore, V., M. Belardinelli, L. Guerra, F. Buonocore, A. M. Fausto, N. Ubero-Pascal, and R. Fochetti. 2009. Do really all stonefly nymphs have hemocyanin? Further data on the presence of hemocyanin in the larval stages of Plecoptera species. Insect Mol. Biol. 18:203-11.

Amore, V and R. Fochetti. 2009. Present knowledge on the presence of hemocyanin in stoneflies (Insecta: Plecoptera). Aquat. Insects. 31: 577-583.

Beintema, J. J., W. T. Stam, B. Hazes and M. P. Smidt. 1994. Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). Mol. Biol. Evol. 11:493–503.

Beresford, P.J., J.M. Basinski-Gray, J.K. Chiu, J.S. Chadwick and W.P. Aston. 1997. Caractherization of hemolimphic and cytotoxic Gallysins: a relationship with arylforins. Dev. Comp. Immunol. 21: 253-266.

Bradford, M.M. 1976. A rapid and sensitive method for quantification of microgram quantities of proteins using the principle of protein dye binding. Anal. Biochem. 72: 248–254.

Braun, R.P. and G.R. Wyatt. 1996. Sequence of the hexameric juvenile hormone-binding rotein from the hemolymph of *Locusta migratoria.* J. Biol. Chem. 271: 31756-31762.

Burmester, T. 2001. Molecular evolution of the arthropod hemocyanin superfamily. Mol. Biol. Evol. 18: 184-195.

Burmester, T. 2002. Origin and evolution of arthropod hemocyanins and related proteins. J. Comp. Physiol. B. 172: 95–117.

Burmester, T. 2004. Evolutionary history and diversity of arthropod hemocyanins. Micron. 35: 121–122.

Burmester, T. 2007. Diversity of stonefly hexamerins and implication for the evolution of insect storage proteins. Insect. Biochem. Mol. Biol. 37: 1064–1074.

Burmester, T and T. Hankeln. 2007. The respiratory proteins of insects. Review. J. Insect Physiol. 53: 285-94.

Burmester, T. and K. Scheller. 1996. Common origin f arthropod tyrosinase, arthropod hemocyanin, insect hexamerin, and dipteran arylphorin receptor. J. Mol. Evol. 42: 713-728.

Decker, H. and T. Rimke. 1998. *Tarantula* hemocyanin shows phenoloxidase activity, J. Biol. Chem. 273: 25889–25892.

Decker, H. and F. Tuczek. 2000. Tyrosinase/catecholoxidasse activity of hemocyains: structural basis and molecular mechanism. Trends Biochem Sci. 25: 392-397.

Decker, H., M. Ryan, E. Jaenicke and N. Terwinlliger. 2001. SDS induced phenoloxidase activity of hemocyanin from *Limulus polyphemus, Eurypelma californicum, Cancer magister*. J. Biol. Chem. 276: 17796-17799.

Decker H, H. Hellmann, E. Jaenicke, B. Lierb, U. Meissnerand and J. Marki. 2007. Recent progress in hemocyanin research. I.C.B. 1-14.

Destoumieux-Garzò, D., D. Saulnier, J. Garnier, C. Jouffrey, P. Bulet and E. Evelyne Bachère. 2001. Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. J. Biol. Chem. 276: 47070–47077.

Enderle, U., G. Käusser, L. Renn, K. Scheller and J. Koolman. 1983. Ecdysteroids in the hemolymphs of blowfly are bound to calliphorin. In Scheller K. (ed). The larval serum proteins of insects: function, biosyntesis, genetic. Thieme, Stuttgart. 40-49.

Fochetti, R., M. Belardinelli, L. Guerra, F. Buonocore, A.M. Fausto and C. Caporale. 2006. Cloning and Structural Analysis of a haemocyanin from the Stonefly *Perla grandis.* Protein Journal. 25: 443-454.

Fochetti, R. and J.M. Tierno de Figueroa. 2008. Plecoptera. Vol. XLIII of Fauna D'Italia. Calderini Eds. Bologna, Italia.

Hagner-Holler, S., A. Schoen, W. Erker, J.H. Marden, R. Rupprecht, H. Decker, and T. Burmester. 2004. A respiratory hemocyanin from an insect. P.N.A.S. 101: 871–874.

Hagner-Holler, C. Pick, S. Gingernrath, J. H. Marden and T. Burmaster. 2007. Diversity of stonefly hexamerins and implication for the evolution of insect storage proteins. Insect Biochem. Mol. Biol. 37: 1064-1074.

Haunerland, N.H. 1996. Insect storage protein: gene families and receptors. Insect Biochem. Mol. Biol. 26: 755-765.

Hayakawa, Y. 1994. Cellular immunosuppressive protein in the plasma of parasitized insect larvae. J. Biol. Chem. 269: 14536-14540.

Hellmann, N., J. Hörnemann, E. Jaenicke and H. Decker. 2004. Urate as effector for crustacean hemocyanins. Micron. 35: 109-110.

Jaenicke, E, R. Föll and H. Decker. 1999. Spider hemocyanin binds ecdysone and 20-OH-ecdysone. J. Biol. Chem. 274: 34267-71.

Kusche, K. and T. Burmester. 2001a. Diplopod Hemocyanin sequence and the phylogenetic position of the Myriapoda. Mol. Biol. Evol. 18: 1566–1573.

Kusche, K. and T. Burmester. 2001b. Molecular cloning and evolution of lobster hemocyanin. Biochem. Biophys. Res. Commun. 282: 887–892.

Lee, S.Y., B.L. Lee and S. Söderhäll. 2002. Processing of an Antibacterial Peptide from Hemocyanin of the Freshwater Crayfish *Pacifastacus leniusculus*. J. Biol. Chem. 7: 7927–7933.

Lee, S.Y., B.L. Lee, and K. Söderhäll. 2004. Processing of crayfish hemocyanin subunits into phenoloxidase. Biochem. Biophys. Res. Commun. 322: 490–496.

Magee, J., N. Kraynack, H. C. Jr Massey and W. H. Telfer. 1994. Properties and significance of a riboflavin-binding hexamerin in the hemolymph of *Hyalophora cecropia*. Arch. Insect Biochem. Physiol. 25: 137-157.

Mangum, C.P. 1983. On the distribution of lactate sensitivity among the hemocyanins. Mar. Bio. letters. 4: 139-149.

Mangum, C.P. 1985. Oxygen transport in invertebrates. A.J.P. Regu. Physiol. 248: 505–514.

Markl, J. 1986. Evolution and function of structurally diverse subunits in the respiratory protein hemocyanin from arthropods. Biol. Bull. 14: 266-276.

Markl, J. and H. Decker. 1992. Molecular structure of the arthropod hemocyanins. Adv. Comp.Environ. Physiol. 13: 325–376.

Nagai, T. and S. Kawabata. 2000. A link betweencoagulation and prophenol oxidase activation in Arthropod host defense. J. Biol. Chem. 29264–29267.

Pan, J-Y., Y-L. Zhang, S-Y. Wang and X-X. Peng. 2008. Dodecamer is required for agglutination of *Litopenaeus vannamei* hemocyanin with bacterial cells and red blood cells. Mar Biotechnol. 10: 645–652.

Pick, C., M. Schneuer and T. Burmester. 2009. Ontogeny in the ovoviviparous cockroach *Blaptica dubia* suggests an embryo-specific role in oxygen supply. J. Insect Physiol. doi:10.1016/j.jinsphys.2009.04.004.

Pless, D.D., M.B. Aguilar, A. Falcòn, E. Lozano-Alvarez and E.P. Heimer de la Cotera. 2003. Latent phenoloxidase activity and N-terminal amino acid sequence of hemocyanin from *Bathynomus giganteus*, a primitive crustacean, Arch. Biochem. Biophys. 409: 402–410.

Pick, C., S. Hagner-Holler and T. Burmester. 2008. Molecular characterization of hemocyanin and hexamerin from the firebrat *Thermobia domestica* (Zygentoma), Insect Biochem. Mol. Bio. 1–7.

Sánchez, D., M.D. Ganfornina, G. Gutiérrez and M.J. Bastiani. 1998. Molecular characterization and phylogenetic relationships of a protein with potential oxygen-binding capabilities in the grasshopper embryo. A hemocyanin in insects? Mol. Biol. Evol. 15: 415–426.

Shevchenko, A., M. Wilm, O. Vorm and M. Mann. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68: 850–858.

Telfer, W.H. and J. C. Kunkel. 1991. The function and evolution of insect storage hexamers. Ann. Rev. Entomolog. 36: 205-228.

Tierno de Figueroa, J.M., A. Sanchez-Ortega, P. Membiela Iglesias and J.M. Lúzon-Ortega. 2003. Plecopera. Vol. 22. Fauna Iberica. Ramos M.A, Eds. Museo Nacional de Ciencias Naturales. Madrid. Spain.

van Holde, K.E., K.I. Miller, H. Decker. 2001. Hemocyanins and invertebrate evolution. J. Biol. Chem. 276: 563–566.

Voit R, G. Feldmaier-Fuchs, T. Schweikard, H. Decker and T. Burmester. 2000. Complete sequence of the 24-mer hemocyanin of the tarantula *Eurypelma californicum.* Structure and intramolecular evolution of the subunits. J. Biol. Chem. 275: 39339-44.

Fig. 1. discontinuous gel (16-5%) electrophoresis. Band analised in nano-RP-HPLC–ESI–MS are visible. (*P. mar*: *Perla marginata*; *D. cep.*:*Dinocras cephalotes).*

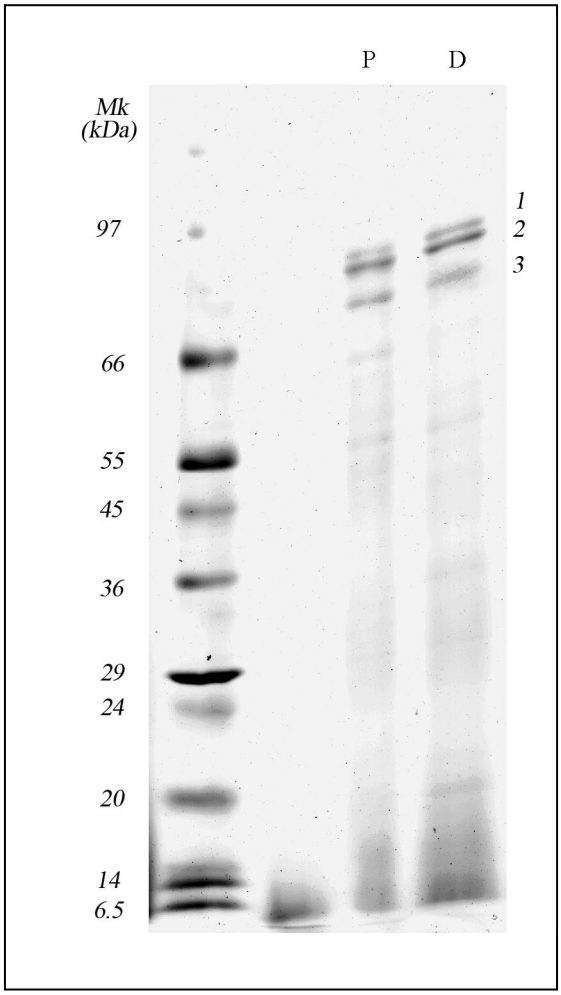

Table 1. List of proteins recognized in Mascot program for each analized band (*P. marginata* : P1, P2, P3; *D. cephalotes*: D1, D2, D3). Mw: molecular weight; Ip = isoelectric point.

	Theoretical Mw, kDa	Ip predict	No. of peptides identified	Mascot Score	NCBI Accession Number	Protein identified
P1	82698	5.72	7	413	gi|145207345	hexamerin 1 [Perla marginata]
	42783	5.20	5	233	gi|145207347	hexamerin 2 [Perla marginata]
P2	82698	5.72	17	1092	gi|145207345	hexamerin 1 [Perla marginata]
	79371	5.80	8	385	gi|40067356	hemocyanin subunit 1 [Perla marginata]
	42783	5.20	2	159	gi|145207347	hexamerin 2 [Perla marginata]
P3	78105	6.21	5	301	gi|40067358	hemocyanin subunit 2 [Perla marginata]
	72074	5.88	3	130	gi|145207351	hexamerin 4 [Perla marginata]
D1	82698	5.72	7	402	gi|145207345	hexamerin 1 [Perla marginata]
	42783	5.20	4	207	gi|145207347	hexamerin 2 [Perla marginata]
D2	82698	5.72	29	1547	gi|145207345	hexamerin 1 [Perla marginata]
	72074	5.88	8	348	gi|145207351	hexamerin 4 [Perla marginata] alcuni peptidi nuovi
	42783	5.20	7	310	gi|145207347	hexamerin 2 [Perla marginata] alcuni peptidi nuovi
	79371	5.80	6	236	gi|40067356	hemocyanin subunit 1 [Perla marginata]
D3	72074	5.88	7	359	gi|145207351	hexamerin 4 [Perla marginata]
	78105	6.21	5	243	gi|40067358	hemocyanin subunit 2 [Perla marginata]

Table 2. Hemocyanin (hc) fragments recognized en spectroscopy analysis. It is shown amino acid (aa) position, the mass (Da) and the polipeptide sequence.

hc type	**aa position**	**Mass (Da)**	**recognized sequence**	***species***
hc1	24-35	1304,65	GSVPADQFLTR	*D. cephalotes and P. marginata*
	61-69	950,43	DYDPSVAGK	*P .marginata*
	230-239	1150,55	AAEYGIEKDR	*P. marginata*
	542-557	588,98	KSSESSVTIPDRETTK	*D. cephalotes and P. marginata*
	563-580	694,64	VEHALEGKETLNVNKDER	*D. cephalotes*
	581-587	903,36	HCGYPDR	*P. marginata*
	643-650	939,42	AMGFPFDR	*D. cephalotes*
	656-666	1409,58	EDFFTDNMYTK	*D. cephalotes and P. marginata*
hc2	187-197	1275,65	FTGSIKNPEQR	*P. marginata*
	225-232	982,47	SYDVTKDR	*D. cephalotes*
	393-398	751,36	DPAFFR	*D. cephalotes and P. marginata*
	420-433	1517,76	EELDFPGVTVDAVK	*D. cephalotes and P. marginata*
	477-483	857,43	LNHEAFK	*D. cephalotes*
	532-541	1072,58	LTAGENVITR	*D. cephalotes and P. marginata*
	546-561	1801,89	SVVTIDEPMSFAEIHK	*P. marginata*
	577-583	909,4	HCGFPHR	*P. marginata*
	649-658	1212,3	ISCEESFITK	*P. marginata*

New data on the presence of hemocyanin in the Plecoptera: recomposing a puzzle

Introduction

The study of hemocyanin in insects is at the center of an ongoing scientific debate. Several studies have explored the functional properties of the hemocyanins in Arthropoda and these have led to a plethora of hypothetical functions, that include the oxygen carry (Markl et al., 1979a, 1979b, Markl, 1986; Markl and Decker, 1992) and others non respiratory tasks, as phenoloxidase and antimicrobial activity (Terwilliger, 1998; Bridges, 2001; Decker and Jaenicke, 2004; Jaenicke and Decker, 2004). The study of hemocyanin in Hexapoda reopens the issue of how respiration occurs in insects. After a preliminary and uncertain clue of the presence of this protein in embryonic hemolymph of the grasshopper *Schistocerca americana* (Sánchez et al., 1998), the first evidence of hemocyanin in the stonefly *Perla marginata* (Hanger-Holler et al., 2004) and *P. grandis* (Fochetti et al., 2006), molecular characterizations were carried out in some representative species of Collembola, Zygentoma, Phasmida, Blattodea, Isoptera (Pick et al., 2008; 2009a; 2009b). At present, the resulting state of knowledge regarding hemocyanin through Hexapoda is a complex puzzle of presence/absence, although hemocyanin seems to be missing in all eumetabolous insects (Burmester and Hankeln, 2007; Pick et al., 2009b). In the last years, we focused our study with the stoneflies insect in order to analyze how hemocyanin is expressed within the order and its relationship with the other Hexapoda. The methods of molecular phylogeny have revolutionized our knowledge on animal systematic, as well as the understanding of the evolution of proteins (Pagel, 1999; Swofford et al., 1996). It is well established that proteins that serve different functions may share significant identities at the molecular level. Amino acid sequences and structure elements may survive even if fundamental changes in protein function appear. Such sequence similarities reveal relationships in evolution (Doolittle, 1981; 1989). Groups of proteins that share a common ancestry can be classified in families or superfamilies (Daynoff et al., 1975). The phylogeny of members of such proteins families or superfamilies can be inferred analogous to those of living species (Swofford et al., 1996). Protein function and adaptations at a molecular level cannot be understood without taking into account species phylogeny. In fact, the proteins of an organism share its phylogenetic history with physiological adaptations that have been driven by evolutionary changes in the protein sequences during the emergence of different proteins. The phylogeny of hemocyanin and of its superfamily (HcSF), have to be combined considering the phylogeny of Arthropoda. The

hemocyanin superfamily, includes, besides hemocyanin, four other classes of exclusive arthropod proteins that share significant sequence similarities. Each group performs a specific and distinct function. (Beintema et al., 2004; Burmester and Scheller, 1996; Burmester, 2001). These are: the prophenoloxidase (PPO), activated after serine proteinase cleavage in phenoloxidases (PO: tyrosinases and catecholoxidases), involved in the initial step of the biochemical cascade of melanin biosynthesis for the sclerotization of the cuticle, browning, wound healing and in immune defense (Söderhäll and Ceresius, 1998); the non-respiratory crustacean pseudo-hemocyanins, also called cryptocyanins (Terwinllinger et al., 1999; Burmester, 1999a) implicated in the molting process; the insect hexamerins, mainly of larval and nymph stages, but also present in some adults species that serve as storage proteins (Telfer and Kunkel, 1991; Burmester, 1999b); and the dipteran hexamerin receptors that are responsible for the uptake of the hexamerin from the hemolymph, incorporating them into storage granules of larval fatty bodies and used up during metamorphosis (Burmester and Scheller, 1996). This superfamily includes type 3 copper proteins (phenoloxidases and hemocyanins), able to bind in the active site two copper ions (Decker and Terwilleger, 2000), and others protein families (hexamerins, dipteran hexamenin receptors and pseudo-hemocyanin) that have lost this capacity. Type 3 copper proteins, and its derivatives, are characterized by three principal domains: in domain II there is the active site; thanks to domain I, the enzymatic activity regulation is possible; the domain III is the most variable with an implication in probable immune activity and in molecule cooperativity (Jaenicke and Decker, 2004).

In this study, we report about new identification, with molecular cloning technique, of hemocyanin and hexamerin cDNA from nymphs and adults stoneflies. We inferred an evolutionary history of superfamily hemocyanin in Plecoptera, and at least, we advanced the study of this complex superfamily of proteins in Arthropod phylum.

Material and methods

Sequence analysis

A total of eleven species (six nymphs and seven adults) were collected and conserved in RNA later.

These are:

Perlodidae

- *Dyctiogenus alpimum*, (Pictet, 1842) and *Perlodes intricatus*, (Pictet, 1841). Nymphs. Collected on February 1, 2009. Po river - Pian della Regina – Crissolo, 1800 mt. (Cuneo - Piemonte - Italy);
- *Besdolus ravizzarum* Zwick and Weinzierl, 1995. Nymphs. Collected on February 3, 2009. Curone stream - Val Curone, 320 mt. (Alessandria - Piemonte - Italy). N 44°47′14″; E 9°04′02″;
- *Arcynopteryx compacta*, (McLachlan, 1872). Nymphs. Collected on June 6, 2009. Blue lake, Rosellón, 2530 mt. (Oriental Pireneus department - Languedoc Region, – France). N 42,61554; E 1,96704;
- *Isoperla acicularis*, (Despax, 1936) ssp. *acicularis.* Nymph and adult. Collected on July 2008. Vallarties river, 1390 mt. (Catalonia – Spain). N 42º39'24,07"; E 00º48'10,9";
- *Isoperla viridinervis* (Pictet, 1865). Adult male. Collected on July 2008. Escita Afluent, 1790 mt. (Catanonia-Spain). N 42º34'44,2"; E 01º00'56,0".

Leuctridae

- *Leuctra alosi*, Navás, 1919. Adults. Collected on July 2008. Vallarties river, 1390 mt. (Catalonia– Spain). N 42º39'24,07"; E 00º48'10,9";
- *Pachyleuctra benllochi*, (Navás, 1917). Nymph and adult. Collected on July 2008. Escita afluent, 1790 mt. (Catalonia– Spain). N 42º34'44,2"; E 01º00'56,0".

Nemouridae

- *Amphinemura sulcicollis*, (Stephens, 1836). Adults. Collected on July 2008. Vallarties River, 1390 mt. (Catalonia– Spain). N 42º39'24,07"; E 00º48'10,9";.
- *Nemoura cinerea*, (Retzius, 1783), and *Protonemura tubercolata*, Kempny, 1888. Adults. Collected on July 2008. Peguera river and afluents, Tor Lake drain, 2295 mt. (Catalonia– Spain). N 42º32'43,9"; E 01º02'47,5".

Total RNA was extracted, and various degenerate oligonucleotide primers, according to hemocyanin conserved region (about 600 nucleotides), were used in RT-PCR reaction. PCR fragments of expected size were cloned into pGEM-T easy vector, and sequenced by a commercial service, as described elsewhere (Amore et al., 2009a). Obtained sequences were translated with the tool provided by ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (http://www.expasy.org).

Sequence data and multiple alignment

Two different multiple alignments were performed. The first concerns Plecoptera sequences; in the second Plecoptera sequences are included in a more comprehensive alignment composed by the different groups of arthropod proteins belonging to the hemocyanin superfamily (HcSF).

Superfamily	**Family**	**Subfamily**	***Species***	**type**	**a.n.**	**acronym**
Perloidea	Perlodidae	Isoperlinae	*Isoperla grammatica*	hc1	EU672885	Iso_gr.hc1
				hc2	EU672886	Iso_gr.hc2
			Isoperla acicularis acicularis	hc2	GU121398	Iso_ac.hc1
		Perlodinae	*Dyctiogenus alpinum*	hc1	GU121395	Dyc_al.hc1
			Dyctiogenus alpinum	hc2	GU121396	Dyc_al.hc2
			Arcynopteryx compacta	hc2	GU121393	Arc_co.hc2
			Guadalgenus franzi	hc1	FJ393060	Gua_fr.hc1
			Besdolus ravizzarum	hc2	GU121394	Bes_ra.hc2
			Perlodes intricatus	hx	GU121397	Per_in.hx
	Perlidae	Perlinae	*Perla marginata*	hc1	AJ555403	Per_ma.hc1
				hc2	AJ555404	Per_ma.hc2
				hx1	AJ690365	Per_ma.hx1
				hx2	AJ690366	Per_ma.hx2
				hx3	AJ690367	Per_ma.hx3
				hx4	AJ690368	Per_ma.hx4
			Perla grandis	hc1	DQ118369	Per_gr.hc1
				hc2	DQ118369	Per_gr.hc2
			Dinocras cephalotes	hc1	FJ415315	Din_ce.hc1
				hc2	EF218621	Din_ce.hc1
			Caroperla sp.	hc1	GU121400	Car_sp.hx
			Tetropina sp.	hx	GU121388	Tet_sp.hx
			Togoperla sp.	hx	GU121389	Tog_sp.hx
			Neoperla sp.	hx	GU121390	Neo_sp.hx
			Etrocorema sp.	hx	GU121391	Etr_sp.hx
			Phanoperla sp.	hx	GU121392	Pha_sp.hx
	Chloroperlidae	Chloroperlinae	*Siphonoperla torrentium*	hx	EU6772887	Siph_sp.hx
Pterocarcyoidea	Peltoperlidae		*Cryptoperla sp.*	hx	GU121387	Cry_sp.hx
Nemouroidea	Taeniopterygidae	Taeniopteryginae	*Taeniopteryx stanchovitchi*	hx	EF218622	Tae_st.hx
			Brachyptera risi	hx	EU6772888	Bra_ri.hx
	Nemouridae	Amphinemurinae	*Amphinemoura sulcicollis*	hx	EU715327	Amp_su.hx
			Protonemura ausonia	hx	EU6772890	Pro_au.hx
		Nemourinae	*Nemoura hesperiae*	hx	EU6772889	Nem_he.hx
			Nemoura sp.	hx1	AM690369	Nem_sp.hx1
			Nemoura sp.	hx2	AM690370	Nem_sp.hx2
			Nemoura sp.	hx3	AM690371	Nem_sp.hx3
	Capniidae		*Capnia bifrons*	hx	FJ384672	Cap_bi.hx
			Allocapnia vivipara	hx	EF617597	All_vi.hx
	Leuctridae		*Leuctra fusca*	hx	EF218620	Leu_fu.hx
			Pachyleuctra benllochy	hx	GU121399	Pac_be.hx
Eusthenioidea	Diamphipnoidae		*Diamphipnopsis samali*	hx	EF620538	Dia_sa.hx

Table 3.5.1. List of stonefly species included in phylogenetic analysis.

Multiple alignment: Plecoptera. The protein sequences of 31 stonefly species, 14 hemocyanins, 6 of subunit1 (hc1) and 8 of subunit 2 (hc2), and 27 hexamerins, were deduced from cDNA sequences as per our studies, and from Genbank database. In Tab. 3.5.1 the sequences utilized for the alignment are listed. Myriapoda (6 hemocyanin sequences: *Scutigera coleopatra* AJ344359, AJ344360, AJ431378, AJ431379, AJ512793 and *Spirostreptus sp.* AJ297738) were chosen as outgroups on the base of the hemocyanin phylogenetic analysis where Myriapoda are in ancestral position (Kusche and Burmester; 2001b). The final alignment includes of 46 sequences and 202 amino acids positions.

tiype	**Subphylum**	***Specie***	**Acronym**	**accession number**
PPO	Crustacea	*Peneus monodon*	Pen_mo.PPO	AF099741
		Fenneropenaeus chinensis	Fen_ch.PPO	EU015060
		Pacifastacus leniusculus	Pac_le.PPO	X83494
	Hexapoda	*Tenebrio molitor*	Ten_mo.PPO	AB020738
		Locusta migratoria	Loc_m.PPO1	FJ771025
		Locusta migratoria	Loc_m.PPO2	FJ771024
		Bombix mori	Bom_m.PPO1	D49370
		Bombix mori	Bom_m.PPO2	D49371
hc	Chelicerata	*Eurypelma californicum*	Eur_ca.hcb	AJ290429
		Eurypelma californicum	Eur_ca.hcc	AJ277489
		Eurypelma californicum	Eur_ca.hcd	AJ290430
		Eurypelma californicum	Eur_ca.hcf	AJ277491
		Eurypelma californicum	Eur_ca.hcg	AJ277492
		Limulus polyphemus	Lim_po.hc2	AM260213
		Limulus polyphemus	Lim_po.hc3	AM260214
		Limulus polyphemus	Lim_po.hc4	AM260215
		Limulus polyphemus	Lim_po.hc6	AM260216
	Crustacea	*Cancer magister*	Can_ma.hc6	U48881
		Peneaus vannamei	Pen_va.hc	X82502
		Fenneropenaeus chinensis	Fen_ch.hc	FJ594414
		Homarus americanus	Hom_am.hcb	EF095142
		Homarus americanus	Hom_am.hca	AJ272095
		Pacifastacus leniusculus	Pac_le.hc1	AF522504
		Pacifastacus leniusculus	Pac_le.hc2	AY193781
	Myriapoda	*Scutigera coleopatra*	Scu_co.hcA	AJ344359
		Scutigera coleopatra	Scu_co.hcD	AJ344360
		Scutigera coleopatra	Scu_co.hcB	AJ512793
		Scutigera coleopatra	Scu_co.hcC	AJ431379
		Scutigera coleopatra	Scu_co.hcX	AJ431378
		Spirostreptus sp.	Spi_sp.hc	AJ297738
	Hexapoda	*Folsomia candida*	Fol_ca.hc1	FM242650
		Sinella curviseta	Sin_cu.hc1	FM242638
		Lepisma saccharina	Lep_sa.hc1	FM165291
		Lepisma saccharina	Lep_sa.hc2	FM165292

Table 3.5.2. List of arthropod species (except Plecoptera), included in the Plecoptera and arthropod HcSF multiple alignment. Protein type, systematic position (subphylum and specie) and Genbank accession number are shown. PPO: prophonoloxidases; hc: hemocyanin; hx: hexamerin; CC: cryptocyanins or pseudo-hemocyanin. Continue.

type	Subphylum	*Specie*	Acronym	accession number
hc	Hexapoda	*Thermobia domestica*	The_do.hc1	FM165288
		Thermobia domestica	The_do.hc2	FM165289
		Machilis germanica	Mac_ger.hc1	FM242639
		Schistocerca americana	Sch_am.EHP	AF038569
		Locusta migratoria	Loc_mi.hc1	FM242651
		Carausius morosus	Car_mo.hc1	FM242640
		Chelidurella acanthopygia	Che_ac.hc1	FM242641
		Chelidurella acanthopygia	Che_ac.hc2	FM242654
		Hierodula membranacea	Hie_me.hc2	FM242643
		Hierodula membranacea	Hie_me.hc1	FM242642
		Blaptica dubia	Bla_du.hc1	FM242646
		Blaptica dubia	Bla_du.hc2	FM242647
		Periplaneta americana	Per_am.hc2	FM242649
		Periplaneta americana	Per_am.hc1	FM242648
		Shelfordella lateralis	She_la.hc2	FM242653
		Shelfordella lateralis	She_la.hc1	FM242652
		Cryptotermes secundus	Cry_se.hc2	FM242645
		Cryptotermes secundus	Cry_se.hc1	FM242644
CC	Crustacea	*Homarus americanus*	Hom_a.Phc1	AJ132141
		Homarus americanus	Hom_a.Phc2	AJ132142
		Cancer magister	Can_ma.CC1	AF091261
hx	Hexapoda	*Thermobia domestica*	The_do.hx	FM165290
		Locusta migratoria	Loc_m.JHBP	U74469
		Periplaneta americana	Per_am.hx	L40818
		Blaberus discoidalis	Bla_di.hx	U31328

Table. 3.5.2. Continued.

Multiple alignment: Arthropod HcSF. The alignment of Plecoptera sequences was completed with others sequences of the arthropod hemocyanin superfamily, retrieved from GenBank database. The alignment is composed of Crustacean prophonoloxidases (PPO), Insect prophenoloxidases (PPO), Crustacean cryptocyanins (CC) or pseudohemocyanins (Phc), Crustacean hemocyanins (hc)

Myriapoda hemocyanins (hc), Insect hemocyanins (hc), and Insect hexamerins (hx).

The hexamerin receptors were ignored in this study because only a little part of the sequences aligned well with the hemocyanin conserved region we analyzed. A list of sequences, other than Plecoptera, used in this study are provided in Tab. 3.5.2. The final alignment comprises 100 sequences and 199 amino acid positions.

Sequence alignment and phylogenetic interference. Multiple alignment of amino acid sequences was constructed with the MAFFT online version (Katoh et al., 2005), matrix BLOSUM62. Further manipulation was carried out with BioEdit version 7.9 (Hall, 1999). Long gap regions, as well as some highly divergent regions, were removed from the final data set. The appropriate model of amino acid sequence evolution was selected by ProtTest

(Abascal et al., 2005) using the Akaike Information Criterion (AIC). Tree constructions were performed by BIO Neighbour Joining (BIONJ) (Gascuel, 1997) and Maximum Likelihood (ML) methods. ML analyses were performed with PhyML (http://www.atgc-montpellier.fr/phyml/) (Guindon and Gascuel, 2003), and the reliability of the trees was tested by bootstrap analysis (Felsenstein, 1985) with 100 replications. Distances between pairs of protein sequences were calculated according to LG model (Le and Gascuel, 2008) assuming gamma distribution of substitution rate.

Results

Sequence analysis. The designed primers were applied to cDNA of studied species. When these primers were applied on nymphs, they produced fragments of the expected size. We amplified two sequences for *D. alpinum* a.n. GU121395, GU121396, one sequence for *B. ravizzarum* a.n. GU121394, *A. compacta* a.n. GU121393, *P. intricatus* a.n. GU121397, *I. acicularis acicularis* a.n. GU121398 and *P. benllochi* a.n. GU121399. The amplified fragments are about 600 nucleotides long, the translated amino acid sequences result in about 195 amino acids, except for *P. intricatus* whose amplified fragment is of 893 nucleotides and the translated sequence of 297 amino acids (Tab.3.5.1). The same primers applied to adult specimens gave no resulting band in PCR analyses.

Both BLAST (Blastn and Blastp) and phylogenetic analyses (see below) unequivocally identified the sequences of *D. alpinum* (Dyc_al.hc1; Dyc_al.hc2), *B. ravizzarum* (Bes_ra.hc2), and *A. compacta* (Arc_co.hc2) as insect hemocyanins. The five histidines (His) of the studied fragment, crucial for O_2-binding, are present in all subunits (Fig. 3.5.1), while the sequence of *P. intricatus* (Per_in.hx) and *P. benllochi* (Pac_be.hx) resembles a hexamerin.

```
Iso_gr.hc1      VAYLGEDVGLNSHHSHWHMDFPFWWKAAEYGIEKDRKGELFYYMHHQMIARYDLERLSNH
Iso_gr.hc2      VAYLGEDIGVNSHHAHWHMDFPFWWKRT-YDVTKDRRGELFFYMHHQMVSRFDAERLSNN
Iso_ac.hc2      VAYLGEDIGVNSHHAHWHMDFPFWWKRT-YDVTKDRRGELFFYMHHQMVNRFDAERLSND
Bes_ra.hc2      VAYLGEDIGVNSHHAHWHMDFPFWWKKT-YDVTKDRRGELFFYMHHQMVNRFDAERLSNN
Arc_co.hc2      VAYFGEDIGVNSHHAHWHMDFPFWWKAT-YDVTKDRRGELFFYMHHQMTNRFDAERLSNN
Dyc_al.hc1      VAYLGEDVGLSSHHAHWHMDFPFWWKATEYGIEKDRKGELFYYMHHQMIARYDLERLSNH
Dyc_al.hc2      VAYLGEDIGVNSHHAHWHMDFPFWWKKT-YDITKDRRGELFFYMHHQMVNRFDAERLSNN
Per_in.hx       ISYFTEDVGLNAFHTYWNLDYPFWANSKNYNLKFDRRGELFYYTQHQLMARYYLERLSNG
Sip_to.hx       VAYFGEDVGVNTFNTYWHLDYPFWMNSAKYNMHFDRRGELFYYTQHQLLARYYLERISNG
Pac_be.hx       VAYLGEDVGLSTFHTYWNMDYPFWANHKTYGIKWDRTGELFYYTQHQILARYYLERLSNG
Leu_fu.hx       VAYLGEDVGLSTFHTYWNMDYPFWANSKYYNLKFDRDGELFYYTQDQILARYYLERLSNG
                ::*: **:*:.:.:::*::*:*** :   *.:  ** ****:* :.*:  *:  **:**
```

Figure 3.5.1. Multiple sequence alignment (BLOSUM62) of hemocyanins conserved aminoacid sequences (hc) and correspondent hexamerins sequences (hx). His (yellow) and Phe (green) residues involved in the oxygen-binding site are indicated. The residues involved in the trimer (blue) and dimer (grey) contacts are also shown.

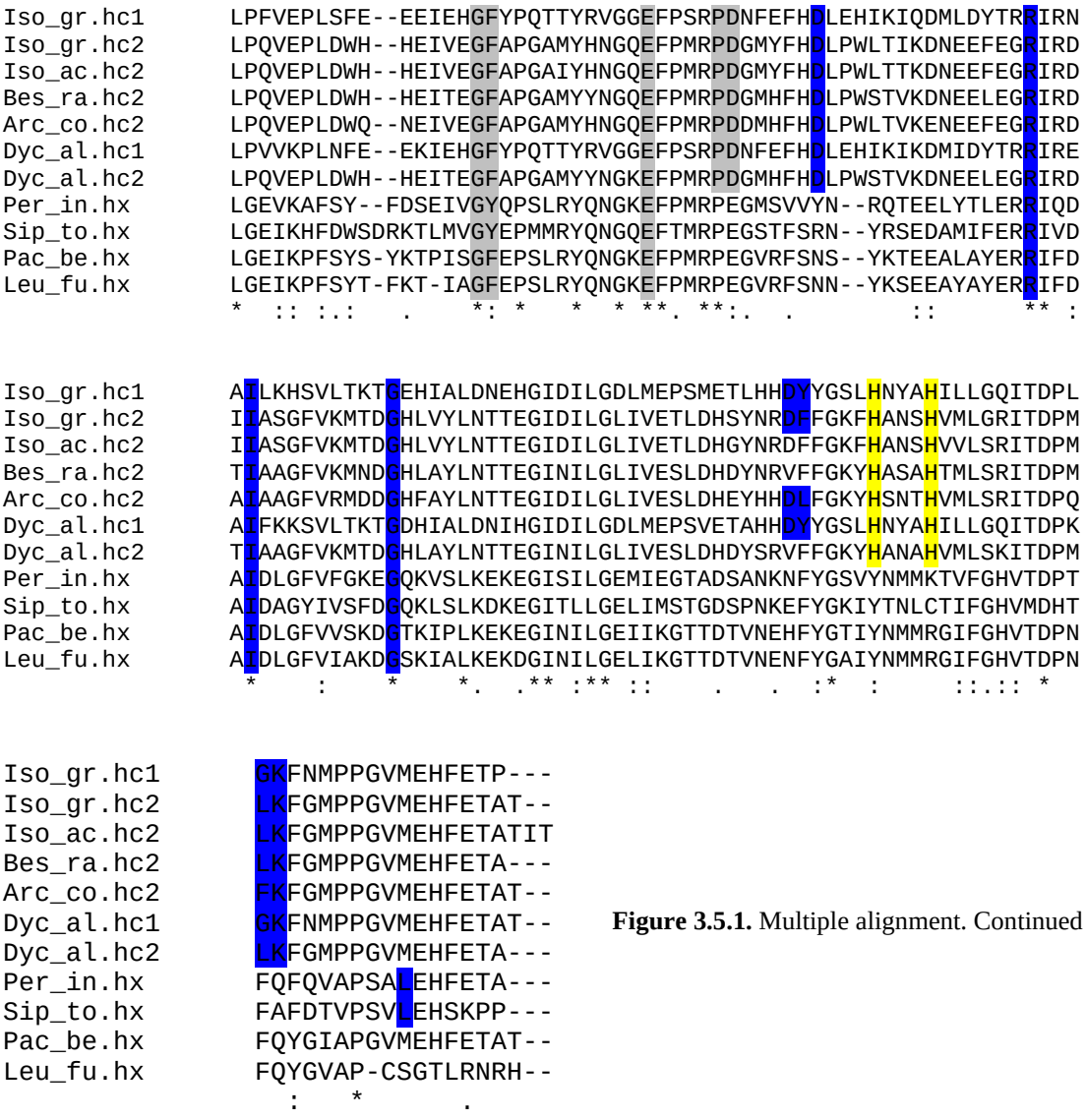

Figure 3.5.1. Multiple alignment. Continued

The nucleotides and amino acid sequences were further compared to the hemocyanins of the stoneflies *I. grammatica* (Iso_gr.hc1 and Iso.gr.hc2; Amore et al. 2009) (Tab. 3.5.3). As expected, *D. alpinum* hemocyanin subunit 1 (Dyc_al.hc1) shows the highest degree of identity with the type 1 hemocyanin subunits (0.90 aminoacidic and 0,85 nucleotidic), while lower scores were obtained when compared to type 2 subunits (0.53-0.55 aminoacidic and 0.61-0.63 nucleotidic). Subunit 2 of *D. alpinum* (Dyc_al.hc2), *B. ravizzarum* (Bes_ra.hc2), *A. compacta* (Arc_co.hc2), *I. acicularis acicularis* (Iso_ac.hc2) and stonefly hemocyanin subunit 2 of *I. grammatica* display 0.86-0.95 identical amino acids, and 0.86-0.94 identical nucleotides, while lower identity scores were observed with other type 1 subunits (0.53-0.54 amino acidic and 0.60-0.62 nucleotitic).

Seq.	Iso_gr.hc1	Iso_gr.hc2	Iso_ac.hc2	Bes_ra.hc2	Arc_co.hc2	Dyc_al.hc1	Dyc_al.hc2	Per_in.hx	Sip_to.hx	Pac_be.hx	Leu_fu.hx
Iso_gr.hc1	**ID**	0,62	0,60	0,60	0,60	0,85	0,59	0,55	0,54	0,57	0,57
Iso_gr.hc2	0,54	**ID**	0,94	0,86	0,84	0,62	0,86	0,55	0,54	0,58	0,55
Iso_ac.hc2	0,53	0,95	**ID**	0,84	0,82	0,60	0,83	0,55	0,53	0,57	0,54
Bes_ra.hc2	0,53	0,89	0,87	**ID**	0,84	0,61	0,95	0,54	0,54	0,56	0,54
Arc_co.hc2	0,54	0,86	0,85	0,86	**ID**	0,63	0,84	0,54	0,53	0,57	0,54
Dyc_al.hc1	0,90	0,55	0,54	0,53	0,55	**ID**	0,61	0,54	0,53	0,57	0,56
Dyc_al.hc2	0,54	0,88	0,87	0,96	0,85	0,54	**ID**	0,53	0,53	0,56	0,55
Per_in.hx	0,38	0,39	0,39	0,39	0,38	0,39	0,38	**ID**	0,69	0,73	0,76
Sip_to.hx	0,35	0,39	0,37	0,38	0,38	0,34	0,37	0,59	**ID**	0,71	0,70
Pac_be.hx	0,45	0,43	0,42	0,42	0,42	0,46	0,43	0,66	0,59	**ID**	0,86
Leu_fu.hx	0,40	0,36	0,35	0,36	0,35	0,40	0,36	0,66	0,58	0,83	**ID**

Table 3.5.3. Nucleotidic (up) and amino acidic (down) identity. Species acronyms are the same used in phylogenetic analysis. Seq.: sequences.

In the *P. intricatus* and *P. benllochi* hexamerins only one of the four Cu-binding histidines are conserved. Comparison with hc1 and hc2 are in the range of 0.38-0.46 for amino acids and 0.53-0.58 for nucleotides, while identity value is higher among Plecoptera hexamerins (0.58-0.83 amino acid and 0.69-0.86 nucleotide). *P. benllochi* showed a major relationship with *Leuctra fusca* (0.83 amino acid and 0.86 nucleotide) with whom shares the same systematic family.

Phylogenetic analysis. Both types of analyses (BIONJ and ML) gave similar tree topologies. Here we show ML results (Fig. 3.5.2 and Fig. 3.5.3).

Plecoptera. The Myriapoda sequences were used to root the tree for visualization purposes. In the analysis, three well supported monophyletic clades were formed. Dyc_al.hc1 joined the clade with the previously identified Plecoptera hemocyanin subunit 1; 100% bootstrap support (Fig. 3.5.2). The Dyc_al.hc2; Bes_ra.hc2, Iso_ac.hc2, and Arc_co.hc2 group with the previously identified Plecoptera hemocyanin subunit 2; 100% bootstrap support. Hexamerins, where Per_in.hx and Pac_be.hx grouped, formed a third clade; 100% bootstrap support.

Within hemocyanin subunit type 1, the Perlodidae sequences (Iso_gr.hc1; Guad_fr.hc1; Dyc_al.hc1) are monophyletic and derived from Perlidae; 87% bootstrap support. Within the clade of hemocyanin subunit 2, Perlidae and Perlodidae formed two clades in the relation of sister groups, even if Perlodidae clade is supported by a 50% bootstrap value. Hemocyanin subunit 2 shares a common ancestor with all Plecoptera hexamerins.

HcSF: Given the ancient origin of prophenoloxidases, these can be assumed as the outgroup of this analysis (Fig. 3.5.3). Within the hemocyanins, three distinct clades emerge in accordance to divergent separation of arthropod subphyla. In ML and BIONJ analysis, the first branch represents Chelicerata hemocyanin (98% bootstrap support); the second Myriapoda hemocyanin (100% bootstrap support), the third includes crustacean and insect hemocyanin, crustacean cryptocyanins and insect hexamerins (100% bootstrap support). Myriapoda hemocyanin is in a sister group position with respect to crustacean and insect hemocyanins, hexamerins and cryptocyanins, (99% bootstrap support) In ML, within hemocyanins, it is evident one clade for insect hemocyanin subunit 1, one clade for insect hemocyanin subunit 2, and one for crustacean hemocyanins, strictly related to cryptocyanin. All insect hexamerins formed a unique clade. The macro-clade that includes insect and crustacean hemocyanin, hexamerins and cryptocyanins, presents a low bootstrap support and. Anyway, hexamerins always join in the same clade. Whitin subunit 1, the hemocyanins from Zygenthoma (Ter_do.hc1 and Lep_sa.hc1) form the sister group of the pterygote proteins (97% bootstrap support). Collembola (Sin_cu.hc1 and Fol_ca.hc1) is basal to the ectognathan subunits (38% bootstrap support). Within the hemocyanin subunit 2, phylogeny resembles that of subunit 1. *Machilis germanica*, Zygenthoma (Mac_ge.hc1), is in an ambiguous position and cluster within hexamerins.

In BIONJ analysis, the hexamerins are a sister group of arthropod hemocyanin and cryptocyanins.

Discussion

Nymphs. In the last year we focused our research on the searching of hemocyanin protein in the Plecoptera. We had already studied nymphs and adults of European fauna, a group of Oriental Perlidae and some Notonemouridae. (Amore et al., 2009a; Amore and Fochetti, 2009; Amore et al., 2009b). To understand this issue and the complex framework that was emerging, we used ecological (habitat type, medium water temperature) and autoecological parameters (life cycle, animal size, trophic role) as work tools to infer generalizations.

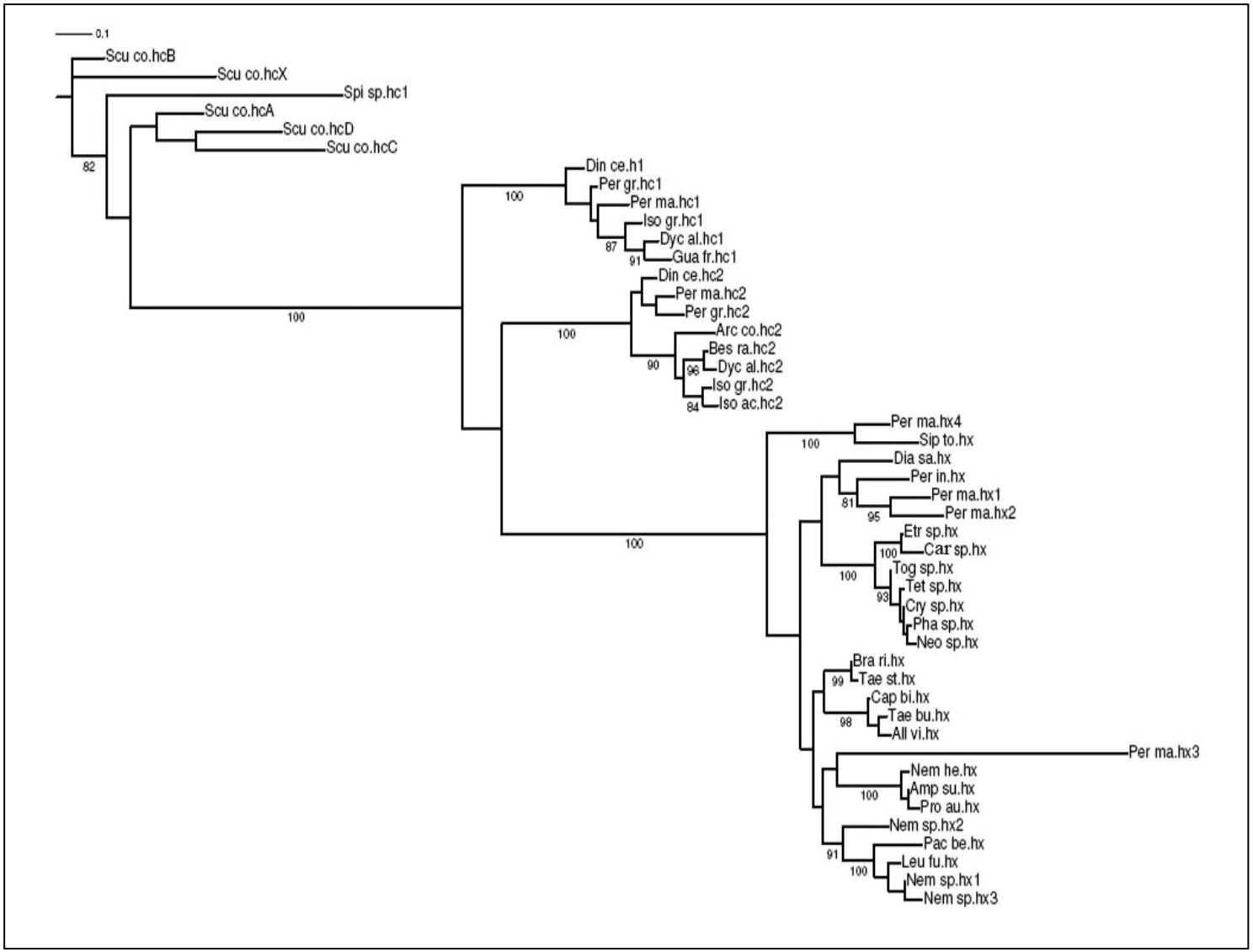

Figure 3.5.2. ML analysis plecoptera hemocyanin (hc). The number rappresent the bootstrap support. The bar equals 0.1 substitutions per site.

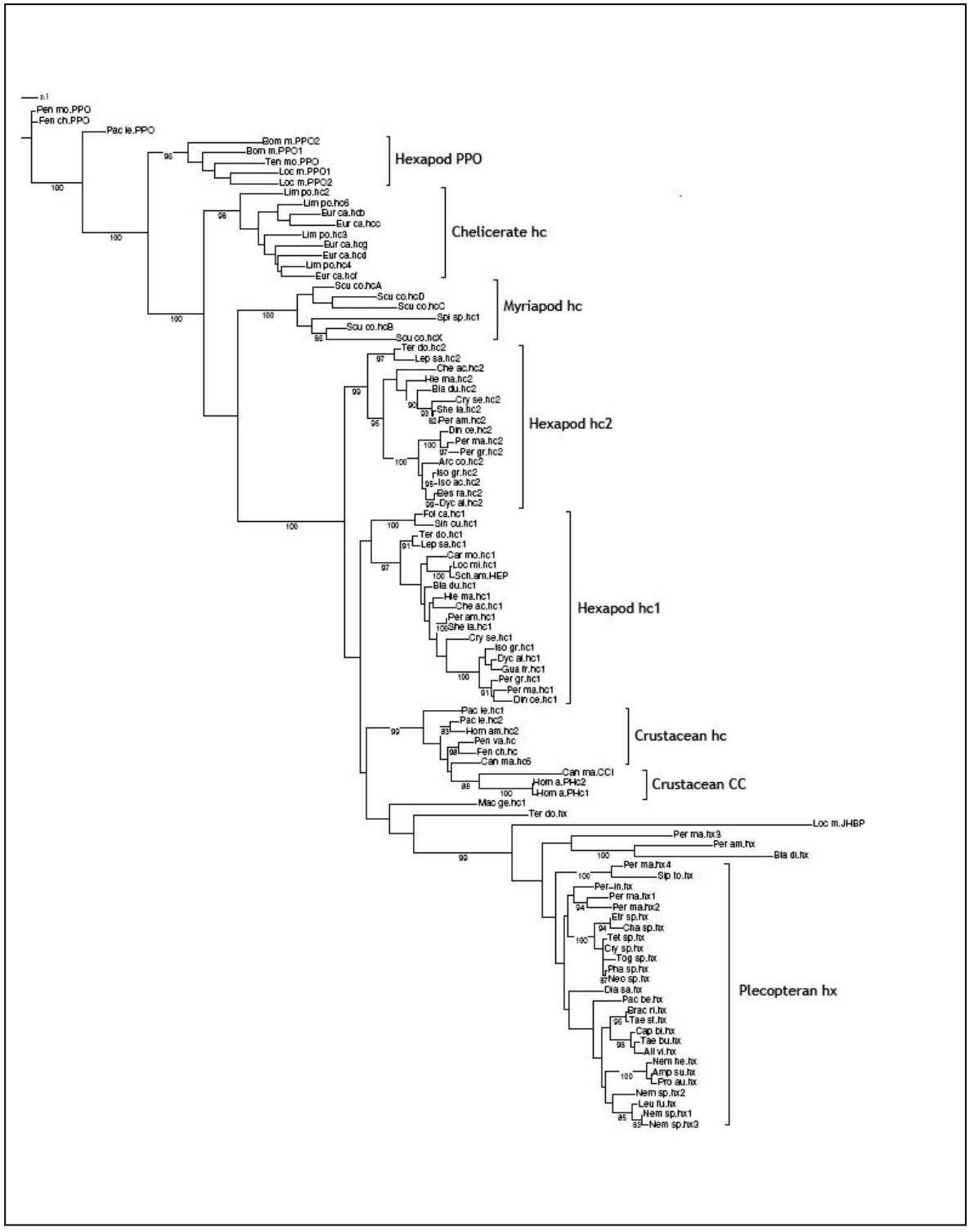

Figure 3.5.3. ML analysis of HcSf. The number rappresent the bootstrap support. The bar equals 0.1 substitutions per site.

In this study we analyzed six new species of Plecoptera fauna, at the nymph stage, to cover the biodiversity of the order and their environmental adaptations, as possible. We studied nymphs of orophilous species, of subalpine, or mountain habitats as the Perlodidae *D. alpinum*, *P. intricatus*, *A. compacta*, *I. acicularis acicularis* or Pyrenees endemism as the Leuctridae *P. benllochi.* These species were collected in mountain rivers, in rhithron zones, or lakes in February and June where water temperatures remain low (some degree up 0 °C and 8 °C) and, as a consequence dissolved oxygen value is high (11-13 mg/L). We sequenced hemocyanin conserved region in all nymphs, except for *P. intricatus* and *P. benllochi.* It is worthy to note that *P. intricatus* and *D. alpinum* were collected in the same river site and on the same date. Both belongs to Perlodidae, are of medium size, presumably both are semivoltine, and are overall predators (Fochetti and Tierno de Figueroa, 2008b), but they display a different physiological response on hemocyanin production. On the other hand, *B. ravizzarum*, a Perlidae living in at lower altitude, in a potamon river zone, express hemocyanin in his mRNA repertory.

In Amore and Fochetti (2009), we compared Perlodidae (*Guadalgenus franzi* and *I. grammatica*) and Taeniopterygidae (*Brachyptera risi* and *B. vera*) species of Italian perennial rivers and Mediterranean seasonal stream of the southern Iberian Peninsula. Presumably, species living in seasonal waters have to face major stress compared to species living in a perennial river. Seasonal streams are formed annually and expand during a short period as a consequence of melting snow and spring rains. It is presumed that life history strategies of these species are greatly influenced by the characteristics of their environment (Lopez-Rodriguez et al., 2009). Even if *G. franzi*, a specie of seasonal stream, as other Perlodidae, harbors hemocyanin, we found no difference between the two *Brachyptera* species. We also analyzed six oriental species of Perlinae (Perlidae), and one Peltoperlidae of medium size comparable to the one of *Isoperla* or *Perla*. Tropical streams are characterized for seasonal and daily temperature stability of about 20 °C (Dudgeon, 1999). At these conditions, the quantity of oxygen dissolved in water is less if compared with ecological conditions of mountain European and perennial rivers. Contrarily to what we expected, hemocyanin was not expressed in hemolymph of Oriental species (Amore et al., 2009b).

Reviewing all we have done until now (Tab. 3.5.4), we can affirm that, in nymphs, hemocyanin expression does not depend on size or trophic role. Environmental adaptation to ecological condition might have led to the loss of the protein in some groups. In evolution, only structures with an essential biological function undergo to a strong evolutive pressure

that permits them to perpetuate highly conserved frames (Ridley, 2004). It is conceivable that independent adaptations to local conditions caused a decrease in hemocyanin requirement, a precondition to generate variability. Cumulative mutations and divergent evolution probably caused significant change in domain II and disabled copper-binding sites and oxygen affinity, leading to ancestor-like hexamerin proteins.

Adults. Plecoptera are hemimetabolous insects whose ecological medium completely changes when they become adults. While nymphs dwell in aquatic habitat, adult stoneflies emerge from the streams, lakes or rivers. They have reduced flight ability and in some cases, males are brachypterous (*D. cephalotes* and *I. viridinervis*), and can generally be found on the banks next to their previous habitat. The quantity of oxygen availability in the air compared with the oxygen dissolved in water is very different. Anyway it was proven that, even insects adapted to terrestrial medium possess respiratory proteins. In fact, hemoglobin genes were found in holometaboulos species as *Drosophila* (Hankeln et al., 2002) and *Apis* (Hankeln et al., 2006), and some Coleoptera Hemiptera and Lepidoptera that live in normoxic conditions (Burmester and Hankeln, 2007).

On the other hand, in Plecoptera, adults' and nymphs' activities are very diverse. Nymphs have the task of feeding (predator, grazing phytophagous or detritivourus) and dealing with various molting, they face considerable physiological stress (Fochetti and Tierno de Figueroa, 2008b; Tierno de Figueroa et al.; 2003). Adults mainly dedicate themselves to mating (Tierno de Figueroa et al., 2006; Tierno de Figueroa and Fochetti, 2001a; Tierno de Figueroa and Luzón-Ortega, 1999; 2002), and in some cases, they do not feed at all (*P. marginata, P. grandis. D. cephalotes*) (Tierno De Figueroa and Fochetti, 2001b) (Fig. 3.5.4).

Preliminary data on the presence of hemocyanin in adults were reported in Amore and Fochetti, 2009. Here we extend the study to all European families covering a representative sample of the biodiversity of the order. We studied Pyrenees endemism as *I. viridinervis,* a species whose adult male is brachypterous, *P. benllochi, and P. tubercolata* and *Leuctra alosi,* and wide distribution species such as *A. sulcicollis and N. cinerea* (Tab. 3.5.4). Hemocyanin was recorded for *P. marginata* (Hagner-Holler, 2004) and *P. grandis* (Fochetti et al., 2006), but in our previous and present studies, we never detected hemocyanin in the adults, even in species where hemocyanin was sequenced in nymphs. Hexamerins were sequenced in nymph and adults only in *Capnia bifrons*, an ovoviviparous species (Hynes, 1941; Fochetti and Tierno de Figueroa, 2008b).

Superfamily	Family	Species	stage of growth	Size (mm) animal	diet	altitudal range (mt)	habitat	Life cycle	ecological category	Stream type	Corology	hc	hx
Pteronarcyoidea	Peltoperlidae	*Cryptoperla sp.*	nymph		detritivourus	?	?	semivoltine	?	permanent	OR	-	yes
Systellognatha	Perlidae	*Perla marginata*	1st, 2nd, 3rd	*16 -33*	detritivourus and predator	160-2800	rhithron	semivoltine	rheophilous, stenotherm	permanent	M-S-EU; MAG	hc1; hc2	yes
			adult		no feeding				-			hc1; hc2	-
		Perla grandis	1st, 2nd, 3rd	*23 -31*	predator	465-2500	hyporhithron	semivoltine	rheophilous, stenotherm	permanent	M-S-EU	hc1; hc2	-
			adult		no feeding				-			hc1; hc2	
		Dinocras cephalotes	nymph	*14 - 31*	predator	40-2800	hyporhithron	semivoltine	rheophilous, stenotherm	permanent	EU	hc1; hc2	yes
			adult		no feeding				-			no	-
		Neoperla sp.	nymph		*?*	?	?	semivoltine	?	permanent	OR	-	yes
		Togoperla sp.	nymph		*?*	?	?	semivoltine	?	permanent		-	yes
		Etrocorema sp.	nymph		*?*	?	?	semivoltine	?	permanent		-	yes
		Phanoperla sp.	nymph		*?*	?	?	semivoltine	?	permanent		-	yes
		Caroperla sp.	nymph		*?*	?	?	semivoltine	?	permanent		-	yes
	Perlodidae	*Isoperla grammatica*	nymph	*11 – 16*	predator	10-2000	rhithron	univoltine	rheophilous. stenotherm	permanent	EU	hc1; hc2	-
		Isoperla rivulorum	adult	*10 - 15*	predator		rhithron	univoltine	orphilous	-	M-S-EU	no	
		Isoperla viridinervis	adult male	*10 – 12*	predator	1000-2400	rhithron	semivoltine		-	PYR	no	
		Isoperla acicularis acicularis	nymph	*14 - 16*	phytophagous and detritivourus	430-2300		univoltine		permanent		hc2	
			adult						-			no	
		Guadalgenus franzi	nymph	*11 – 18*	predator	100-1660	-	semivoltine	thermophilous	temporal	IB	hc1	-
		Perlodes intricatus	nymph	*15 – 25*	predator	800-2700	rhithron	semivoltine?	orophilous	permanent	EU	-	yes
		Dyctiogenus alpinum	nymph	*16 - 24*	detritivourus and predator	570-2700	hyporhithron	semivoltine	rheophilous - orophilous	permanent	EU	hc1; hc2	
		Besdolus ravizzarum	nymph	*15 - 19*	phytophagous	220-520	potamon	univoltine		temporal	M-S-EU	hc2	
		Arcynopteryx compacta	nymph	*15 – 22*	predator	950-2475	rhithron and mountain lakes	semivoltine	orophilous - stenotherm	permanent	OL	hc2	
	Clhoroperlidae	*Siphonoperla torrentium*	nymph	*7 – 9*	predator and phytophagous	30-2000	rhithron	univoltine	rheophilous -orophilous	permanent	M-EU	-	yes
			adult		predator and phytophagous				-			-	-

Table 3.5.4. Resume of studied species. (33 species). Systematic position, autoecological factors (size, altitudinal range, habitat type and stream type, ecological category, corology), hemocyanin (hc) and hexamerins presence are shown in nymph and adult stage.
Corology: AF: Africa; EU: Europe; EU-AS: Euroasiatic; IB: Iberian peninsula; IT: Italian peninsula; PAL: paleartic (Europe+Asia); PYR: Pyrenean chain; MAG: Maghreb; OL: Oloartic; OR: Oriental
M: medium; S: South; N:North.
Temperature request regard eggs needs: eurytherm, stenotherm. (From Berthélemy 1966; Fochetti and Tierno de Figueroa 2008b). Continue.

Superfamily	Family	Species		Size (mm) animal	diet	altitudal range (mt)	habitat	Life cycle	ecological category	Stream type	Corology	hc	hx
Eulognatha	Taeniopterygidae	*Taeniopteryx stanckovitchi*	nymph	*8 – 12,5*	phytophagous	250-1800	rhithron	univoltine	rheophilous	permanent	S-EU	-	yes
		Brachyptera risi	nymph	*8 – 12*	phytophagous	100-1100	rhithron	univoltine	rheophilous - orophilous	permanent		-	yes
		Brachyptera vera	nymph	*8.5 – 10.5*	phytophagous		-	univoltine		temporal	IB	-	-
	Leuctridae	*Leuctra fusca*	nymph	*6 – 8*	phytophagous		ubiquitous	univoltine	rheophilous, mesotherm	permanent	EU-AS	-	yes
		Leuctra alosi	adult	*5 – 7*	phytophagous		rhithron	univoltine	rheophilous	permanent	PYR	-	-
		Pachyleuctra benllochi	nymph	*11 – 12*	phytophagous and detritivourus	1000-2500	rhithron	semivoltine	stenotherm	permanent	PYR	-	yes
			adult						-			-	-
	Nemouridae	*Nemoura hesperiae*	nymph	*6 - 9*	phytophagous		rhithron	univoltine	rheophilous	permanent	IT	-	yes
		Nemoura cinerea	adult	*6 – 10*	detritivourus	85-2410	ubiquitous	univoltine	reophilous, eurytherm	-	PAL	-	
		Protonemura ausonia	nymph	*7 -11*	phytophagous	500-2000	crenon	univoltine	stenotherm	permanent	IT	-	yes
		Protonemura tuberculata	adult	*7.5 – 10,5*	phytophagous and detritivourus	1000-2350		univoltine	-	-	PYR	-	-
		Amphinemura sulcicollis	nymph	*4 – 8*	phytophagous	240-2100	-	univoltine	reophilous, eurytherm	permanent	EU	-	yes
			adult						-			-	-
	Capniidae	*Capnia bifrons*	nymph	*6 - 9*	phytophagous and detritivourus			univoltine	reophilous	permanent	OL	-	yes
			adult		phytophagous				-			-	yes
	Notonemouridae	*Afronemura anhatolae*	nymph			-	-	univoltine		-	S-AF	no	-
			adult									no	-
		Aphanicella bullata	adult					univoltine		-	S-AF	no	-

Table 3.5.4. Continued.
Corology: AF: Africa; EU: Europe; EU-AS: Euroasiatic; IB: Iberian peninsula; IT: Italian peninsula; PAL: paleartic (Europe+Asia); PYR: Pyrenean chain; MAG: Maghreb; OL: Oloartic; OR: Oriental
M: medium; S: South; N:North.

It is interesting to note that hexamerins are usually proteins expressed at high concentrations in larval and nymphal stages and rarely in adults (Beintema et al., 1994). Insect hexamerins exhibit significant similarities in structure and sequence to arthropod hemocyanins (Markl et al., 1992; Beintema et al., 1994; Burmester and Scheller, 1996; Markl and Winter, 1989). Hexamerins serve mainly as sources of amino acids during non-feeding periods, in larval molting or adult development (Telfer and Kunkel, 1991; Haunerland, 1996; Beintema et al. 2004), but they can also work as carrier proteins for small organic compounds, like steroid hormone, riboflavin and juvenile hormones (Enderle et al., 1983; Magee et al., 1994; Braun and Wyatt, 1996), or may be involved in immune response (Hayakawa, 1994; Beresford et al., 1997). It was demonstrated that in some Crustacea, hemocyanin function resembles to the one of hexamerins. Its concentration is associated with a molting cycle, suggesting a specific utilization during starvation (Depledge and Bjeregaard, 1989), and under special circumstances, hemocyanin is metabolically recycled and employed as a source of energy from amino acids (Zuckerkandl, 1960; Hagerman, 1983).

These considerations, together with the different presence in nymphs and adults we found in Plecoptera, allow us to suppose that hemocyanin has not only a respiratory function, and that the physiological need of hemocyanin may changes during the life cycle.

Phylogeny implications.

Plecoptera. Our results suggest differences in the presence of hemocyanin expression among nymphs of stonefly species. Starting from the hypothesis that all Plecoptera had hemocyanin as ancestral condition, it may be possible that hemocyanin has been lost several times during the evolution of the order. A first time might have happened in a Nemouroidea ancestor, since we never find hemocyanin in Nemouroidea species analysed. Secondarily in Perloidea, hemocyanin might have been independently lost within families (Chloroperlidae) or genus (*Perlodes*). This idea is in accord with the accepted theory that, even if Plecoptera is a very ancient order (fossils from early Permian), present families do not seem to be very old, and recent and repeated phenomena of speciation and extinction are described. Poor flight capability of these insects means that their dispersion on a large scale is unlikely (Zwick, 2000). In species where we did not sequence hemocyanin, we only found hexamerins. Hemocyanins and hexamerins share many characteristics in terms of structure and sequence, but due to the degeneration of the Cu-binding active site, hexamerins do not bind oxygen. It is accepted that hexamerins evolved from hemocyanins in the early steps of insect evolution, so they are paralogous proteins. Our data would indicate that hexamerins evolved from subunit 2

(hc2), even though the analysis of a more complete dataset led by Burmester and Hankeln (2007) hypothesizes hc1 as the probable closest subunit.

It is remarkable that the hemocyanin studied conserved region acts like a phylogenetic molecular marker within Plecoptera. There are always two hemocyanin subunits (hc1 and hc2) and the phylogenetic pattern obtained by using of hemocyanin evolution matches the accepted scheme of traditional phylogeny based on morphology and anatomy. Hexamerins follows more loosely the systematic relationship, indicating a lower evolutionary pressure that permitted to accumulate mutations and distinct types of amino acids (Telfer and Kunkel, 1991; Burmester et al., 1998).

The use of hemocyanin as molecular marker could be interesting to study in depth taxa whose systematic position in still uncertain, and verify phenomena of speciation and adaptation.

On the other hand, our study mainly focuses on Arctoperlaria species, and above all on European fauna. The unique sequence of Antarctoperlaria (*Dhiamphipnopsis samali*, Eustenoidea) included in our phylogenetic analysis, derived from a specific study on Plecoptera hexamerin (Hagner-Holler et al., 2007). Enlarging the study to Antactoperlaria would give a wider general overview to the problematic investigation of hemocyanin in Plecoptera.

HcFS. Although hemocyanins, pseudo-hemocyanins, prophenoloxidases and hexamerins form a functionally highly diversified protein superfamily, most sequences and structural core elements are strikingly conserved, allowing to trace the evolutionary history of these proteins. The relationships among different members of the hemocyanin superfamily can be deduced by comparing their sequences using molecular phylogenetic methods, as reported in literature (Beintema et al., 1994; Burmester and Scheller, 1996; Durstewitz and Terwillinger, 1997; Burmester et al., 1998; Burmester, 1999a, 1999b; 2001; Kusche and Burmester, 2001b; Pick et al., 2009b).

Tyrosinase as well as catecholoxidase, belong to the group termed phenoloxidases. Tyrosinase incorporates oxygen into organic compounds by hydroxylation. This enzyme catalyzes two reactions, the hydroxylation of phenolic compounds in the ortho position (cresolase activity) and subsequently oxidation of diphenolic products (catecholase activity) (Solomon et al., 2004; Sánchez-Ferrer et al., 1995). Their presence in all phyla of living organisms demonstrates their early origin in the history of life.

Chelicerata hemocyanins form a separate clade. It has been noted phenoloxidases activity of some subunit of chelicerata hemocyanin (Decker et al., 2001), therefore these subunit types may be considered as transitional structures between phenoloxidases and hemocyanins.

The Myriapoda hemocyanins clade is the sister group of insect and crustacean hemocyanin and their derivates (insect hexamerins and crustacean cryptocyanins). Assuming that protein phylogeny reflects species evolution, the unique clade for crustacean and hexapod hemocyanins and descendents, strongly supports the Pancrustacea hypothesis, where all crustaceans and hexapods are comprised in a unique monophyletic taxon, in contrast to the Atelocerata hypothesis in which Myriapoda and Hexapoda are sister taxa, and Crustacea are only more distantly related (Brusca and Brusca, 2002).

Hexamerins and cryptocyanins undergo a parallel evolution. The hexamerins form a monophyletic clade which is the sister group of the known Insect and Crustacean hemocyanin, while cryptocyanins derived from crustacean hemocyanins.

In an evolutionary perspective, the first ancestor of the HcSF could be identified with a hypothetical essential mini-phenoloxidades constituted by the second domain (domain II) of today's arthropod hemocyanins, with uncontrolled access to phenolic substrates, in a time when oxygen was only a poisonous trace element in the reducing atmosphere. Thus, this mini-phenoloxidase was always active, detoxifying dioxygen in the cell and keeping oxygen concentrations low. This mini phenoloxidase would have acquired additional functions in other essential physiological processes such as in primary immune response, for example, by encapsulation of microbial invaders, sclerotization of the cuticle after molting, wound healing, and protective coloration (Jaenicke and Decker, 2004). In the course of evolution, and the consequent change of oxygen concentration in the atmosphere, it became advantageous to regulate this enzymatic activity. A mechanism of regulation was acquired by the fusion with another α-helical protein at the N-terminal part (domain I) (Decker and Terwilliger, 2000). This domain regulated enzymatic activity by controlling access to the active site for bulky phenolic substrates by conformational changes. Modern phenoloxidases, present a third domain (domain III), whose function is still unclear. Jaenicke and Decker (2004), proposed that the function of domain III, the most variable, is to complement the enzymatic activity of phenoloxidase in immune response and wound healing by mediating a binding reaction to surfaces. The conversion of phenoloxidase to hemocyanin implied that phenoloxidase activity had to be permanently, or almost permanently, inactivated, so that the active site was no longer accessible to any ligands larger than gas molecules such as oxygen. To achieve these properties, only small changes in domain I were necessary, such as the removal of proteolytic cleavage site for activation of prophenoloxidase between the first and second domains, the improvement of reverse oxygen binding and the reduction of enzymatic activity. Subsequently, degeneration of hemocyanin active sites and a consequent relaxation of

selective pressure led to the variety of hexamerin proteins existing today, characterized by distinct types of amino acid frequencies. Hexamerins are rich in highly aromatic amino acids that may have been favored by positive selection because they are necessary constituents during sclerotization or molting (Telfer and Kunkel, 1991)(Fig 3.5.4.).

Further considerations

Hemocyanins, together with hemoglobins and hemerythrins, are considered the unique animal proteins able to bind oxygen for sustaining the production of ATP in the respiratory chain of mitochondria (Truchot, 1992), but alternative physiological functions for insect hemocyanins may also be considered.

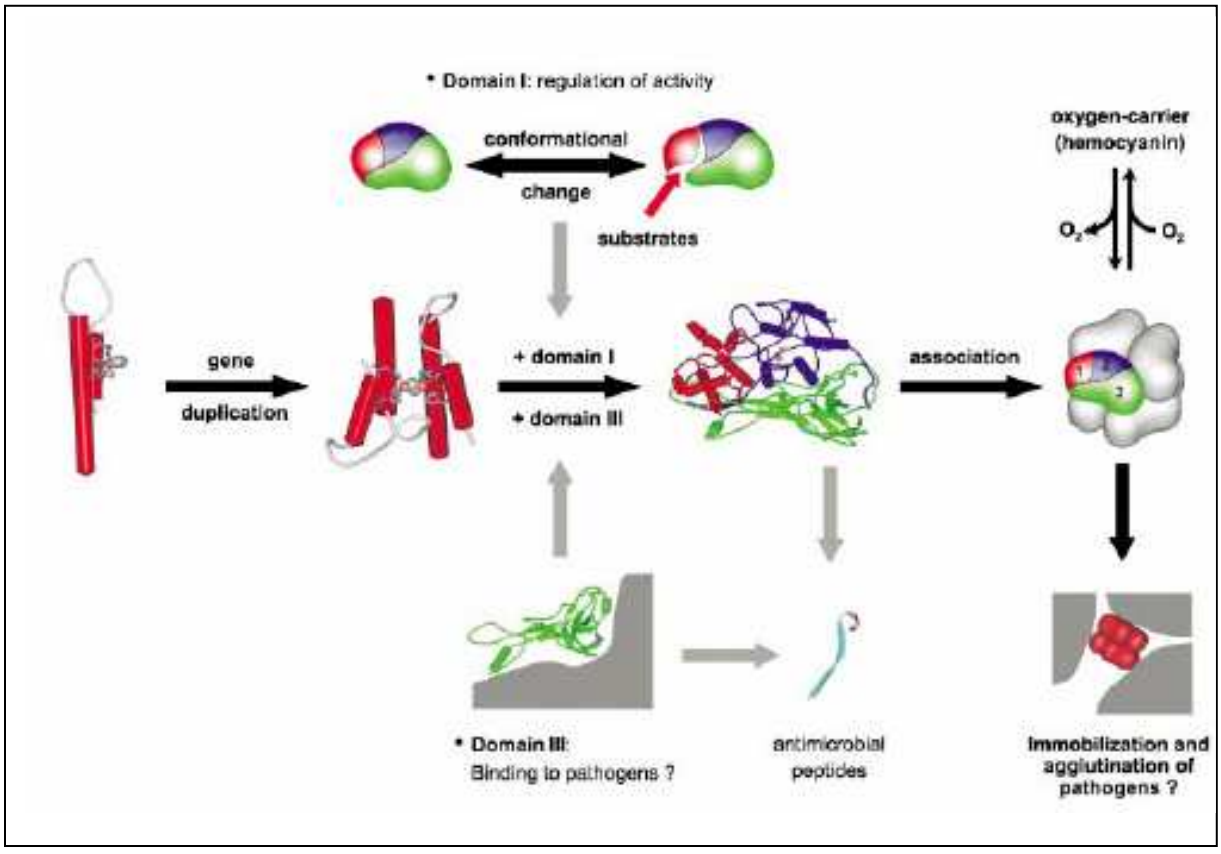

Figure 3.5.4. Structural evolution of the arthropod hemocyanin superfamily. The first type 3 copper proteins developed by gene duplication from mononuclear copper proteins which were already able to bind dioxygen. These minimal phenoloxidases detoxified oxygen but lacked regulation. They would have been comparable to domain II of present-day hemocyanin. Later domain I was added at the N-terminus by gene fusion. Domain I regulates activity by controlling access of bulky phenolic substrates to the active site. Another gene fusion at the C-terminus added domain III with an immunoglobulin folding motif which is a putative binding site to various surfaces and also the source of antimicrobial peptides in hemocyanins. Phenoloxidases aggregated to hexamers by self-assembly. This increased the concentration of active sites on pathogen surfaces and putatively enabled agglutination of pathogens. From this structure, cooperative oxygen carriers evolve (from Janicke and Decker, 2004).

This hypothesis arises by an interesting feature of the hemocyanin molecule of Chelicerates, which lacks phenoloxidases, suggesting that evolution has developed a double function for this molecule. In fact, recent studies about chelicerate hemocyanins suggest that hemocyanin acquires a phenoloxidase activity after proteolitic cleavage at the amino-terminal part (Decker and Rimbke, 1998, Decker and Tuczek, 2000). Hemocyanins of *Euryplma californicum*, *Limulus polyphemus* and *Tachypleus tridentatus*, are comparable to phenoloxidases based on activation mechanisms, substrate specificity and inhibition (Nagai and Kawabata, 2000; Nagai et al., 2001).

The role of hemocyanins in immune response seems to be not only present in chelicerates but also in crustaceans. Under normal conditions the hemocyanin functions as an oxygen carrier protein, but it may convert to phenoloxidases after microbial infections. In crustaceans, antimicrobial peptides can be cleaved from the C-terminal domain of hemocyanin. The specificity of the antimicrobial peptides seems to depend on the species. While peptides originating from hemocyanin of *Penaeus vannamei* and *P. stylirostris* (Destoumieux-Garzò et al., 2001) are antifungal, those from *Pacifastacus leniusculus* are antibacterial (Lee et al., 2003). Furthermore, cooperative properties of this protein could be made of a whole hemocyanin macromolecule, an antibacterial substance in the insect hemolymph. Binding of a subunit to a pathogen not only attaches the active site of one subunit on the pathogen, but it brings the five other active sites of the hexameric hemocyanin into the close vicinity of the pathogen, thus increasing enzymatic activity, analogous to the IgM-pentamer. When two subunits of a hexamer bind to different pathogen, then agglutination of pathogens will occur (Pan et al., 2008). Hemocyanin could play a role even in wound repair and molting as demonstrated in Chelicerata (Nagai et al. 2001; Paul et al., 1994).

Open questions

Hemocyanin seems to deserve the name of putative multifunctional protein. One may still ask whether the phenoloxidase activity of hemocyanins induced *in vitro* by matters such as lipophilic substances, detergents (SDS), proteases, alcohols, or salts, is an experimental artifact or if it can also be found *in vivo.* In the last case, it might be induced by physiological substances with the same charactheristic of the artificial ones, for example by lysolecithin, a lipophilic compound involved in wound repair (Janicke and Decker, 2004). However, *in vitro* or *in vivo* studies on alternative functions to respiration, have not been made in the Hexapoda yet.

Another outstanding issue concerns plasticity of hemocyanin with respect to an environmental context. The capacity of flexible oxygen transport of hemocyanin refers to the ability to form hexamers by self-assembly. Cooperative binding of oxygen to hemocyanins had to be established to ensure efficient and flexible oxygen transport together with ability to assume at least two conformational states (oxy and deoxy) (Decker and Jaenicke, 2004). The variability and plasticity of hemocyanin can be attributed to the existence of distinct subunit types, which contribute differently to the structural and physiological properties of the whole hemocyanin molecule. Different aggregation states are related to modified oxygen binding properties (Markl and Decker, 1992). As generally observed for respiratory proteins, a fundamental physiological property of hemocyanin is its competence to bind oxygen with different affinity in response to allosteric effectors. The oxygen concentration and the concentration of various hemocyanin allosteric factors as inorganic and organic ions, pH, sulphide, thiosulphate, neurohormones, lactate and carbon monoxide (Mangum, 1983; Richley et al., 1985; Morris, 1990; Burnett, 1992; Bridges, 2001), are related to the physiological response of the animals in the ecolological environment where they live. Changes in hemocyanin expression can affect the total concentration of hemocyanin in the hemolymph and/or can modify the level of expression of a single subunit with respect to the others. Experiments aimed to monitoring adaptive physiology of Plecoptera in response to environmental stimuli, at the level of protein expression modulation and subunit ratio, are in progress with quantitative real-time PCR (qRT-PCR). If oxygen affinity and cooperativity of hemocyanin, and consequently the capacity of oxygen-transport are adapted to environmental conditions, possessing hemocyanin represents, for animals, a potential adaptative capacity in global warming. In this futuristic context, the presence of hemocyanin, and its variability in subunits type and multimeric formation, may represent a focal aspect to be also analyzed from an ecological selection perspective (Schluter, 2001).

In any case, the aspects of the present research focused primarily on products of protein transcription and translation. The study of the hemocyanin gene could provide a better understanding even in species where currently hemocyanin seems to be absent.

Acknowledgement

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4. Work in progress

4.1 Immune-detection approach to for hemocyanin research in Plecoptera order

Introduction

Hemocyanins are copper binding protein able to combine a oxygen molecule. Their mainly function is to transport oxygen close to cells sustaining the production of ATP in mitochondria. Together with hemoglobins and hemerythrins, hemocyanins are the only respiratory proteins known in animal kingdom (Mangum, 1985). Although the first insect hemocyanin like (Hc-like) protein was identified in embryonic hemolymph of the grasshopper *Schistocerca americana* (Sánchez et al., 1986), its functionality and expression in later developmental stages remained uncertain, lacking demonstration of O_2 binding. Hemocyanin was known for many arthropod subphyla like Crustacea, Chelicerata, Onychophora and Myriapoda (Burmester, 1999a; van Holde et al., 2001; Kusche and Burmester, 2001b; Kusche et al., 2002), and only recently it was detected in the Plecoptera *Perla marginata* (Hagner-Holler et al., 2004). It was characterized as a multimeric protein (nx6), organized as dimers of trimers (Fochetti at al., 2006) of two different subunits (hc1 and hc2) of about 75 kDa each. This finding was a novelty respect to the accepted theory that gas exchanges in insect is mediated trough a highly branched tracheal system that enables diffusion of oxygen to the metabolically active tissue (Brusca and Brusca, 1990). *Perla marginata* hemocyanin gave a cue to a deeper research in respiratory proteins across insect orders (Pick et al., 2008; 2009a; 2009b), and especially in Plecoptera order, as regards our research group (Fochetti et al., 2006; Amore et al., 2009a, 2009b; Amore and Fochetti, 2009).

The degree of complexity of this study is increased by the presence of hexamerin proteins. These proteins are similar to the hemocyanins and belong to the same superfamily, but serve different functions (Burmester, 2002). Even if hexamerins basically share the same sequence motif, they do not bind oxygen because the copper-binding histidines residues of the active site are replaced by other amino acids with different biochemical properties (Beintema et al., 1994; Burmester, 2001).

Like arthropod hemocyanins, hexamerins usually consist of six identical or similar subunits, in the range of 75-85 kDa (Telfer and Kunkel, 1991; Hagner-Holler et al., 2007), and they can

accumulate in the hemolymph till up to high concentrations (Haunerland, 1996). Hexamerins are thought to serve mainly as storage proteins, which are used as a source of energy and amino acids during non-feeding periods, such as pupal and molting stages and adult development (Telfer and Kunkel, 1991; Burmester, 1999b).

From sequence analysis (total mRNA extraction, RT-PCR and cloning) we evidenced that hemocyanin is not homogenously present in all Plecoptera species (Amore et al., 2009a ; Amore and Fochetti, 2009; Amore et al., 2009 b). To verify the real translation of hemocyanin mRNA in protein, once sequenced conserved fragments in *Dinocras cephalotes*, we verified the expression of the protein in mass spectrometry (nano-RP-HPLC–ESI–MS) (Amore et al., 2009c). In this subsequent step of the study, we directly analyzed, with the immuno-detection technique, the presence of the protein in a sample of representative species of the two subfamilies of European Plecoptera fauna, during different moment of their life cycle (nymph and adult).

Materials and methods

Studied Species. 11 species of Plecoptera belonging to the two European superfamilies Perloidea and Nemouroidea were collected by Kick method (nymphs) and insect net (adults) and they was conserved liquid nitrogen, in the field and subsequently at -80 °C.

These are:

Nymphs

Perloidea: *Perla marginata* (Panzer, 1799); *Dinocras cephalotes* (Curtis, 1827); *Isoperla nevada* Aubert 1952; *Isoperla curtata* Navás, 1924; *Guadalgenus franzi* (Aubert, 1963).

Nemouroidea: *Rhabdiopteryx thienemanni* Illies, 1957; *Brachyptera vera* Berthélemy and González del Tanago, 1983, *Capnioneura mitis* Despax, 1932; *Leuctra* Stephens, 1836 *sp.*

Adults:

Nemouroidea*: Leuctra hippopus* Kempny, 1899; *Capnioneura gelesae* Berthélemy and Baena, 1984.

Protein biochemistry. Total proteins were extracted from nymphs and adults by homogenizing specimens that had been conserved at -80 °C using an antiproteasic cocktail (Radio-Immunoprecipitation Assay Buffer, and Protease inhibitor Cocktail SIGMA) and 10% SDS. Cell debris was removed by 30min centrifugation at 13,000 r.p.m. at 4ºC. The total protein concentration was determined according to the method of Bradford (1976) with the Quanti Pro BCA Assay Kit, SIGMA.

SDS-PAGE. Denaturing SDS/PAGE was performed on 10% polyacrylamide gels (Invitrogen) according to standard procedures. For Western Blotting, the proteins were transferred to a PVDF membrane (BIORAD). Non specific binding site were blocked 1h at room temperature by 5% non fat dry milk in Phosphatase-buffered saline/Tween 20. Incubation with anti-hc antiserum of *Limulus polyphemus* (Arthropoda, Chelicerata), Abcam 54132, diluted 1:400 in 5% nonfat dry milk in PBS/Tween (washing solution), was carried out overnight at 4 °C.

The filters were washed in Washing Buffer, incubated for 1 h with the anti-goat conjugated IgGs-HRP (Abcam 5755), 1:5000 with 5% non fat dry milk in Phosphatase-buffered saline/Tween 20. The membranes were washed in washing solution and detection was carried out with Supersignal West Dura Extended duration Substrate (Thermo Scientific). After electrophoresis, a gel was fixed in ethanol/acetic acid and stained with 0.1% Coomassie Brilliant Blue R-250 to test the transference efficiency (Fig. 4.1)

Result

Immuno-blotting results are apparently in disagreement with the sequence data derived from PCR products. In all samples of studied species we obtained the expected band of about 75 kDa, typical of hemocyanin or hemocyanin-like proteins (Fig. 4.2). Furthermore, to optimize the performance of the commercial antiboby anti-hc on *L. polyphemus*, we utilized a 1:400 dilution, in order to force its reactivity.

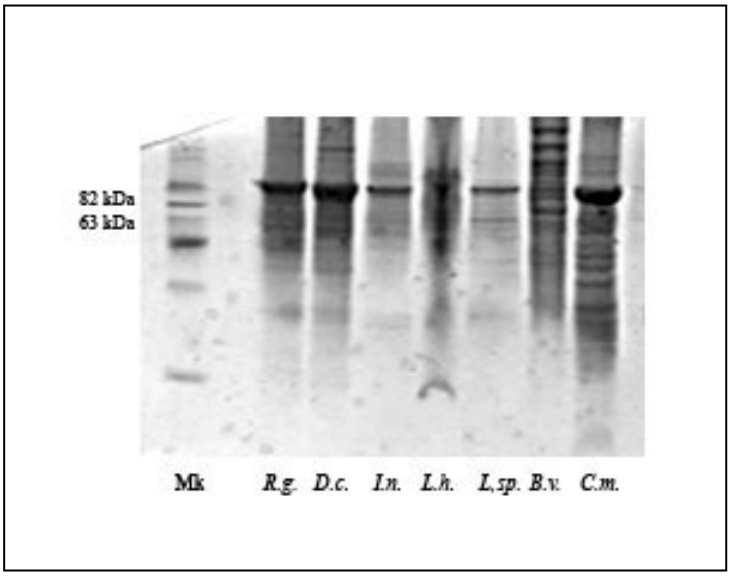

Figure 4.1.
Separation of total protein in SDS-P AGE (10%) and stained with Coomassie brilliant blue.
Mk: marker; *R.g.: Rhadiopteryx thienmanni; D.c.: Dinocras cephalotes; I.n.: Isoperla nevada; L.h.: Leuctra hippopus; L.sp.: Leuctra sp.; C.m.: Capnioneura mitis.*

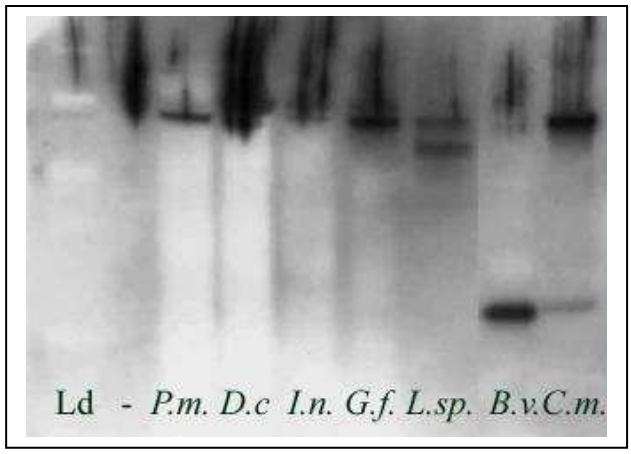

Figure 4.2.
Identification with Limulus anti-hc, The molecular mass standard is given on the left side (Ld), P.m.: Perla marginataM D.c.: Dinocras cephalothes; I,n.: Isoperla nevada; G.f.: Guadalgenus franzi; L. sp.: Leuctra sp.; B.v.: Brachyptera vera; C.m.: Capnioneura mitis.

Discussion

As already stated, immuno-blotting results are apparently in contrast with the sequence data derived from PCR. In fact, all analyzed species gave the expected band of about 75 kDa of the hemocyanin-like proteins. However, we suspect that the commercial antibody used, caused cross reactions with others similar proteins, i.e. hexamerins. Even if it is supposed that hexamerin is a group of protein common to many insect orders (Telfer and Kunkel, 1991; Beintema et al., 1994), Plecoptera, together with Zygenthoma (Pick et al., 2008) is the only insect order known to possess both types of hexameric proteins (Hagner-Holler et al., 2007). The relationship between insect hexamerins and hemocyanins is strong: both belong to the same superfamily of proteins (Burmester, 2001); they share more or less the same sequence motif, three-dimensional organization in hexamers (Burmester, 2002) and molecular weight of about 75-80 kDa. In *Perla grandis* hc1 mass is estimated of 77 kDa, and hc2 of 76.3 kDa, while hexamerins molecular weight is of about 75-80 kDa (Hanger-Holler et al., 2007). Hexamerins probably evolved from a hemocyanin subunit and consequently diverged, accumulating mutations, including aromatic residues and losing copper binding capacity (Burmester and Hankeln, 2007).

In many insect species hexamerins accumulate in the hemolymph at high concentrations (Haunerland, 1996), while we have reasons to suppose, from semi-quantitative PCR, that hemocyanin is expressed at very low concentrations.

Due to the possible presence of multiple epitopes in the commercial antibody (*L. polyphemus*) the presence of hemocyanin must be confirmed by using western blotting with a specific antibody. We believe that, due to cross-reactivity in insects, commercial antibodies do not provide any additional information. The production of a more informative polyclonal antibody is the goal to overcome the difficulty found in the present study.

Work in progress:

Material and methods

Specific Antibody. From the conclusion of the previous immuno-detection experiments, we headed for the production of a specific antibody. Our purpose was to generate a specific hemocyanin antibody able to screen the presence of both subunit types of hemocyanin (hc1 and hc2) to use across the whole Plecoptera order. In order to focus the epitope region we performed a multiple alignment of Plecoptera hemocyanin sequences deposited in EMBL/GeneBank and described as whole complete mRNA *(Perla marginata* hc1, hc2; AJ555403 AJ555404; *Perla grandis* hc1, hc2; DQ118369, DQ118370). We compared good alignment zones with complete mRNA hexamerins sequences (*Perla marginata* hx1, hx3, hx4 AM690365, AM690367, AM690368; *Taeniopteryx bursci* hx EF617598) to visualize the sequence difference between these two proximal classes of proteins. We selected the antibody epitope in the characteristic region of hemocyanins, where sequences only aligned with hemocyanins, and there was no identity with hexamerins.

This fragment (TRDPAFFRLHKYIDNLFK) covering the amino acid position 381-398, includes two phenylalanine and one histidine of the active site, in the domain 2 of the subunit. The polyclonal antibody is on process in rabbit (2 specimens) by AtibodyBcn (Barcelona).

Protein biochemistry and SDS-PAGE Total protein extraction and SDS-PAGE procedure were performed as described above only for *Perla marginata*, the stonefly species used as positive control. To optimize resolution for the protein of expected size, SDS-PAGE was performed on a 7.5% polyacrylamide gel. At present we proved the first, the second and the third bleedings of both rabbits in order to check the quality of immune response in rabbits. The three bleedings of two rabbits were tasted at different concentrations (1:500; 1:1000; 1:2000), with anti-rabbit conjugated IgG-HRP1:5000 (R1364HRP Acris antibodies). The detection were carried out with Supersignal West Dura Extended duration Substrate (Thermo Scientific).

Results

The presence of the hemocyanin in *P. marginata* hemolymph was established by Western blotting employing bleeding raised against a recombinant peptide that covers amino acids 381-398 of hc1 and hc2. Immune response was generated only in one rabbit bleeding. For these experiments the second bleeding, at a 1:1000 dilution, was the best. This bleeding recognizes four distinct bands in total protein extraction. A low and slight unspecific band (about 50 kDa), two band at expected size for hemocyanin hc1 and hc2 (about 75 kDa), and a

higher band at about 140 kDa, a multiple of molecular weight of the subunit are evidenced on the filter (Fig. 4.3).

Discussion

Bleedings are poorly purified products, they are just used to check the status of the work in progress. We suppose that in the final antibody the light band at 50 kDa should disappear. Reasonably, the two bands at about 75 kDa are the two hemocyanin subunits. The molecular masses of the hemocyanins are in the range of 75 kDa and thus are in agreement with those predicted from the translated cDNA sequences. The high band, at about 140 kDa, is just at a multiple molecular weight of the single subunit. It is possible that the antibody is conjugated with a percent fraction of dimers of hemocyanin subunits, where disulphide bridge were not broken, probably due to protein extraction procedures. Once purified this anti-hc antibody could be a direct tool to use in SDS-PAGE and Immune detection analysis, to verify the presence of hemocyanin in various species of stoneflies, or to test the production of this protein in different moment of biological cycle, or environment conditions.

It is known that the hemocyanin is freely dissolved in the hemolymph (Mangum, 1985), but it is our purpose to use the anti-hc antibody in immune histochemical analysis to identify body regions with different hemocyanin concentrations.

Finally, in the last months, research on hemocyanins in Hexapoda point out that hemocyanin is present in more taxa than thought before (Pick et al., 2009a; 2009b).

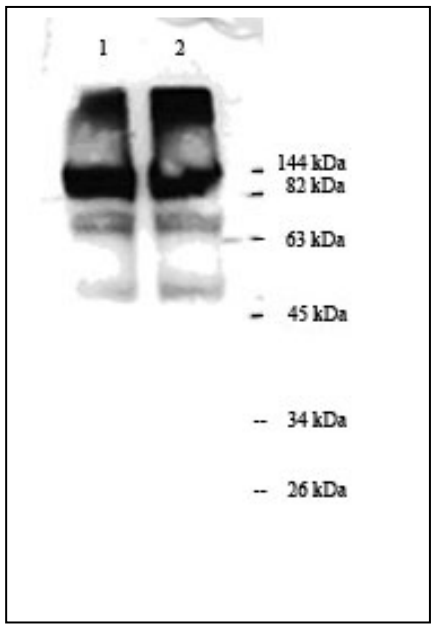

Figure 4.3: Identification in *Perla marginata* proteins (1th and 2nd well) detected with the 2nd bleeding for the productoin of an antibody raised against a recombinant hemocyanin peptide. On the right side, the position of standard molecular weight

A comparative analysis shows that the epitope chosen for the antibody, is a very conservative amino acid sequence not only in Plecoptera, but also in all known hemocyanin sequences known till now. These sequences belong to Collembola, Zygentoma, Phasmida, Blattodea, Isoptera. Starting from these bibliographic informations, the anti-hc antibody that we are producing, has the potentiality to be a good tool for a rapid screening about the presence of hemocyanin throughout species in many Hexapoda orders.

Acknowledgments

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4.2 Studies on stonefly (Plecoptera) hemocyanin under stress conditions

Plecoptera hemocyanin is a macro and multimeric molecule (nX6) composed of monomers of about 75 kDa. Two different subunits type of hemocyanin had been sequenced, even if subunits ratio is still unknown (Hagner-Holler et al., 2004; Fochetti et. al., 2006; Amore et al., 2009b). It is possible that the different contributions in terms of subunit ratio, concur to macromolecule changes and dynamicity in terms of oxygen affinity and cooperativity. In the hemolymph of *Portunus trituberculatus* (Crustacea, Malacostraca), hemocyanin 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).

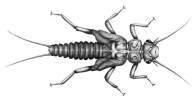
As studied for Crustacea (Giomi and Beltramini, 2007), the adaptive potentiality of the hemocyanin in the physiological response, can depend by several mechanisms, defines as extrinsic and intrinsics.

- Extrinsic mechanisms are considered the different external modulators, as the allosteric effectors, that affect the protein oxygen binding properties. These are: inorganic and organic ions, pH, sulphide, thiosulphate, and neurohormones, lactate, urate (Mangum, 1983; Morris, 1990; Burnett, 1992; Bridges, 2001; Hellmann et al., 2004).
- Intrinsic mechanisms concerning protein structure, such as changes in the ratio between different hemocyanin oligomers and phenotypic modifications through the regulation of expression levels for distinct subunits (Giomi and Beltramini, 2007).

The variability and plasticity of hemocyanin refers to the existence of distinct subunit types, which contribute differently to the structural and physiological properties of the whole hemocyanin molecule. Different aggregation states are related to modified oxygen binding properties (Markl and Decker, 1992). As generally observed for respiratory proteins, a fundamental physiological property of hemocyanin is its competence to bind oxygen with different affinity in response to allosteric effectors, including hydrogen ions. Furthermore, changes in hemocyanin expression can affect the total concentration of hemocyanin in the hemolymph and/or the level of expression of a single subunit with respect to the others.

At the present we are processing experiments with quantitative real-time PCR (qRT-PCR) to contribute to the understanding of the adaptive physiology of Plecoptera in response to environmental stimuli at level of protein expression, modulation and subunit ratio. We forced stonefly nymphs at stress condition of hypoxia, anoxia and toxicity. Our purpose is to compare levels of hemocyanin expressions in different conditions. The results of these

experimental work could supply new evidences to sustain the importance of hemocyanin as respiratory protein, or its hypothetical role in immune response.



5. Conclusions and perspectives

In this research, about the presence of hemocyanin in Plecoptera, we studied a total of 33 species, including nymphs and adults. We covered a representative sample of European stoneflies biodiversity and we tested some taxa of Oriental and Southern African fauna living in environments with different ecological features respect to those of the Palaearctic streams.

Considering the outcomes, we can conclude that not all stonefly nymphs have hemocyanin in their mRNA repertory in the hemolymph. We analyzed species with different ecological (seasonal vs perennial streams, mountain and tropical rivers and lakes) and autoecological adaptations (size, life cycle, trophic role), and we deduced that the patchy presence of hemocyanin across the Plecoptera order is not dependent on the size or on the life cycle. Environmental adaptations, independent loss of hemocyanin in some taxa (superfamily, family or genus level) and subsequent species radiation could lead to the actual complex framework.

In adults, we never found hemocyanin, even if the presence of hemocyanin in adult of *Perla grandis* and *P. marginata* is described in literature. We suggest that the physiological need of hemocyanin changes during the life cycle.

The irregular presence of hemocyanin in Plecoptera questions the functionality of the protein. On the base of an extensive literature regarding studies on Chelicerata and Crustacea, we hypothesize that the hemocyanin in Plecoptera could have not exclusively, or not primarily, a respiratory function. It is considered a possible role in the immune response of insect hemocyanin (antifungal or antimicrobial), in would repair and molting.

The study of the conserved region of hemocyanin (domain II) revealed that it works as a good phylogenetic molecular marker because, in phylogenetic analysis, the pattern of hemocyanin evolution follows the accepted scheme of traditional phylogeny based on morphology and anatomy. Hemocyanin conserved fragment could be used as a tool to solve nodes where phylogenetic relations between groups are still unclear on the morphological ground.

We verified the expression of the protein and we found that, as well as for *P. marginata*, hemocyanin is expressed in *Dinocras cephalotes* hemolymph too, suggesting that in Plecoptera species, where hemocyanin is detected as transcript mRNA sequence, this is really translated into protein to be ready for physiological demand of the animals.

In this study, we mainly focused on Arctoperlaria species. To widen the study to Antactoperlaria species should give a wider general overview to the all hemocyanin problematic investigation in Plecoptera. So far we have sequenced the most conserved fragment of hemocyanin subunits, the active site region in domain II. It could be interesting to extend the amplification to the whole subunit, with RACE-pcr technique, since domain I and III, presumably play an important function in the regulation and flexible plasticity of the protein on the one hand, and capability to generate an immune response on the other hand.

The specific antibody anti-hc, currently on production, will allow us a rapid screening of various species at different stages of life cycle with western blotting experiments. Even if it is known that hemocyanin is freely dissolved in the hemolymph, the use of the antibody in immune histochemistry analysis could detect different protein concentrations in special regions of the body.

Change in hemocyanin concentration can be directly related to environmental stimuli. Hemocyanin provides an adaptive potentiality toward alteration of ecological conditions and could be a successful tool during a process of colonization of diverse habitat. Experiments aimed to monitoring adaptive physiology of Plecoptera in response to environmental stimuli are in progress with quantitative real-time PCR (qRT-PCR). If oxygen affinity and cooperativity of hemocyanin, and so oxygen-transport capacity, is a tool for adaptation to the environmental conditions, hemocyanin means, for animals, a potential adaptative capacity in global warning. Hemocyanin may represent a pre-adaptative tool and this aspect could be also analyzed in view of ecological selection studies.

Finally, the present research dealt primarily with protein transcription and translation products. The study of hemocyanin gene could provide a better understanding even in species where currently hemocyanin is absent as transcript product.

6. Attachment

Cloned sequences:

Perlodidae:
Isoperla grammatica hc1. Accesion number: EU672885
bp 5'-
GTGGCCTACCTGGGTGAAGACGTCGGTCTTAACTCCCACCACTCCCACTGGCACA
TGGACTTCCCCTTCTGGTGGAAGGCAGCTGAATACGGCATCGAGAAGGACCGCA
AGGGAGAACTCTTCTACTATATGCATCATCAGATGATCGCCCGTTACGACCTTGA
GCGTCTGTCCAATCACCTCCCCTTCGTCGAGCCACTCTCTTTCGAGGAAGAAATCG
AGCACGGCTTCTATCCTCAGACCACTTACAGGGTTGGAGGAGAATTCCCCAGCAG
GCCCGACAACTTCGAGTTCCATGACCTTGAGCATATTAAGATCCAGGACATGCTT
GACTACACCAGAAGGATCAGAAACGCGATCCTTAAACACTCTGTGCTTACCAAGA
CCGGCGAGCACATCGCTCTGGACAACGAGCACGGTATCGACATCCTCGGAGATCT
GATGGAACCCTCGATGGAGACCCTCCACCACGACTACTACGGCTCTCTGCACAAC
TACGCCCACATTCTCCTCGGACAGATCACCGACCCCCTGGGCAAGTTTAACATGC
CCCCAGGAGTGATGGAACACTTTGAGACGCCACA-3'

aa
V A Y L G E D V G L N S H H S H W H M D F P F W W K A A E Y G I E K D R K G E L
F Y Y M H H Q M I A R Y D L E R L S N H L P F V E P L S F E E E I E H G F Y P Q T T
Y R V G G E F P S R P D N F E F H D L E H I K I Q D M L D Y T R R I R N A I L K H S
V L T K T G E H I A L D N E H G I D I L G D L M E P S M E T L H H D Y Y G S L H N
Y A H I L L G Q I T D P L G K F N M P P G V M E H F E T P

Isoperla grammatica hc2. Accession number: EU672886
bp 5'-
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CTCTCGAACAATCTTCCTCAGGTGGAGCCCCTAGACTGGCATCATGAGATTGTGG
AGGGCTTCGCCCCTGGAGCGATGTACCACAACGGCCAAGAGTTCCCCATGAGACC
CGATGGGATGTACTTCCACGACCTTCCCTGGCTGACAATCAAGGACAACGAGGAG
TTTGAGGGACGTATCAGGGACATCATCGCTTCTGGATTCGTCAAGATGACCGACG
GCCACCTCGTGTACCTGAACACGACCGAGGGCATCGACATTCTGGGTCTGATTGT
CGAGACCCTGGACCACAGCTACAACCGAGACTTCTTCGGCAAGTTCCACGCCAAC
TCCCACGTCATGCTCGGCAGAATCACCGACCCCATGCTCAAGTTCGGGATGCCCC
CAGGAGTGATGGAGCACTTCGAGACTGCCACA- 3'

aa
V A Y L G E D I G V N S H H A H W H M D F P F W W K R T Y D V T K D R R G E L F
F Y M H H Q M V S R F D A E R L S N N L P Q V E P L D W H H E I V E G F A P G A M
Y H N G Q E F P M R P D G M Y F H D L P W L T I K D N E E F E G R I R D I I A S G F
V K M T D G H L V Y L N T T E G I D I L G L I V E T L D H S Y N R D F F G K F H A N
S H V M L G R I T D P M L K F G M P P G V M E H F E T A T

Isoperla acicularis acicularis hc2. Accesion number GU121398
bp 5'-
AATTCGATTGTCGCTTATCTTGGCGAGGACATCGGCGTCAACTCCCACCACGCTC
ACTGACACATGGACTTCCCCTTCTGGTGGAAGAGAACCTATGACGTCACTAAGGA
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GCCGAGCGACTCTCGAACGATCTTCCTCAGGTGGAGCCCCTAGACTGGCACCATG
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CATGAGACCCGATGGGATGTACTTCCACGACCTTCCTTGGCTGACTACCAAGGAC
AACGAGGAGTTTGAGGGACGTATAAGGGACATCATCGCTTCTGGATTCGTCAAGA
TGACCGACGGCCACCTCGTGTACCTAAACACGACCGAGGGCATCGACATCCTGGG
TCTGATTGTCGAGACCCTGGACCACGGCTACAACCGAGACTTCTTCGGCAAGTTC
CACGCCAACTCCCATGTCGTGCTCAGCAGAATCACCGACCCCATGCTCAAGTTCG
GGATGCCTCCAGGAGTGATGGAACACTTCGAGACCGCTACAATCACTAG-3'

aa
I G V N S H H A H W H M D F P F W W K R T Y D V T K D R R G E L F F Y M H H Q
M V N R F D A E R L S N D L P Q V E P L D W H H E I V E G F A P G A I Y H N G Q E
F P M R P D G M Y F H D L P W L T I K D N E E F E G R I R D I I A S G F V K M T D G
H L V Y L NT T E G I D I L G L I V E T L D H G Y N R D F F G K F H A N S H V M L S
R I T D P M L K F G M P P G V M E H F E T

Guadalgenus franzi hc1. Accesion number: FJ393060
bp 5'-
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AGCACGGTTTCTACCCCCAGACTACCTACAGGGTTGGAGGAGAATTCCCCAGCAG
GCCCGACAATTTCGAGTTCCATGATCTTGATCACATTAAGATCAAGGACATGATT
GACTACACCAGGCGCATCAGAGAGGCCATCTTCAAGAAGTCTGTACTCACCAAG
AACGGTGACCACATTTCTCTGGACAACATGCACGGTATCGACATCCTCGGCGACC
TGATGGAACCTTCGATGGAGACCGTCCACCAAGACTACTACGGATCTCTGCACAA
CCACGCCCACATCTTACTGGGACAGATCACGGACCCCAAGGGAAGGTTCAACAT
GCCCCCTGGTGTGATGGAGCATTTCGAAACTGCTAC-3'

aa
V A Y L G E D V G L N S H H A H W H M D F P F W W K A A E Y G V E K F R K G E
L F Y Y M H H Q M I A R Y D L E R L S N H L P V V K P L S F E E E I E H G F Y P Q T
T Y R V G G E F P S R P D N F E F H D L D H I K I K D M I D Y T R R I R E A I F K K S
V L T K N G D H I S L D N M H G I D I L G D L M E P S M E T V H Q D Y Y G S L H N
H A H I L L G Q I T D P K G R F N M P P G V M E H F E T A

Perlodes intricatus hx. Accesion number: GU121397
bp 5'-
CCTGTCCGTCGCCACCCTCCAGCGTGAGGACTGCAAGGACTTCGTCCTCCCCCCC
GCGTACGAAGTCTACCCCCACCTTTTCCTCAACAACGAGGTCATCCAAAAGGCTT
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TCGACCAGACTTATTACATCCCCGCAAACTACTCTGGTGGGTACTACACTCAGTTC
CCCGAACAGTTCATTTCCTACTTCACCGAAGATGTTGGTCTCAACGCCTTCCACAC
CTACTGGAACTTGGACTATCCCTTCTGGGCCAACTCCAAGAACTACAACCTCAAG
TTCGACCGTCGTGGAGAGCTCTTCTACTACACCCAGCACCAGCTGATGGCCCGTT

ACTACCTTGAGCGTCTGTCTAACGGACTCGGAGAGGTCAAGGCCTTCTCTTACTTC
GACAGCGAGATTGTTGGATACCAGCCATCTCTGCGTTACCAGAACGGCAAGGAGT
TCCCCATGCGTCCCGAGGGAATGTCCGTCGTATACAACCGTCAGACCGAGGAACT
CTACACACTCGAGAGGAGGATCCAAGACGCAATCGACCTTGGATTCGTCTTCGGC
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GAGATGATCGAAGGTACCGCAGATTCCGCCAACAAGAACTTCTATGGCTCCGTCT
ACAACATGATGAAGACCGTCTTCGGCCACGTCGCCGATCCCACTTTCCAATTCCA
GGTTGCTCCCAGCGCACTTGAGCACTTCGAGACCGCACTCAGGGACCCCGCGTAC
TACACTCTGTACAAGCGCATCGACTCCCTCTTCAAGTCTTACAAGAACTTGATGCC
CGAGTACACCT-3’

aa
L S V A T L Q R E D C K D F V L P P A Y E V Y P H L F L N N E V I Q K A Y E V R M
Q G E H Y T A V D H V Y K V D Q T Y Y I P A N Y S G G Y Y T Q F P E Q F I S Y F T
E D V G L N A F H T Y W N L D Y P F W A N S K N Y N L K F D R R G E L F Y Y T Q
H Q L M A R Y Y L E R L S N G L G E V K A F S Y F D S E I V G Y Q P S L R Y Q N G
K E F P M R P E G M S V V Y N R Q T E E L Y T L E R R I Q D A I D L G F V F G K E G
Q K V S L K E K E G I S I L G E M I E G T A D S A N K N F Y G S V Y N M M K T V F
G H V A D P T F Q F Q V A P S A L E H F E T A L R D P A Y Y T L Y K R I D S L F K S
Y K N L M P E Y T

Dyctiogenus alpinum hc1. Accession number: GU121395
bp 5’-
GTGGCCTATTTGGGGGAGGATGTCGGCCTTAGCTCCCATCACGCCCACTGGCACA
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GAGCACGGCTTCTATCCTCAGACCACTTACAGGGTTGGAGGAGAGTTCCCCAGCA
GGCCAGACAACTTCGAGTTCCATGACCTCGAGCACATTAAGATCAAGGACATGAT
TGACTACACCAGGCGGATCAGAGAGGCCATCTTCAAGAAGTCTGTACTCACCAAG
ACTGGCGATCACATTGCCCTGGACAATATCCACGGTATCGACATCCTCGGGGATC
TTATGGAGCCCTCGGTAGAGACAGCTCACCACGATTACTACGGCTCACTTCACAA
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CCACCTGGTGTGATGGAACACTTTGAAACCGCCACA-3’

aa
V A Y L G E D V G L S S H H A H W H M D F P F W W K A T E Y G I E K D R K G E L
F Y Y M H H Q M I A R Y D L E R L S N H L P V V K P L N F E E K I E H G F Y P Q T T
Y R V G G E F P S R P D N F E F H D L E H I K I K D M I D Y T R R I R E A I F K K S V
L T K T G D H I A L D N I H G I D I L G D L M E P S V E T A H H D Y Y G S L H N Y A
H I L L G Q I T P K G K F N M P P G V M E H F E T A T

Dyctiogenus alpinum hc2. Accesion number: GU121396
bp 5’-
GTGGCCTATCTGGGTGAGGACATTGGCGTCAACTCGCACCACGCCCACTGGCACA
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CACCTGGTGTGATGGAACATTTCGAAACTGCCAC-3’

aa
V A Y L G E D I G V N S H H A H W H M D F P F W W K K T Y D I T K D R R G E L F
F Y M H H Q M V N R F D A E R L S N N L P Q V E P L D W H H E I T E G F A P G A M
Y Y N G K E F P M R P D G M H F H D L P W S T V K D N E E L E G R I R D T I A A G
F V K M T D G H L A Y L N T T E G I N I L G L I V E S L D H D Y S R V F F G K Y H A
N A H V M L S K I T D P M L K F G M P P G V M E H F E T A

Besdolus ravizzarum hc2. Accession number: GU121396
bp 5’-
GTGGCTTATCTGGGGGAAGACATTGGCGTCAACTCGCACCACGCCCACTGGCACA
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CGGACGGAATGCACTTCCACGATCTACCCTGGTCGACGGTCAAGGACAACGAGG
AACTAGAGGGTCGTATCAGAGACACCATCGCTGCTGGATTCGTCAAGATGAACG
ACGGCCACCTCGCCTACCTTAACACTACCGAGGGCATCAACATCCTGGGTCTCAT
AGTGGAGTCACTGGATCACGACTACAACCGAGTGTTCTTCGGAAAGTACCACGCC
AGCGCTCACACCATGCTGAGCAGGATCACCGACCCAATGCTCAAGTTCGGGATGC
CACCAGGTGTGATGGAACACTTCGAAACTGCCAC-3’

aa
V A Y L G E D I G V N S H H A H W H M D F P F W W K K T Y D V T K D R R G E L F
F Y M H H Q M V N R F D A E R L S N N L P Q V E P L D W H H E I T E G F A P G A M
Y Y N G Q E F P M R P D G M H F H D L P W S T V K D N E E L E G R I R D T I A A G
F V K M N D G H L A Y L N T T E G I N I L G L I V E S L D H D Y N R V F F G K Y H
A S A H T M L S R I T D P M L K F G M P P G V M E H F E T A

Arcynopteryx compacta hc2. Accession number: GU121393
bp 5’-
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CATGTTGGCTGTCCAGGGAAACATGCTCGCCGCTCTTAGTTAGGACGGACTTCTT
GAGGATAGACCCTCTAATACGCCGGGTGAAGTCAAGCATATCCTTGATTTTTATG
TCGTCAAGGTCATGGAATGCGAAGTGGTCAGGCCTGCTGGGGAATTCTCCTCCAA
TCCTGTAAGTAGTTTGGGGATAGAACCCATGCTCAATATCCTCGTCGAAATACAT
AGGTTTGACGAAGGGGAGGTGATTGGACAGTCGCTCCAGGTCGTAACGGGCGAT
CATTTGGTGGTGCATGTAGTAAAAGAGTTCTCCTTTACGGTCCTTCTCGACGCCGT
ATTCAGTCGCCTTCCACCAGAAGGGGAAGTCCATGTGCCAGTGAGCGTGATGAGA
GTTGAGTCCGACATCCTCACCCAAATAAGCGACA-3’

aa
V A Y F G E D I G V N S H H A H W H M D F P F W W K A T Y D V T K D R R G E L F
F Y M H H Q M T N R F D A E R L S N N L P Q V E P L D R Q N E I V E G F A P G A M
Y H N G Q E F P M R P D D M H F H D L P W L T V K E N E E F E G R I R D A I A A G

F V R M D D G H F A Y L N T T E G I N I L G L I V E S L D H E Y H H D F F G K Y H S
N T H V M L S R I T D P Q F K F G M P P G V M E H F E T A T

Perlidae

Dinocras cephalotes hc1. Accession number: FJ415315
bp 5'-
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ACGGCGAGCGAGTTCCCCTGGACGCCGTCCACGGAATCGACATTCTCGGCGACCT
GATTGAGCCATCCGTGGAGAGCGTGCATGAGAACTTCTACGGTTCACTGCACAAC
TACGCCCATATCATGCTGGGCAAAATCACCGACCCTCACGGCAAGTTCGATTTGC
CCCCAGGTGTGATGGAGCACTTTGAAACTGCCACGATGGAGCACTTTGAAACTGC
CAC-3'

aa
V A Y F G E D V G L N S H H A H W H M D F P F W W K A A E Y G I E K D R K G E L
F Y Y M H H Q M I A R Y D L E R L S A W L H F V E P L S F E D K I E H G F Y P Q T
T Y R V G G E F P A R P D N F Y F H D L E D I K I K D M L D Y T K R I R N A I Y K Q
G V L T K D G E R V P L D A V H G I D I L G D L I E P S V E S V H E N F Y G S L H N
Y A H I M L G K I T D P H G K F D L P P G V M E H F E T A

Dinocras cephalotes hc2. Accession number: EF218621
bp5'-
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CACCACTGAAGGCATCGACATCCTGGGTCTTATTGTCGAGTCCTTGGATCACGAC
TACAACCGCCACTACTTCGGCAAATTCCACTCCAACGCTCACGTCCTGCTCTCTAA
GATTACCGACCCTGAGCAGAAGTTTGGAATGCCTCCAGGTGTGAT-3'

aa
V A Y F G E D V G L N S H H S H W H M D F P F W W K K S Y D V T K D R R G E L F
F Y M H H Q M V N R F D A E R L S N W L P Q V E P L G W H H E I E E G F A P A A
M Y F N G Q E F P M R P D G M H F H D M P W F T V K D T E D Y E D R I R D V I A
K G Y V K T N D F H K V Y L N T T E G I D I L G L I V E S L D H D Y N R H Y F G K F
H S N A H V L L S K I T D P E Q K F G M P P G V M E H F E T A

Caroperla sp. hx. Accession number: GU121400
bp 5'-
CCCTCACCCAAGTAAGCGACAGTGTGGCGGTCTCGAAGTGTTCCACACCTACTGG
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GGAACGTTTGTCCAACGGACTCGGAGAAGTCAAGCCCTTCTCTTACTTCACCCAC
GAGACCAACATCGAAGGGTTCGAGCCCAGCCTCCGCTACCAGAACGGCAAGGAG
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GAGCTGATCGAAGGCACCGGCGATTCTGCCAACAAGAACTTCTACTGCTCTCTCT
ACAACATCATCAGGACCGTCTTTGGACACATCACCGACCCCACCTACCAGCACAC
CATTGCCCCCACTGCTCTGGAACACTTGAAACCGCTAC-3’

aa
P H P S K R Q C G G L E V F H T Y W N F D Y P F W A E S K H Y N L K F D R R G A L
F Y Y T Q H Q L M A R Y Y L E R L S N G L G E V K P F S Y F T H E T N I E G F E P S
L R Y Q N G K E F P M R P E G L S F V D S Y K T E E V I V L E R L I R D A T D L G F
V V G K E G Q K I S L K D K E G I T L L G E L I E G T G D S A N K N F Y C S L Y N I I
R T V F G H I T D P T Y Q H T I A P T A L E H L K P L

Tetropina sp. hx. Accession number: GU121388
bp 5’-
GTAGCGGTTTCAAAGTGCTCCACACCTACTGGAACTTTGACTATCCCTTCTGGGCC
GAATCCAAGCACTACAACCTCAAGTTCGACCGCCGCGGAGAGCTGTTCTACTACA
CTCAGCACCAGCTGATGGCACGCTACTACCTGGAACGTTTGTCCAACGGACTCGG
AGAAGTCAAGCCCTTCTCTTACTTCACCTACAAGTCAAAGATTGAAGGTTTCGAG
CCCTCCCTCCGCTACCAGAACGGCAAGGAGTTCCCCATGCGTCCTGAAGGAGCTT
CCTTCGTCGACTCTCTCAAGACCGAGGACCTGATCGTCTTCGAGAGGAGGATCCA
CGACGCCATCGACCTTGGATTCGTCTCCGGTAAGGAGGGCCAGAAGATTTCCTTG
AAGGAGAAGGAAGGTATCGCCATCCTGGGAGAAATGATCGAAGGCACCGGAGAT
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TTCGAAACCGCTACA-3’

aa
S G F K V L H T Y W N F D Y P F W A E S K H Y N L K F D R R G E L F Y Y T Q H Q L
M A R Y Y L E R L S N G L G E V K P F S Y F T Y K S K I E G F E P S L R Y Q N G K E
F P M R P E G A S F V D S L K T E D L I V F E R R I H D A I D L G F V S G K E G Q K I
S L K E K E G I A I L G E M I E G T G D S V N K N F Y G S L Y N L I K T V F G H V T
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Togoperla sp. hx. Accesion number: GU121389
bp 5’-
GTGGCGGTTTCAAAGTGCTCCACACCTACTGGAACTTTGACTATCCCTTCTGGGCC
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GGAAGTCAAGCCCTTCTCTTACTTCACCCACAAGACCAACATCGAAGGGTTCGAG
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TCCGTCAACAAGAACTTCTATGGTTCCCTCTACAACCTGATCAAGACCGTCTTCGG
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TTTCGAAACTGCCACA-3’

aa
G G F K V L H T Y W N F D Y P F W A E S K H Y N L K F D R R G E L F Y Y T Q H Q
L M A R Y Y L E R L S N G L G E V K P F S Y F T H K T N I E G F E P S L R Y Q N G K
E F P M R P E G A S F V D S L K T E D L I V F E R R I H D A I D L G F V F G K E G Q
K I S L K E K E G I A I L G E M I E G T G D S V N K N F Y G S L Y N L I K T V F G H
V T D V T Y Q H T V A P Q C S G A F R N C H

Neoperla sp. hx. Accesion number: GU121390
bp 5'-
GTGGCGGTCTCAAAGTGCTCCACACCTACTGGAACTTTGACTATCCCTTCTGGGCC
GAATCCAAGCACTACAACCTCAAGTTCGACCGCCGCGGAGAGCTGTTCTACTACA
CTCAGCACCAGCTGATGGCACGCTACTACCTGGAACGTTTGTCCAACGGACTCGG
AGAAGTCAAGCCCTTCTCTTACTTCACCTACAAGTCAAAGATTGAAGGTTTCGAG
CCCTCCCTCCGCTACCAGAACGGCAAGGAGTTCCCCATGCGTCCTGAAGGAGCTT
CCTTCGTCGACTCTCTCAAGACCGAGGACCTGATCGTCTTCGAGAGGAGGATCCA
CGACGCCATCGACCTTGGATTCGTCTTCGGTAAGGAGGGCCAGAAGATTTCCTTG
AAGGAGAAGGAAGGTATCGCCATCCTGGGAGAAATGATCGAAGGCACCGGAGAT
TCCGTCAACAAGAACTTCTATGGTTCCCTCTACAACCTGATCAAGACCGTCTTCGG
CCACGTAACTGACGTCACCTACCAGCACACCGTTGCCCCCAGTGCTCTGGAACAC
TTTGAGACCGCTACA-3'

aa
G G L K V L H T Y W N F D Y P F W A E S K H Y N L K F D R R G E L F Y Y T Q H Q
L M A R Y Y L E R L S N G L G E V K P F S Y F T Y K S K I E G F E P S L R Y Q N G K
E F P M R P E G A S F V D S L K T E D L I V F E R R I H D A I D L G F V F G K E G Q
K I S L K E K E G I A I L G E M I E G T G D S V N K N F Y G S L Y N L I K T V F G H
V T D V T Y Q H T V A P S AL E H F E T A T

Etrocorema sp. hx. Accession number: GU121391
bp 5'-
GTGGCGGTTTCAAAGTGTTCCACACCTACTGGAACTTTGACTATCCCTTCTGGGCC
GAATCCAAGCACTACAACCTCAAGTTCGACCGCCGCGGAGAGCTGTTCTACTACA
CTCAGCACCAGCTGATGGCACGCTACTACCTGGAACGTTTGTCCAACGGACTCGG
AGAAGTCAAGCCCTTCTCTTACTTCACCCACAAGACCAACATCGAAGGGTTCGAG
CCCAGCCTCCGCTACCAGAACGGCAAGGAGTTCCCGATGCGCCCCGAAGGTCTCT
CTTTCGTTGACTCTTACAAGACCGAGGAGGTCATAGTTTTGGAGAGGAGAATCCG
TGACGCTATTGACCTTGGGTTCGTTGTTGGCAAGGAAGGCCAGAAGATCTCCCTG
AAGGACAAGGAAGGAATCACCCTCCTTGGGGAGCTGATCGAAGGCACCGGCGAT
TCTGCCAACAAGAACTTCTACTGCTCTCTCTACAACATCATCAGGACCGTCTTTGG
ACACATCACCGACCCCACCTACCAGCACACCATTGCCCCCACTGCTCTGGAGCAT
TTCGAGACTGCCACA-3'

aa
G G F K V F H T Y W N F D Y P F W A E S K H Y N L K F D R R G E L F Y Y T Q H Q
L M A R Y Y L E R L S N G L G E V K P F S Y F T H K T N I E G F E P S L R Y Q N G K
E F P M R P E G L S F V D S Y K T E E V I V L E R R I R D A I D L G F V V G K E G Q
K I S L K D K E G I T L L G E L I E G T G D S A N K N F Y C S L Y N I I R T V F G H I
T D P T Y Q H T I A P T A L E H F E T A T

Phanoperla sp. hx. Accesion number: GU121392
bp 5'-
GTGGCAGTTTCGAAATGTTCCACACCTACTGGAACTTTGACTATCCCTTCTGGGCC
GAATCCAAGCACTACAACCTCAAGTTCGACCGCCGCGGAGAGCTGTTCTACTACA
CTCAGCACCAGCTGATGGCTCGTTACTACCTGGAACGTTTGTCCAACGGACTCGG
AGAAGTCAAGCCCTTCTCTTACTTCACCTACAAGTCAAAGATTGAAGGTTTCGAG
CCCTCCCTCCGCTATCAGAACGGCAAGGAGTTCCCCATGCGTCCTGAAGGAGCTT
CCTTCGTCGACTCTCTCAAGACCGAGGATCTGATCGTCTTCGAGAGGAGGATCCA
CGACGCCATCGACCTTGGATTCGTCTTCGGTAAGGAGGGCCAGAAGATTTCCTTG
AAGGAGAAGGAAGGTATCGCCATCCTGGGAGAAATGATCGAAGGCACCGGAGAT
TCCGTCAACAAGAACTTCTATGGTTCCCTCTACAACCTGATCAAGACCGTCTTCGG
CCACGTAACTGACGTCACCTACCAGCACACCGTTGCCCCCAGTGCTCTGGAACAT
TTCGAAACCGCCACA-3'

aa
G S F E M F H T Y W N F D Y P F W A E S K H Y N L K F D R R G E L F Y Y T Q H Q L
M A R Y Y L E R L S N G L G E V K P F S Y F T Y K S K I E G F E P S L R Y Q N G K E
F P M R P E G A S F V D S L K T E D L I V F E R R I H D A I D L G F V F G K E G Q K I
S L K E K E G I A I L G E M I E G T G D S V N K N F Y G S L Y N L I K T V F G H V T
D V T Y Q H T V A P S A L E H F E T A T

Chloroperlidae
Siphonoperla torrentium hx. Accession number: EU6772887
bp 5'-
ATGTTGGTGTCAACACCTTCAACACCTACTGGCACTTGGACTACCCCTTCTGGATG
AACTCTGCTAAATACAACATGCATTTCGACCGTCGTGGAGAGCTCTTCTACTACA
CCCAGCACCAGCTGTTGGCCCGTTACTACCTTGAGCGTATTTCCAACGGACTTGG
AGAGATCAAGCACTTTGACTGGAGCGATAGGAAGACATTGATGGTTGGATATGA
ACCTATGATGCGTTACCAAAACGGACAGGAATTCACCATGCGCCCCGAGGGATCT
ACTTTCTCCAGGAACTACAGGTCCGAGGATGCTATGATCTTCGAGAGGAGAATCG
TTGATGCTATTGATGCTGGATACATTGTTTCCTTTGACGGACAGAAGCTGTCTCTT
AAGGACAAGGAAGGTATCACCCTCCTCGGAGAGTTGATCATGTCTACCGGTGATT
CCCCCAACAAGGAATTCTACGGCAAGATCTACACCAACTTGTGCACCATCTTCGG
ACACGTTATGGACCACACCTTCGCCTTCGATACCGTTCCCAGCGTGCTG-3'

aa
V A Y F G E D V G V N T F N T Y W H L D Y P F W M N S A K Y N M H F D R R G E L
F Y Y T Q H Q L L A R Y Y L E R I S N G L G E I K H F D W S D R K T L M V G Y E P
M M R Y Q N G Q E F T M R P E G S T F S R N Y R S E D A M I F E R R I V D A I D A
G Y I V S F D G Q K L S L K D K E G I T L L G E L I M S T G D S P N K E F Y G K I Y T
N L C T I F G H V M D H T F A F D T V P S V L E H S K P P

Peltoperlidae
Cryptoperla sp. hx. Accession number: GU121387
GTGGCGGTTTCAAAGTGTTCCACACCTACTGGAACTTTGACTATCCCTTCTGGGCC
GAATCCAAGCACTACAACCTCAAGTTCGACCGCCGCGGAGAGCTGTTCTACTACA
CTCAGCACCAGCTGATGGCTCGTTACTACCTGGAACGTTTGTCCAACGGACTCGG
AGAAGTCAAGCCCTTCTCTTACTTCACCTACAAGTCAAAGATTGAAGGTTTCGAG

CCCTCCCTCCGCTACCAGAACGGCAAGGAGTTCCCCATGCGTCCTGAAGGAGCTT
CCTTCGTCGACTCTCTCAAGACCGAGGATCTGATCGTCTTCGAGAGGAGGATCCA
CGACGCCATCGACCTTGGATTCGTCTTCGGTAAGGAGGGCCAGAAGATTTCCTTG
AAGGAGAAGGAAGGTATCGCCATCCTTGGAGAAATGATCGAGGGCACCGGAGAT
TCCGTCAACAAGAACTTCTATGGTTCCCTCTACAACCTGATCAAGACCGTCTTCGG
CCACGTAACTGACGTCACCTACCAGCACACCGTTGCCCCCAGTGCTCTGGAACAT
TTCGAAACCGCTACA-3’

aa
G G F K V F H T Y W N F D Y P F W A E S K H Y N L K F D R R G E L F Y Y T Q H Q
L MA R Y Y L E R L S N G L G E V K P F S Y F T Y K S K I E G F E P S L R Y Q N G K
E F P M R P E G A S F V D S L K T E D L I V F E R R I H D A I D L G F V F G K E G Q
K I S L K E K E G I A I L G E M I E G T G D S V N K N F Y G S L Y N L I K T V F G H
V T D V T Y Q H T V A P S A L E H F E T A T

Taeniopterygidae
Taeniopteryx stankovitchi hx. Accession number: EF218622
bp 5’-
GATGTCGGTCTTAACGCCTTCCACACCTACTGGAACATGGACTATCCCTTCTGGGC
CAACTCCAAATACTACAACCTCAAGTTCGACCGACGCGGAGAGCTGTTCTACTAC
ACCCAGCACCAGCTGATGGCCCGTTACTACTTGGAGCGTCTGTCCAACGGACTCG
GAGAAGTCAAGCCTTTCTCTTACTCATACAAGACCCCCATTGCCGGATTCGAGCC
ATCTCTTCGCTACCAGAACGGAAAGGAATTCCCGATGCGCCCCGAATTCGCCAGA
TTCTCCAACAGTTACAAGACCGAAGAGGCCCTCGCTTTCGAGAGGAGGATTTACG
ATGCTATCGATCTTGGATACGTCATCAACAAGGAAGGAGCTAAGATTTCCCTGAG
GGAGAAGGATGGTATCAGTCTTCTCGGAGAGATTATTGAAGGAAGCTGGGACTCT
ACCAACAAGGACTTCTACGGAGCCCTCTACAACATCATGAGGACCATCTTCGGTC
ACGTCACCGACCCTACCTACCAATACGGCGTTGCCCCCGGTGTGTT-3’

aa
V A Y L G E D V G L N A F H T Y W N M D Y P F W A N S K Y Y N L K F D R R G E L
F Y Y T Q H Q L M A R Y Y L E R L S N G L G E V K P F S Y S Y K T P I A G F E P S L
R Y Q N G K E F P M R P E F A R F S N S Y K T E E A L A F E R R I Y D A I D L G Y V
I N K E G A K I S L R E K D G I S L L G E I I E G S W D S T N K D F Y G A L Y N I M R
T I F G H V T D P T Y Q Y G V A P G V L E H F E T A

Brachyptera risi hx. Accession number: EU6772888
bp 5’-
GTAGCGGTTTCAAAGTGCTCCCTCCCCGAGATAAGCGACAGTGTGGCAGTTTCGA
AATGCTCCCTCTCCCAGGTAAGCGACAGTGTTGCAGTTTCGAAGTGCTCCTTCCCC
CAAGTAGGCGACAATGTGGCTGTTTGAAATGTTCCTCCCCCAGGTAAGCGACAGT
GTAGCGGTCTCAAAGTGCTCCTTCACCCAGATAAGCGACAGTGTGGCAGTTTCAA
AGTGTTCCCCCCCCCTTCCCCAAAATAAGCGACAGCGTTGCGGTTTCGAAGTGCT
CCCTCTCCAAGATAAGCCACAGTGTAGCGGTTTCGAAGTGTTCCCTCCCCCAGGT
AAGCCACAGTGTGGCAGTTTCAAAGTGTTCCTTCCCCCAAGTAAGCGACAGTGTG
GCGGTTTCGAAGTGTTCCCCCCCTCGCCCAGTAAGCGACAGTGTGGCAGTTTCGA
AATGCTCCCTCTCCCAGGTAAGCGACA-3’

aa
V A Y F G E D V G L N A F H T Y W N M D Y P F W A N S K Y Y N L K F D R R G E L
F Y Y T Q H Q L M A R Y Y L E R L S N G L G E V K P F S Y S Y K T I A G F E P S L R
Y Q N G K E F P M R P E F A R F S N S Y K T E E A L A F E R R I Y D A I D L G Y V I
N K E G A K I S L R E K D G I S L L G E I I E G S W D S T N K D F Y G A L Y N I M R
T I F G H V T D P T Y Q Y G V A P C V G T F R N R H

Nemouridae
Nemoura hesperiae hx. Acccession number: EU6772889
bp 5'-
CAGACCTACAACCTCAAGTTCGACCGACGTGGTGAGCTCTTCTACTACACCCAGT
CGCAGCTGATGGCCCGCTACTACCTTGAGCGCCTGTCCAACGGACTTGGAGAGGT
CAAGCCCTACTCGTACTCCTTCAAGACTCCCATTGCTGGCTTCGAGTCGTCCCTGC
GTTACCAGAGCGGCAAGGAATTCCCCAGCCGTCCCGAAGGAATCAAGTTCTTCGC
CAACTACTTCACCGAGAAGGCTCTGTCTCTGGAATCCAGAATCTTGAACGCCATC
GACATTGGATTCGTCTGGACCAAGGACGGACAGAAGTACGCTCTTAAGGACAAG
GAAGGCATCAATCTTCTGGGAGAGATGATCAGCGGAGTCAGCGACTCAGTGAAC
AAGGACTTCTACGGAAACCTGTACAACCTGATGAGGACGATCTTCGGCCACGTTA
CCGACCCCAACTTCAAATACGGAGTTGCCCCCGGCGTGATGGAACATTTCGAAAC
T-3'

aa
Q T Y N L K F D R R G E L F Y Y T Q S Q L M A R Y Y L E R L S N G L G E V K P Y S
Y S F K T P I A G F E S S L R Y Q S G K E F P S R P E G I K F F A N Y F T E K A L S L
E S R I L N A I D I G F V W T K D G Q K Y A L K D K E G I N L L G E M I S G V S D S
V N K D F Y G N L Y N L M R T I F G H V T D P N F K Y G V A P G V M E H F E T

Amphinemura sulcicollis hx. Accession number: EU715327
bp 5'-
CAGACCTACAACCTCAAGTTCGACCGACGTGGTGAGCTCTTCTACTACACCCAGT
CGCAGCTGATGGCCCGCTACTACCTTGAGCGCCTGTCCAACGGACTTGGAGAGGT
CAAGCCCTACTCTTACTCCTTCAAGAACGCCATCTCTGGCTTTGAATCATCCCTGC
GTTACCAGAGCGGCAAGGAATTCCCCAGCCGTCCCGAGGGAGTCAAGTTCTTCAA
CAACTACTACACCGAGAAAGCACTGTCCCTGGAATCCAGAATCCTGAACGCTATC
GACATCGGATTTGTCTGGACCAAGGACGGACAGAAATACGCTCTTAAGGACAAG
GAAGGCATCAACCTTCTGGGAGAGATGATCAGCGGAGTCAGCGACTCTGTCAAC
AAGGACTTCTACGGAAACCTGTACAACCTGATGAGATCGATCTTCGGCCATGTCA
CCGACCCCAACTTCAAATACGGAGTCGCCCCCGGCGTCATGGAACATTTTGAAAC
-3'

aa
Q T Y N L K F D R R G E L F Y Y T Q S Q L M A R Y Y L E R L S N G L G E V K P Y S
Y S F K N A I S G F E S S L R Y Q S G K E F P S R P E G V K F F N N Y Y T E K A L S
L E S R I L N A I D I G F V W T K D G Q K Y A L K D K E G I N L L G E M I S G V S D
S V N K D F Y G N L Y N L M R S I F G H V T D P N F K Y G V A P G V M E H F E

Protonemura ausonia hx. Accession number: EU6772890
bp 5'-
GTGGCCTATCTCGGTGAGGATGTCGGTGTCAACGCTTTCCACACCTACTGGAACA

TGGACTACCCCTTCTGGGCCAACTCCAAGACCTACAACCTCAAGTTCGACCGACG
TGGAGAGCTCTTCTACTACACCCAGTCGCAGCTGATGGCCCGTTACTACCTTGAG
CGCCTGTCCAACGGACTTGGAGAGGTCAAGCCTTACTCGTACTCGTTCAAGAACG
CCATCTCTGGATTCGAGTCTTCCCTGCGTTACCAGAGCGGCAAGGAGTTCCCCAG
CCGTCCCGAGGGTGTCAAGTTCTTCAACAACTACTACACCGAGAAGGCTCTCTCT
CTTGAATCCAGAATCTTGAACGCCATCGACATTGGATTCATCTGGACCAAGGACG
GACAGAAGTTCGCTCTTAAGGATAAGGAAGGCATCAACCTTCTGGGAGAGATGA
TCAGTGGAGTCAACGACTCTGTATGCATGGACTACTACTGAAACCTTTACAACCT
GACGAGGAAAATCTCCGACCACCTTACCGACCCCAACGTTAGGGGGGGAGTCAC
ACATGGTTTGATGGAACCTTTTGAAACCGCGTA-3’

aa
V A Y L G E D V G V N A F H T Y W N M D Y P F W A N S K T Y N L K F D R R G E L
F Y Y T Q S Q L M A R Y Y L E R L S N G L G E V K P Y S Y S F K N A I S G F E S S L
R Y Q S G K E F P S R P E G V K F F N N Y Y T E K A L S L E S R I L N A I D I G F I W
T K D G Q K F A L K D K E G I N L L G E M I S G V N D S V C M D Y Y

Capniidae
Capnia bifrons hx. Accession number: FJ384672
bp 5’-
CATACAACATGAAATTTGACCGACGTGGAGAACTCTTCTACTACACCCAGAGCCA
GCTGTTGGCCCGTTATACCCTTGAGCGTCTGTCCAACGGACTTGGAGAGGTCAAG
CCTTTCTCTTACGCCTACAAGACACCCGTTGCCGGATTCGAGCCATCTCTGCGTTA
CCAGAACGGTAAGGAGTTCCCCATGCGTCCCGAAGGTTCCAAGTTCTTCAAGAGC
TTCAAGACCGAAGTTGCCCTTGCCTACGAGAGGAGGATCTACGACGCTATTGATC
TTGGATTCGTGTGGACCAAGGAAGGCCAGAAGGTTGCATTGAAGGAGAAGGAAG
GTATCAACATTCTGGGAGAGATGATCGAGGGAAGCTACGACTCTGTCAACAAGC
AGTTCTACGGAACACTCTACAACATCATGAGGACGATCTTCGGACACGTCACTGA
CCCCACCTTCCAATACGGTGTTGCCCCCGGTGTTTTGGAACATTTCGAAACTGC-3’

aa
Y N M K F D R R G E L F Y Y T Q S Q L L A R Y T L E R L S N G L G E V K P F S Y A
Y K T P V A G F E P S L R Y Q N G K E F P M R P E G S K F F K S F K T E V A L A Y E
R R I Y D A I D L G F V W T K E G Q K V A L K E K E G I N I L G E M I E G S Y D S V
N K Q F Y G T L Y N I M R T I F G H V T D P T F Q Y G V A P G V L E H F E T

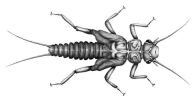
Leuctridae
Leuctra fusca hx. Accession number: EF218620
bp 5’-
ACGTCGGTCTGAGCACCTTCCACACCTACTGGAACATGGACTATCCCTTCTGGGC
CAACGCCAAATACTACAACCTGAAGTTCGACCGCGATGGAGAGCTCTTCTACTAC
ACTCAGGATCAGATCCTGGCCCGCTACTACCTGGAACGTCTGTCTAACGGACTTG
GAGAGATCAAGCCGTTCTCATACACTTTCAAGACACCCATTTCTGGATTCGAGCC
ATCGCTCCGTTACCAGAACGGCAAGGAGTTCCCCATGCGCCCAGAGGGTGTCCGA
TTCTCCAACAACTACAAGAGCGAGGAAGCCTACGCCTACGAGAGGAGGATCTTC
GATGCTATTGACCTTGGCTTCGTCATTTCCAAAGACGGATCAAAGATTTCTCTGAA
TGAGAAGGAAGGCATCAACATTCTCGGCGAGCTGATCAAGGGAACCACAGACAC

CGTCAACGAGTACTTCTACGGCACCATCTACAACATGATGCGAGGAATCTTCGGC
CACGTGACCGATCCCAACTTCCAATATGGAGTTGCCCCCGGTGTTTT-3’

aa
V A Y F G E D V G L S T F H T Y W N M D Y P F W A N A K Y Y N L K F D R D G E L
F Y Y T Q D Q I L A R Y Y L E R L S N G L G E I K P F S Y T F K T P I S G F E P S L R
Y Q N G K E F P M R P E G V R F S N N Y K S E E A Y A Y E R R I F D A I D L G F V I
S K D G S K I S L N E K E G I N I L G E L I K G T T D T V N E Y F Y G T I Y N M M R
G I F G H V T D P N F Q Y G V A P G V L E H F E T A

Pachyleuctra bellonchi hx. Accession number: GU121399
bp 5’-
GTGGCTTATCTGGGCGAGGACGTCGGTTTGAGCACCTTCCACACCTACTGGAACA
TGGACTATCCCTTCTGGGCCAACCACAAAACTTATGGCATCAAGTGGGATCGAAC
TGGAGAGCTTTTCTACTACACGCAGCACCAGATCTTGGCTCGCTACTACCTGGAG
CGTCTGTCTAACGGACTTGGAGAGATCAAGCCTTTCTCATACTCTTACAAGACAC
CCATTTCTGGATTCGAGCCATCTCTGCGTTACCAGAACGGCAAGGAGTTCCCCAT
GCGACCCGAGGGCGTCCGATTCTCCAACAGCTACAAGACCGAGGAAGCTCTGGC
TTACGAGAGGAGGATCTTTGATGCCATCGATCTTGGCTTCGTTGTTTCCAAAGAC
GGAACAAAGATTCCTCTGAAGGAGAAGGAAGGCATCAATATCCTCGGCGAGATC
ATCAAGGGCACCACCGACACCGTCAACGAACACTTCTACGGCACAATCTACAAC
ATGATGCGCGGAATCTTCGGGCACGTAACTGACCCTAACTTCCAATACGGAATTG
CCCCCGGCGTAATGGAACACTTCGAAACCGCCACA-3’

aa
V A Y L G E D V G L S T F H T Y W N M D Y P F W A N H K T Y G I K W D R T G E L
F Y Y T Q H Q I L A R Y Y L E R L S N G L G E I K P F S Y S Y K T P I S G F E P S L R
Y Q N G K E F P M R P E G V R F S N S Y K T E E A L A Y E R R I F D A I D L G F V V
S K D G T K I P L K E K E G I N I L G E I I K G T T D T V N E H F Y G T I Y N M M R G
I F G H V T D P N F Q Y G I A P G V M E H F E T A T



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E come sempre, la mamma ed il papà, senza i quali nulla sarebbe stato possibile.

8. Bibliography

A

- Abascal F., Zardoya R., Posada D. (2005). ProtTest: selection of best-fit models of protein evolution. *Bioinformatics.* 21: 2104-2105.

- Amore V., Belardinelli M., Guerra L., Buonocore F., Fausto A.M., Ubero-Pascal N., and Fochetti R. (2009). Do really all stonefly nymphs have hemocyanin? Further data on the presence of hemocyanin in the larval stages of Plecoptera species. *Insect Molecular Biology*. 18: 203-11.

- Amore V., Fochetti R. (2009a). Present knowledge on the presence of hemocyanin in stoneflies (Insecta: Plecoptera). *Aquatic Insects*. 31(4): 577-583.

- AmoreV., Gaetani B., Fochetti R. (2009b). Lack of hemocyanin in Oriental Perlidae and Perlodidae (Plecoptera) suggests multifunctionality of the protein in stoneflies larvae. *Submitted to Entomological Science.*

- Amore V., Puig García M.A., Timperio A.M., Egidi G., Ubero-Pascal N., Fochetti R. (2009c). Preliminary data on comparative proteomic analysis of hemocyanins in the stoneflies: *Dinocras cephalotes* and *Perla marginata* (Plecoptera). *Submitted to Environmental Entomology*.

-Ashida M., Yoshida H. (1998). Limited proteolisis of phenoploxidase during activation by microbial product in insect plasma and effect of phenoloxidase on electrophoretic mobility of plasmaprotein. *Insect Biochemestry*. 18: 11-19.

- Àspan A., Söderhäll K. (1991). Purification of prophenoloxidase from crayfish blood cell, and its activation by an endogenous serine protease. *Insect Biochemestry*. 21: 363-373.

B

- Beintema J.J., Stam W.T., Hazes B., Smidt M.P. (1994). Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). *Molecular Biology and Evolution*. 11: 493–503.

- Beresford P.J., Basinski-Gray J.M., Chiu J.K., Chadwick J.S., Aston W.P. (1997). Caractherization of hemolimphic and cytotoxic Gallysins: a relationship with arylforins. *Developmental and Comparative Immunology*. 21: 253-266.

- Bentélemy C. (1966) Recherches écologiques et biogéographiques sur les Plécoptères et Coléoptères d'eau courante *(Hydraena* et Elminthidae) des Pyrénées. *Annales de Limnologie. 2* (2):227-458.

- Beutel R.G., Gorb S.N. (2001). Ultrastucture of attachment of exapods: evolutionary patterns inferred from a revised ordinal phylogeny. *Journal of Zoological Systematics and Evolutionary Research.* 39: 177–207.

- Bradford M.M. (1976). A rapid and sensitive method for quantification of microgram quantities of proteins using the principle of protein dye binding. *Analytical Biochemistry*. 72: 248–254.

- Braun R.P., Wyatt G.R. (1996). Sequence of the hexameric juvenile hormone-binding protein from the hemolymph of *Locusta migratoria*. *Journal of Biological Chemistry.* 271: 31756-31762.

-Bridges C.R., 2001. Modulation of haemocyanin oxygen affinity: properties and physiological implications in a changing world. *Journal of Experimental Biology*. 204: 1021–1032.

- Brusca R.C., Brusca G.J. (2002). Invertebrates, second ed. *Sinauer Associates, Sunderland M.A.*

- Burmaster T. (1999a). Identification, molecular cloning and phylogenetic analysis of a non-respiratory pseudo-hemocyanin of *Homarus americanus*. *Journal of Biological Chemistry*. 274: 13217–13222.

- Burmester T. (1999b). Evolution and function of the insect hexamerins. *European Journal of Entomology*. 96: 213–225.

- Burmester T. (2001). Molecular evolution of the arthropod hemocyanin superfamily. *Molecular Biology and Evolution*. 18: 184-195.

- Burmester T. (2002). Origin and evolution of arthropod hemocyanins and related proteins. *Journal of Comparative Physiology* B*: Biochemical, Systemic, and Environmental Physiology*. 172: 95–117.

- Burmester T., Scheller K. (1996). Common origin of arthropod tyrosinase, arthropod hemocyanin, insect hexamerin, and dipteran arylphorin receptor. *Journal of Molecular Evolution.* 42: 713–728.

- Burmester T., Massey H.C. Jr., Zakharkin S.O., Beneš H. (1998). The evolution of hexamerins and the phylogeny of insects. *Journal of Molecular Evolution.* 47: 93-108.

- Burmester T., Hankeln T. (1999). A globin gene of *Drosophila melanogaster*. *Molecular Biology and Evolution.* 16: 1809–1811.

- Burmester T., Hankeln T. (2007). The respiratory proteins of insects. Review. *Journal of Insect Physiology.* 53: 285-94.

- Burnett, L.E. (1992). Integrated function of the respiratory pigment hemocyanin in crabs. *American Zoology.* 32: 438–446.

C

- Cuff M., Miller K., van Holde K., Hendrickson W. (1998). Crystal structure of a functional unit from *Octopus* hemocyanin. *Journal of Molecular Biology*. 278: 855-70.

D

- Daynoff M.O., McLaughlin P.J., Barker W.C., Hunt L.T. (1975). Evolution of sequences within protein superfamilies. *Naturwissenschaftten.* 62:154-161.

- Deavis R.G. (1988). Outlines of entomology, 7th edition (Lineamenti di entomologia). *Zanichelli S.p.a.* Bologna.

- Decker H., Rimke T. (1998). *Tarantula* hemocyanin shows phenoloxidase activity. *Journal of Biological Chemistry.* 273: 25889–25892.

- Decker H., Terwilliger N.B. (2000). Cops and robbers: putative evolution of copper oxygenbinding proteins. *Journal of Experimental Biology.* 203: 1777–1782.

- Decker H., Tuczek F. (2000). Tyrosinase/catecholoxidasse activity of hemocyains: structural basis and molecular mechanism. *Trends in Biochemical Sciences.* 25: 392-397.

- Decker H., Ryan M., Jaenicke E., Terwinlliger N. (2001). SDS induced pheneloxidase activity of hemocyanin from *Limulus polyphemus*, *Eurypelma californicum*, *Cancer magister*. *Journal of Biological Chemistry.* 276: 17796- 17799.

- Decker H., Jaenicke E. (2004). Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins. *Developmental and Comparative Immunology.* 28: 673-87.

- Decker H., Hellmann H., Jaenicke E., Lieb B., Meissner U., Markl J. (2007). Recent progress in hemocyanin research. *Integrative and Comparative Biology.* 1-14.

- Destoumieux-Garzò D., Saulnier D., Garnier J., Jouffrey C., Bulet P., Evelyne Bachère E. (2001). Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challange. *The Journal of Biological Chemistry.* 276: 47070–47077.

- Dickerson R.E., Geis I. (1983). Hemoglobin: Structure, Function, Evolution, and Pathology. *Benjamin/Cummings Publ. Co., California.* 176 pp.

- Doolittle R.F. (1981). Similar amino acid sequences: change or common ancestry? *Science.* 214: 149-159.

- Doolittle R.F. (1989). Similar amino acid sequences revisited. *Trends in Biochemical Sciences.* 14: 244-245.

- Dudgeon D. (1999). Tropical Asian streams: zoobenthos, ecology and conservation. *Hong Kong University Press.* 812 pp.

- Durstewitz G., Terwilliger N.G. (1997). cDNA cloning of a developmentally regulated hemocyanin subunit in the crustacean *Cancer magister* and phylogenetic analysis of the hemocyanin gene family. *Molecular Biology and Evololution*. 14: 266-276.

E

- Enderle U., Käusser G., Renn L., Scheller K., Koolman J. (1983). Ecdysteroids in the hemolymphs of blowfly are bound to calliphorin. *In Scheller K. (ed). The larval serum proteins of insects: function, biosyntesis, genetic.* Thieme, Stuttgart. 40-49.

- Ertas B., von Reumont B.J., Wägele J.W., Misof B., Burmester T. (2009). Hemocyanin a close relationship of Remipeda and Hexapoda. *Molecolar Biology and Evolution*. 26(12):2711-18. Epub 2009 Aug 19.

F

- Fochetti R., Belardinelli M., Guerra L., Buonocore F., Fausto A.M. Caporale C. (2006). Cloning and Structural Analysis of a haemocyanin from the Stonefly *Perla grandis. Protein Journal*. 25: 443-454.

- Fochetti R., Tierno de Figueroa J.M. (2004). Plecoptera. Fauna Europaea Web Service. http://www.faunaeur.org (Fauna Europaea project, ref. EVR1-CT-1999–2001).

- Fochetti R., Tierno de Figueroa J.M. (2008). Global diversity of stoneflies (Plecoptera; Insecta). *Hydrobiologia*. 594: 365-377.

- Fochetti R., Tierno de Figueroa J.M. (2008). Plecoptera. Vol. XLIII of Fauna D'italia. *Calderini Eds.* 339 pp.

- Felsenstein J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 39: 783–791.

G

- Gascuel O. (1997). BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Molecular Biology and Evolution*. 14: 685-695.

- Giomi F., Beltramini M. (2007). The molecular heterogeneity of hemocyanin: its role in the adaptative plasticity of Crustacea. *Gene.* 15: 192-201.

- Guindon S., Gascuel O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology.* 52: 696-704.

- Gu X. (1998). Early metazoan divergence was about 830 million years ago. *Journal of Molecular Evolution.* 47: 369–371.

H

- Hagerman L. (1986). Haemocyanin concentration in the shrimp *Crangon crangon* (L.) after exposure to moderate hypoxia. *Comparative Biochemistry and Physiology. Part A: Molecular & Integrative Physiology*. 85: 721–724.
- Hagner-Holler S., Schoen A., Erker W., Marden J.H., Rupprecht R., Decker H., Burmester T. (2004). A respiratory hemocyanin from an insect. *Proceedings of the National Academy of Sciences USA*. 101: 871–874.
- Hagner-Holler S., Pick C., Gingernrath S., Marden J.H. and Burmaster T. (2007). Diversity of stonefly hexamerins and implication for the evolution of insect storage proteins. *Insect Biochemistry and Molecular Biol*ogy. 37: 1064-1074.
- Hall T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series.* 41: 95-98.
- Hankeln T., Jaenicke V., Kiger L., Dewilde S., Ungerechts G., Schmidt M., Urban J., Marden M.C., Moens L., Burmester T. (2002). Characterization of *Drosophila* hemoglobin: Evidence for hemoglobin-mediated respiration in insects. *Journal of Biological Chemistry*. 277: 29012–29017.
- Hankeln T., Klawitter S., Krämer M., Burmester T. (2006). Molecular characterisation of hemoglobin from the honeybee *Apis mellifera. Journal of Insect Physiology*. 52: 701–710.
- Haunerland N.H. (1996). Insect storage protein: gene families and receptors. *Insect Biochemestry and Molecular Biology*. 26: 755-765.
- Hayakawa Y. (1994). Cellular immunosuppressive protein in the plasma of parasitized insect larvae. *Journal Biological Chemistry*. 269: 14536-14540.
- Hellmann N., Hörnemann J., Jaenicke E., Decker H. (2004). Urate as effector for crustacean hemocyanins. *Micron*. 35: 109-110.
- Henning W. (1981). Insect phylogeny. *Chichester: Wiley and Sons*, New York. 614 pp.
- Hughes A.L. (1999). Evolution of the arthropod prophenoloxidase/hexamerin protein family. *Immunogenetics*. 49: 106–114.
- Hynes H.B.N. (1941). The taxonomy and ecology of the nymphs of British Plecoptera with notes on the adults and eggs. *Transactions of the Royal Entomological Society of London*. 91: 459-557.
- Hynes H. B. N. (1976). Biology of Plecoptera. *Annual Review of Entomology*. 21: 135–153.

J

- Janicke E., Decker H. (2004). Functional changes in the family of type 3 copper proteins during evolution. *ChemBioChem*. 5: 163-169.

- Jones D.T., Taylor W.R., Thornton J.M. (1992). The rapid generation of mutation data matrices from protein sequences *Computer Applications in the Biosciences.* 8: 275-282.

K

- Katoh K., Kuma K.I., Toh H., Miyata T. (2005). MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research*. 33: 511–518.

- Kestler P. (1985). Respiration and respiratory water loss. In: *Hoffmann, K.H. (Ed.). Environmental Physiologyand Biochemistry of Insects*. Springer, Berlin. 137–186.

- Klippenstein G.L. (1980). Structural aspects of hemerytrin and myohemerytrin. *American Zoologist*. 20: 39-51.

- Kristensen N.P. (1991). Phylogeny of extant hexapods. In *Insects of Australia: A Textbook for Students and Research Workers*. Volume I and II. Second Edn. 125–140.

- Kusche, K., Burmester T. (2001a). Molecular cloning and evolution of lobster hemocyanin. *Biochemical and Biophysical Research Communications.* 282: 887–892.

- Kusche K., Burmester T. (2001b) Diplopod Hemocyanin Sequence and the Phylogenetic Position of the Myriapoda. *Molecular Biology and Evolution.* 18: 1566-73.

-Kusche K., Ruhberg H., Burmester T. (2002). A hemocyanin from the Onychophora and the emergence of respiratory proteins. *Proceedings of the National Academy of Sciences*. 99: 10545–10548.

- Kusche K., Hembach A., Hagner-Holler S., Gebauer W., Burmester T. (2003). Complete subunit sequences, structure and evolution of the 6X6-mer hemocyanin from the common house centipede, *Scutigera coleoptrata. European Journal of Biochemistry*. 270: 2860–2868.

L

- Law J.H., Wells M.A. (1989). Insects as biochemical models. *Journal of Biological Chemistry.* 264: 16335–16338.

- Le S.Q., Gascuel O. (2008). LG: An improved general amino acid replacement matrix. *Molecular Biology and Evolution.* 25: 1307-1320.

- Lee S.Y., Lee B.L., Söderhäll S. (2003). Processing of an Antibacterial Peptide from Hemocyanin of the Freshwater Crayfish *Pacifastacus leniusculus. Journal of Biological Chemistry*. **7**: 7927–7933.

- Lee S.Y., Lee B.L., Söderhäll K. (2004) Processing of crayfish hemocyanin subunits into phenoloxidase. *Biochemical and Biophysical Research Communications.* 322: 490–496.

- Lee S.M, Lee S.B, Park C.H, Choi J. (2006). Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, chironomidae) larvae exposed to various

environmental pollutants: A potential biomarker of freshwater monitoring. *Chemosphere.* 65: 1074–1081.

- Linzen B., Soeter N.M., Riggs A.F., Schneider H.J., Schartau W., Moore M.D., Yokota E., Behrens P.Q., Nakashima H., Takagi T. et al. (1985). The structure of arthropod hemocyanins. *Science*. 229: 19–524.

- López-Rodríguez M.J., Tierno de Figueroa J.M, Fenoglio S., Bo T., Alba-Tercedor J. (2009). Life strategies of 3 Perlodidae species (Plecoptera) in a Mediterranean seasonal stream in southern Europe. *Journal of the North American Benthological Society*. 28: 611-625.

M

- Magee J., N. Kraynack H., Massey C. Jr., Telfer W.H. (1994). Properties and significance of a riboflavin-binding hexamerin in the hemolymph of *Hyalophora cecropia*. *Archives of Insect Biochemistry and Physiology*. 25: 137-157.

- Magnus K.A., Bart Hazes B., Ton-That H.,Bonaventura C., Bonaventura J., Hol W.G.J. (1994). Crystallographic analysis of oxygenated and deoxygenated states of arthropod hemocyanin shows unusual differences. *Proteins: Structure, Function, and Genetics.* 19: 302–309.

- Mangum C.P. (1983). The distribution of lactate sensitivity among the hemocyanins. *Marine Biology Letters.* 4, 139-149.

- Mangum C.P. (1985). Oxygen transport in invertebrates. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology.* 248: 505–514.

- Mangum C.P. (1994). Subunit composition of hemocyanins of *Callinectes sapidus*: phenotypes from naturally hypoxic waters and isolated oligomers. *Comparative Biochemistry and Physiology,*
part B: Biochemistry and Molecular Biology. 108: 537–541.

- Markl J., Markl A., Schartau W., Linzen B. (1979a). Subunit heterogeneity in arthropod hemocyanins. I. Chelicerata. *Journal of Comparative Physiology.* 130: 283–292.

- Markl J., Hofer A., Bauer G., Markl A., Kempter B., Brenzinger M., Linzen B. (1979b). Subunit heterogeneity in arthropod hemocyanins: II. Crustacea. *Journal of Comparative Physiology*. 133: 167–175.

- Markl J. (1986). Evolution and function of structurally diverse subunits in the respiratory protein hemocyanin from arthropods. *The Biololgical Bulletin*. 14: 266-276.

- Markl J., Winter S. (1989). Subunit-specific monoclonal antibodies of tarantula hemocyanin, and a common epitope shared with calliphorin. *Journal of Comparative Physiology.* 159: 139-151.
- Markl J., Decker H. (1992). Molecular structure of the arthropod hemocyanins. *Advantages in Comparative and Environmental Physiology.* 13: 283–292.
- Markl J., Burmester T., Decker H., Sievel-Niemann A., Harris J.R., Sfilino M., Naumann U., Scheller K. (1992). Quaternary and subunit structure of *Calliphora* arylphorin as deduced from electron microscopy, electrophoresis, and sequence similarities with arthropod hemocyanins. *Journal of Comparative Physioogy.* 162: 665-680.
- Martin G., Depoix F., Stohr M., Meissner U., Hagner-Holler S., Hammouti K., Thorsten Burmester T., Heyd J., Wriggers W., Markl J. (2007). *Limulus polyphemus* hemocyanin: 10 Å Cryo-EM structure, sequence analysis, molecular modelling and rigid-body fitting reveal the interfaces between the eight hexamers. *Journal of Molecular Biology*. 366: 1332–1350.
- Matthews P.G.D., Seymour R.S. (2008). Haemoglobin as a buoyancy regulator and oxygen supply in the backswimmer (Notonectidae, *Anisops*). *The Journal of Experimental Biology*. 211: 3790-3799.
- Morris S. (1990). Organic ions as modulators of respiratory function during stress. *Physiological Zoology*. 63: 253–287.

N

- Nagai T., Kawabata S. (2000). A link between blood coagulation and prophenoloxidase by horseshoe crab antimicrobial peptides. *Journal of Biololgical Chemidtry.* 276: 29264-29267.
- Nagai T., Osaki T., Kawabata S. (2001). Functional conversion of hemocyanin to phenoloxidase by horseshoe crab antimicrobial peptides. *Journal of Biololgical Chemidtry*. 276: 27166–27170.
- Nelson C.H. (1984). Numerical cladistic analysis of phylogenetic relationships in Plecoptera. *Annual of Entomological Society of America.* 77: 466-473.

P

- Pagel M. (1999). Inferring the historical patterns of biological evolution. *Nature*. 401: 877-884.
- Pan J-Y., Zhang Y-L., Wang S-Y., Peng X-X. (2008). Dodecamer is required for agglutination of *Litopenaeaus vannamei* hemocyanin with bacterial cells and red blood cells. *Marine Biotechnology.* 10: 645-652.

- Paul R., Bergner B., Pfeffer-Seidl A., Decker H., Efinger R., Storz H. (1994). Gas transport in the haemolymph of an arachnids – Oxygen transport and the physiological role of hemocyanin. *Journal of Experimental Biology*. 188: 25-46.

- Pick C., Hagner-Holler S., Burmester, T. (2008). Molecular characterization of hemocyanin and hexamerin from the firebrat *Thermobia domestica* (Zygentoma). *Insect Biochemistry and Molecular Biology*. 38: 977-983.

- Pick, C., Schneuer M., Burmester T. (2009a). Ontogeny of hemocyanin in the ovoviviparous crockroach *Blaptica dubia* suggest an embryo-specific role in oxygen supply. *Journal of Insect Physiology.* doi:10.1016/j.jinsphys.2009.04.004.

- Pick C., Schneuer M., Burmester T. (2009b). The occurrence of hemocyanin in Hexapoda. *FEBS Journal*. 276: 1930-1941.

- Pless D.D., Aguilar M.B., Falcòn A., Lozano-Alvarez E., Heimer de la Cotera E.P. (2003). Latent phenoloxidase activity and N-terminal amino acid sequence of hemocyanin from *Bathynomus giganteus*, a primitive crustacean. *Archives of Biochemistry and Biophysics*. 409: 402–410.

R

- Richley B., Decker H., Gill J. (1985). Binding of oxygen and carbon monoxide to arthropod hemocyanin: an allosteric analysis. *Biochemestry.* 24:109-117.

- Ridley M. (2004). Evolution. *Blackwell Science.* Ldt. 750 pp.

S

- Sánchez D., Ganfornina M.D., Gutiérrez G., Bastiani, M.J. (1998). Molecular characterization and phylogenetic relationships of a protein with potential oxygen-binding capabilities in the grasshopper embryo. A hemocyanin in insects? *Molecular Biology and Evolution.* 15: 415–426.

- Sánchez-Ferrer A., Rodríguez-López J.N., García-Cánovas F., García-Carmona F. (1995). Tyrosinase: a comprehensive review of its mechanism. *Biochimica et Biophysica Acta.* 1247: 1-11.

- Schluter D. (2001). Ecology and the origin of species. *Trends in Ecology and Evolution.* 16: 372–380.

- Söderhäll K., Cerenius L. (1998). Role of the prophenilosidase-activating system in invertebrate immunity. *Current Opinion in Immunology.* 10: 23-28.

- Solomon E., Tuczek F., Root D., Brown C. (1994). Spectroscopy of binuclear dioxygen complexes. *Chemical Reviews.* 94: 827–856.

- Swofford D.L., Olsen G.J., Waddell P.J., Hillis D.M. (1994). Phylogenic interference. In*: Hillis D.M., Moritz C., Mable B.K. (eds). Molecular systematics. Snauer Associates, Mass*. 407-514.

T

- Telfer H., Kunkel J.G. (1991). The function and evolution of insect storage hexamers. *Annual* Review of *Entomology.* 36: 205-28.

- Terwilliger N.B. (1998). Functional adaptations of oxygen-transport proteins. *Journal of Experimental Biology*. 201: 1085–1098.

- Terwilliger N.B., Dangott L.J., Ryan M.C. (1999). Cryptocyanin, a crustacean molting protein, and evolutionary links to arthropod hemocyanin and insect hexamerins. *Proceedings of the National Academy of Sciences* USA. 96: 2013–2018.

- Terwilliger N.B., Ryan M.C. (2006). Functional and phylogenetic analyses of phenoloxidases from Brachyuran (*Cancer magister*) and Branchiopod (*Artemia franciscana, Triops longicaudatus*) Crustaceans. *Biological Bulletin of Marine Biological Laboratory*. 210: 38–50.

- Tierno De Figueroa J.M., Fochetti R. (2001a). Male drumming call of an Italian population of *Dinocras cephalothes* (Curtis, 1827), (Plecoptera Perlidae). *Fragmenta Entomologica.* 33: 15-19.

- Tierno De Figueroa J.M., Fochetti R. (2001b). On the adult feeding of several European stoneflies (Plecoptera). *Entomological news.* 112: 130-134.

- Tierno de Figueroa J.M., Luzón-Ortega. (2002). The male drumming of *Isoperla bipartita* Aubert 1952 (Plecoptera Perlidae). *Aquatic Insect.* 21: 33-38.

- Tierno de Figueroa J.M., Sánchez-Ortega A., Membiela Iglesia P., Luzón-Ortega J.M. (2003). Plecoptera. *Fauna Ibérica.* (Ramos, M.A. *et al*. eds) Museo Nacional de Ciencias Naturales, CSIC, Madrid. 22: pp. 404.

- Tierno de Figueroa J.M., Luzón-Ortega J.M., López-Rodríguez M.J. (2006). Mating balls in stoneflies (Insecta, Plecoptera). *Zoogical baetica.* 17: 93-96.

- Truchot J.P. (1992). Respiratory function of arthropod hemocyanins. In: *Mangum, C.P. (Ed.), Advances in Comparative and Environmental Physiology. Blood and tissue oxygen carriers, Springer-Verlag, Berlin.* 13: 377–410.

V

- van Holde K.E., Miller K.I., Decker H. (2001). Hemocyanins and invertebrate evolution. *Journal of Biological Chemistry.* 276: 15563–15566.

- Vinogradov S.N. (1985). The structure of invertebrate extracellular hemoglobins (erythrocruorins and chlorocruorins). *Comparative Biochemistry and Physiology. Part B: Biochemistry*. 82: 1–15.

- Volbeda A., Hol W.G. (1989). Crystal structure of hexameric hemocyanin from *Palinurus interruptus* refined at 3.2 Å resolution. *Journal of Molecular Biology*. 209: 249–279.

- Voit R., Feldmaier-Fuchs G., Schweikardt T., Decker H., Burmester T. (2000). Complete sequence of the 24mer hemocyanin of the tarantula *Eurypelma californicum*: structure and intramolecular evolution of the subunits. *Journal of Molecular Biology*. 275: 39339–39344.

W

- Weber R.E., Vinogradov S.N. (2001). Nonvertebrate hemoglobins: functions and molecular adaptations. *Physiological Reviews*. 81: 569–628.

- Willmer P., Stone G., Johnston I. (2000). Environmental Physiology of Animals. *Blackwell, Oxford.* 768 pp.

- Wray G.A., Levinton J.S., Shapiro L.H. (1996). Molecular Evidence for Deep Precambrian Divergences Among Metazoan Phyla. *Science.* 274: 568–573.

Y

- Yoo, B.S., Kim, S.B., Lee J.H., Yang K.H. (1988). The subunit composition of *Portunus trituberculatus* hemocyanin polymers. *Biochemical and Biophysical Research Communications.* 153: 748–752.

Z

- Zuckerkandl E. (1960). Hémocyanine et cuivre chez un Crustacé Décapode dans leurs rapports avec le cycle d'intermue. *Annuaire d'Institute d'Océanographique*. 38: 1–122 (in Frances).

- Zwick P. (1973). Insecta: Plecoptera. Phylogenetisches System und Katalog. Das Tierreich 94. *Walter de Gruyter and Co., Berlin.* 465 pp.

- Zwick P. (2000). Phylogenetic System and Zoogeography on the Plecoptera. *Annual Review of Etomology*. 45: 709-746.