HELGOLÄNDER MEERESUNTERSUCHUNGEN
Helgoländer Meeresunters. 43, 207–223 (1989)

Haemolymph protein composition and copper levels in decapod crustaceans

M. H. Depledge & P. Bjerregaard

Institute of Biology, Odense University; DK-5230 Odense M., Denmark

ABSTRACT: Variations in haemolymph protein composition and concentration, in copper content and copper distribution in the tissue of decapod crustaceans are reviewed. Haemocyanin is the major haemolymph constituent (> 60 %); the remaining proteins (in order of concentration) include coagulogen, apohaemocyanin, hormones and antisomes. Moulting, nutritional state, infection, hypoxia and salinity fluctuations are the major factors affecting the relative proportions and total quantities of the haemolymph proteins. With regard to haemocyanin, the changes in concentration during the moult cycle are principally associated with changes in haemolymph volume, rather than with changes in total haemocyanin content due to synthesis or catabolism. The role of the midgut gland in regulating haemolymph copper and haemocyanin concentration has been re-evaluated. More than 50 % of the whole body copper load is stored in the haemolymph. In contrast, less than 3 % of the copper load resides in the midgut gland. The latter has little potential for regulating haemolymph copper levels, at least in the short term (hours to a few days), though it may be involved in regulating haemocyanin levels over longer periods (weeks to months). The total copper content of the haemolymph remains within a narrow range, except during starvation when levels may decrease. Consequently, variations in the copper content of soft tissues, which constitute only 20 % of decapod dry weight, do not significantly alter whole body copper concentrations. Evidence that copper released following haemocyanin catabolism becomes bound to metallothionein for later use in the resynthesis of haemocyanin is reviewed and found to be inçonclusive. The amount of copper that can be stored in this way is trivial compared with the amount of copper required to permit significant changes in haemolymph haemocyanin concentration. Average tissue copper requirements, calculated during the present study, are approx. 4 times higher than previous theoretical estimates.

INTRODUCTION

The concentration and composition of proteins in the haemolymph of decapods are of interest to researchers in several branches of crustacean biology. The haemolymph oxygen carrier, haemocyanin, has been studied intensively by respiratory physiologists; nutritional and osmoregulatory physiologists have focused their attention on variations in haemolymph protein composition associated with starvation and osmotic stress; and during the last 5 to 10 years, comparative immunologists have begun searching for primitive antibodies in crustacean blood while ecotoxicologists have started to investigate the role of haemolymph proteins in pollutant uptake and transport.

Inevitably, such interest has generated a wealth of publications, which, when examined, give rise to many inconsistencies and contradictions. The purpose of this article is to summarise the most important findings, synthesise the data into a consistent scheme and to stimulate investigations in areas where knowledge is still scanty.

HAEMOLYMPH PROTEIN COMPOSITION

Up to 18 distinct protein bands have been recognised in electrophoretic studies of decapod haemolymph (Ceccaldi, 1968; Claybrook, 1983). These components can be assigned to one of 5 groups.

Haemocyanin and apohaemocyanin usually constitute the dominant protein group. Detailed analysis of the respiratory properties of haemocyanin are beyond the scope of this article and, in any case, have been adequately reviewed elsewhere (Mangum, 1983). However, it is worth noting the heterogeneity of haemocyanins in decapod haemolymph, with 3 or 4 components present. These are subunit aggregations of haemocyanin (Manwell & Baker, 1963; Claybrook, 1983), and range in size from 450×10^3 to 1.7×10^6 daltons (Mangum, 1983). In general, haemocyanins exhibit low oxygen affinity, considerable cooperativity and a large Bohr effect (Mangum, 1983). A critical factor enabling haemocyanin to transport oxygen is the presence of copper in its structure. Copper normally represents 0.17 % of the functional haemocyanin molecule (Ghidalia, 1985). As well as its oxygen-transporting role, haemocyanin has considerable buffering potential. Its buffering power towards carbon dioxide is very high in *Maia squinado* (Kerridge, 1926), while in *Orconectes limosus,* only 18 % of the blood's buffer capacity can be removed by dialysis (Andrews, 1967). Haemocyanin is an important organic nutrient reserve (Hagerman, 1983) and may also be involved in the storage of amino acids liberated by cells during hypo-osmotic shock (Schoffeniels, 1976). Conversely, haemocyanin may contribute to the release of certain amino acids required as osmotic effectors during hyperosmotic stress.

Apohaemocyanin lacks copper and does not function in oxygen transport. Its presence in the haemolymph permits rapid synthesis of new haemocyanin by the addition of copper (Horn & Kerr, 1963) and it too functions as a nutrient store (Uglow, 1969b).

The second major group of proteins is involved in clot formation following injury (Manwell & Baker, 1963; Horn & Kerr, 1963; Durliat, 1974; Vendrely et al., 1979). Dominant among these is fibrinogen (also referred to as coagulogen; Bang, 1983) which was first identified by Fredericq (1879). The amino acid contents of lobster haemocyanin and fibrinogen are distinctly different, with the latter containing approximately 2.5 times as much cysteine (as the dimer, cystine/2; Stewart et al., 1966). This may have some significance for metal transport in the haemolymph as many metals show a high affinity for cysteine (Hammond & Beliles, 1980).

The three remaining groups of proteins occur in low concentrations, but their importance in physiological processes should not be underestimated. For example, proteinaceous hormones in the haemolymph are known to regulate the moult cycle and reproduction, influence activity rhythms and to be involved in the control of gill ventilation, cardiac activity and colouration (Kleinholz, 1985). Many of these processes are associated with changes in other protein constituents of the haemolymph and thus hormones are potential modulators of total protein concentration.

Decapods can produce circulating proteins that inactivate bacteria, protozoans and viruses (Hildemann & Reddy, 1973; Hildemann, 1974). Cantacuzene (1925) first identified these substances in the blood of hermit crabs. *Panulirus argus* immunised with a bacterial species endogenous to the lobster intestine, produce a non-dialysable bactericidin detectable in the haemolymph in less than 24 h (Evans et al., 1968). Hildemann (1974)

refers to two agglutinins in the haemolymph of *Homarus americanus*. Similar agglutinins have been found in the haemolymph of the spiny lobster, *Panulirus interruptus* (Tyler & Metz, 1945), in the blue crab, *Callinectes sapidus* (Pauley, 1974) and in the crayfish, *Procambarus clarkii* (Miller et al., 1972). Agglutinins have more recently been referred to as antisomes and are thought to be analogous to vertebrate antibodies (Bang, 1983).

Lastly, a group of metal-binding proteins (i.e. proteins showing a particularly high affinity for metals) have been detected in decapod haemolymph (Martin, 1973; Guary & Negrel, 1980; Huebers et al., 1982; Ghidalia et al., 1982; Depledge et al., 1986). These proteins assume special significance with regard to the uptake and transport of essential trace metals. For example iron, which is a constituent of cytochromes, catalases and peroxidases, is largely insoluble in the haemolymph and must be transported in a protein-bound form. In *Scylla serrata*, 0.84 μg Fe ml^{-1} haemolymph is found bound to a transferrin-like protein of 142,000 daltons. Experimental loading of the haemolymph with iron indicates that this represents only 25 % of the total iron-binding capacity (Depledge et al., 1986).

HAEMOLYMPH PROTEIN CONCENTRATION

Hagerman (1983) refers to a 15-fold intraspecific variation in haemolymph protein concentration, and brief examination of the literature reveals wide variation in interspecific values also (Table 1). This led Claybrook (1983) to speculate that haemolymph protein concentration is not closely regulated. Despite such variation, most values fall between 40–80 mg ml^{-1}. Mangum (1983) considers haemocyanin concentration (and

Table 1. Haemolymph protein concentrations in some marine decapods

Species	Protein concentration (mg ml^{-1})	Source
Carcinus maenas	35–56	Uglow (1969a)
C. maenas	28	Gilles (1977)
C. maenas	29	Pequeux et al. (1979)
C. maenas	30	Henke (1977)
C. maenas	57	Bjerregaard & Vislie (1986)
Callinectes sapidus	44	Leone (1953
C. sapidus	82	Horn & Kerr (1963)
C. sapidus	49	Pequeux et al. (1979)
Homarus americanus	43	Leone (1953)
H. americanus	30	Stewart et al. (1967a)
H. americanus	63	Stewart et al. (1967a)
H. americanus	45	Castell & Budson (1974)
Homarus vulgaris	40–80	Glynn (1968)
Panulirus longipes	116	Dall (1974)
Penaeus spp.	82	Smith & Dall (1982)
Crangon vulgaris	77	Djangmah (1970)
Cancer magister	45	Leone (1953)
Scylla serrata	45–75	Depledge et al. (1986)
Uca pugilator	85	Spindler-Barth (1976)
Uca minax	222	Pequeux et al. (1979)
Libinia emarginata	41	Leone (1953)

consequently total protein levels) to be rather low in decapods. This she interprets as a limitation imposed by crustacean circulatory systems which are „open" and operate at very low hydrostatic pressures. However, in Man, where blood is circulated in a „closed", high pressure system, the normal blood protein range is 60–80 mg ml^{-1} (Weatherall et al., 1984), a range not very different from that in decapods. Of this, 20–40 mg ml^{-1} is albumin which, in vertebrate plasma, serves to maintain osmotic pressure and to transport metals, ions, fatty acids, amino acids, metabolites, bilirubin, enzymes and hormones (Rothschild & Oratz, 1976). In striking contrast, crustacean haemolymph contains large amounts of haemocyanin, but no albumin. It remains to be seen to what extent haemocyanin performs an analogous role in invertebrates to that of albumin in vertebrates.

The percentage contribution of haemocyanin to the total pool of protein in the haemolymph is a matter of debate and, in any case, is highly variable. Ghidalia (1985) states that during the C_4 stage of the moult cycle haemocyanin may represent 95 % of the haemolymph protein in decapods. Uglow (1969a) gives a value of 90 % for *Carcinus maenas* while Djangmah (1970) reports a range from 60–93 % in *Crangon vulgaris*. Zuckerkandl (1960) found that haemocyanin constituted between 17–70 % of total haemolymph protein in the crab, *Maia squinado* during the moult cycle. For comparison, in the isopod *Sphaeroma serratum*, 38–79 % of blood protein is haemocyanin while in the amphipod *Conilera cylindracea*, the proportion ranges from 25–92 % (Wieser, 1965). Most values for decapods fall within the range 70–95 %. In view of the overriding influence of haemocyanin on total haemolymph protein concentration, the major factors affecting haemocyanin levels will be examined in detail later.

Little is known of the variations in concentrations of the other protein constituents of the haemolymph. From the foregoing discussion it can be deduced that they may constitute up to 83 % of haemolymph protein under some circumstances. Apohaemocyanin may represent up to 25 % of haemolymph proteins in some *C. maenas*, but in others it is not detectable (Uglow, 1969a, b; Busselen, 1970). Stewart et al. (1966) reported that < 10 % of the blood protein is fibrinogen. Changes in fibrinogen content have not been investigated systematically but a number of factors suggest large variations. For example, clotting times for decapod haemolymph are apparently closely correlated with haemocyanin concentration (Glavind, 1948; Stewart et al., 1966), indicating an association between haemocyanin and the clotting mechanism.

With regard to antisome production, there is once again little information to assess. *Carcinus maenas* releases an immunologically reactive protein into the haemolymph when infected with the rhizocephalan parasite, *Sacculina carcini* (Andrieux et al., 1976; Bang, 1983). Consistent with this, Damboviceanu (1928), Drilhon (1936) and Drilhon & Pora (1936) reported that *Sacculina*-infected *C. maenas* had raised haemolymph protein concentrations. Uglow (1969c) found that total protein was unchanged following infection although apohaemocyanin concentration increased. In *Callinectes sapidus* infected with the rhizocephalan, *Loxythylacus texanus*, blood protein levels were elevated (Manwell & Baker, 1963). In contrast, when *C. maenas* and *Uca pugilator* are infected with bacteria, both total protein and copper levels in the haemolymph decrease and coagulation processes are disturbed (Spindler-Barth, 1976). In a more recent detailed study, Henke (1985) found that total protein levels in infected and disease-free *C. maenas* were similar, but protein composition changed with two new components appearing following infection.

In summary, immunological reactions involving protein synthesis do occur but are unlikely to significantly influence overall protein concentration of the haemolymph.

Arumugam & Ravindranath (1986) reported that in growing (ageing) *Scylla serrata*, the quantity of copper-free proteins (apohaemocyanin, fibrinogen, etc.) increases at a greater rate than the quantity of copper-bound proteins (i.e. haemocyanin). This may reflect increasing use of the haemolymph as an organic nutrient reserve and/or the gradual accumulation of fibrinogen and antisomes.

FACTORS AFFECTING HAEMOCYANIN SYNTHESIS

The direct determinants of haemocyanin in the haemolymph are: (1) the rate of haemocyanin synthesis, (2) the haemolymph volume, (3) the rate of haemocyanin breakdown and loss.

Very little is known about the rate of haemocyanin synthesis in decapods. Indeed, there is still controversy surrounding even the site (or sites) of synthesis. Haemocyanin is thought to be made within specialised cells, the cyanocytes, located at the base of the rostrum in Natantia and along the dorsal and lateral borders of the anterior chamber of the foregut in Reptantia (Bauchau & Mengeot, 1978). In *Carcinus maenas*, the cyanoblasts are non-circulating cells occurring in the reticular connective tissue according to Ghiretti-Magaldi et al. (1977). These cells derive from pluripotent stem cells found in the lymphocytogenic nodules of the gut walls. In *Homarus americanus*, haemocyanin is apparently synthesised in the midgut gland (Senkbeil & Wriston, 1981) and there are frequent references elsewhere in the literature to the midgut gland (hepatopancreas) as the site of haemocyanin synthesis (Zuckerkandl, 1957; Djangmah & Grove, 1970; Boone & Schoffeniels, 1979; Engel et al., 1985; Brouwer et al., 1986; Rainbow, 1988). The evidence usually cited in support of this contention is that the midgut gland is a major copper storage site and therefore is likely to be involved in the synthesis of copper-containing haemocyanin. Johnston & Barber (1969) showed that a midgut gland supernatant fraction was able to reconstitute haemocyanin non-enzymatically from apohaemocyanin by the donation of copper. Furthermore, Senkbeil & Wriston (1981) used uptake of [^{14}C] labelled aspartic acid in vitro to demonstrate that haemocyanin can be synthesised in the midgut gland.

Irrespective of the site of synthesis, it is clear that haemocyanin manufacture requires the presence of apohaemocyanin and the availability of sufficient copper from endogenous and/or exogenous sources. The latter sources include copper in the food and in the ambient seawater. It has been suggested that food is the most important source, as seawater copper levels are low (40–340 ng l^{-1}, Windom & Smith, 1979; Bryan, 1984). However, if one considers a 100 g fresh weight *C. maenas* consuming 20 times its body weight in food per year (probably an over-estimate), and if the food consists mainly of the soft parts of *Mytilus edulis* (Adelung & Ponat, 1977), then in unpolluted conditions where mussel soft parts contain < 0.12 µg g^{-1} fresh weight (Phillips, 1976), the crab would be exposed to only 240 µg Cu per year in the food. This constitutes less than 10 % of the crabs total copper content.

Haemolymph volumes in decapod crustaceans have been estimated by a variety of techniques yielding highly variable results. Values for intermoult decapods vary from approx. 17–37 % of the fresh weight (Nicol, 1967; Prosser, 1973; Gleeson & Zubkoff,

1977). Such variability not only reflects the techniques used (dilution techniques, bleeding, etc.) but, as is discussed later, also real changes in haemolymph volume associated with growth and nutritional state. Addition of water to, or loss from the haemolymph pool will inevitably result in dilution or concentration of the protein constituents unless other regulatory processes intervene. Smith & Dall (1982) made a distinction between haemolymph volume (containing haemocyanin) and haemocyanin-free, extracellular fluid volume in the prawn *Penaeus* spp.. Blood volume represented only 10 % fresh weight while the haemocyanin-free extracellular fluid volume represented a further 5–20 % fresh weight.

Great caution should be exercised when basing calculations on estimated haemolymph volumes. This will become apparent later in the discussion but one example serves to illustrate the point. Differences in the theoretical and actual copper requirements in the shrimp *Systellaspis debilis,* were interpreted as being indicative of copper deficiency in juveniles (White & Rainbow, 1987). However, the theoretical haemocyanin copper requirement was based on an estimated haemolymph volume of 37 % fresh weight (f. w.) (White & Rainbow, 1985, 1987). If a more realistic blood volume estimate is 10 % f. w. (Smith & Dall, 1982) then theoretical requirements and measured copper levels fall within the same range. This removes the basis for postulating that copper and haemocyanin deficiency limit the ability of juveniles to perform vertical migrations.

Finally, the rate of breakdown or loss of haemocyanin under normal physiological conditions is unknown. All that can be stated is that some factors accelerate breakdown, the best known of which are starvation (Stewart et al., 1967a, b; Hagerman, 1983) and salinity stress (Schoffeniels, 1976).

HAEMOCYANIN AND THE MOULT CYCLE

Haemocyanin concentration in decapods varies during the moult cycle (e. g. Zuckerkandl, 1960; Djangmah & Grove, 1970). Levels fall to extremely low values in *Maia squinado* after moult but gradually return to normal during the intermoult period (Zuckerkandl, 1960). It was postulated that copper is released by the breakdown of haemocyanin and a proportion stored in the midgut gland to be reutilised later for the resynthesis of haemocyanin (again in the midgut gland) during stage C_4 of the moult cycle (Zuckerkandl, 1960). This phenomenon has also been reported for *Crangon crangon* (Nott & Marvin, 1986). It has been hypothesised that storage of copper in the midgut gland is made possible by the presence of the metal-binding proteins, metallothioneins (Engel & Brouwer, 1984; Engel et al., 1985; Brouwer et al., 1986; Wong & Rainbow, 1986a, b; Rainbow, 1988). Other data do not support this view. For example, in *Crangon vulgaris,* reductions in blood protein and copper after moult were not accompanied by a rise in midgut copper (Djangmah & Grove, 1970). Nor was the reciprocal relationship between blood copper and midgut gland copper confirmed by Kerkut et al. (1961). Brouwer et al. (1986) found that copper metallothioneins were not capable of reconstituting apohaemocyanin to functional haemocyanin, the transfer process being slow and incomplete.

In fact, there is no need to postulate the catabolism of haemocyanin at the time of moulting. Shortly before ecdysis (D_2/D_3), peak values for total haemolymph protein are reached (Djangmah & Grove, 1970). Then, beginning during D_4 and continuing during

stage E, blood volume doubles. Throughout the whole period of ecdysis blood volume may increase 9-fold in *Cancer* spp. (Passano, 1960). In *Carcinus maenas*, the increase in fluid volume represents 70 % of the pre-ecdysis fresh body weight (Robertson, 1937). Carlisle (1954) and Robertson (1960) suggest even higher percentages (72–80 %). Considering a 100 g f. w. *C. maenas* with a premoult blood volume of 37 ml (Nicol, 1967), then at ecdysis a further 70–80 ml of water are taken up resulting in 2–3 times dilution of the haemolymph proteins, including haemocyanin. If lower, more recent estimates of haemolymph volume are used (Prosser, 1973), then a 3–4 times dilution is predicted. Such estimates are entirely consistent with the dilutions measured experimentally. Busselen (1970) reports a 4–5-fold dilution of haemocyanin in *C. maenas* at the time of ecdysis. Glynn (1968) and Smith & Dall (1982) present data indicating a 3-fold dilution of total protein in the lobster, *Homarus vulgaris* and in penaeid prawns. Djangmah's (1970) data reveal a reduction in total protein from 80–90 mg ml^{-1} to approx. 30 mg ml^{-1}, and a reduction in haemocyanin concentration from 55–65 mg ml^{-1} to 20 mg ml^{-1} in the prawn *Crangon vulgaris* during moulting; again a 3-fold dilution. A 4-fold decrease in haemocyanin concentration occurs after moulting in Astacidae (Chaisemartin et al., 1968). Therefore, it seems doubtful that significant quantities of haemocyanin are catabolised during the moult. One further argument may be employed. The midgut gland of *C. maenas* represents approx. 1.2 % of the animals' dry weight (d. w.) and has a copper content of 85 μg g^{-1} dry weight (Jennings & Rainbow, 1979; Bjerregaard & Vislie, 1986). In a 100 g fresh weight specimen (approx. 30 g dry weight) the haemolymph volume is approx. 30 ml and copper concentration is approx. 70 μg ml^{-1} (Prosser, 1973; Bjerregaard & Vislie, 1986). Even if only 25 % of the haemolymph haemocyanin were catabolised this would liberate 525 μg of copper. If 50 % of this copper were taken up in the midgut gland, the concentration would rise 10-fold to more than 800 μg Cu g^{-1} dry weight. Such dramatic rises have not been recorded in *C. maenas*.

Assuming the above interpretation is correct, some other explanation must be sought to account for the rise in midgut gland copper that has occasionally been observed. Several workers have shown that when excess copper is available, it is stored in the midgut gland (Kerkut et al., 1961; Bjerregaard & Vislie, 1986). The question then arises, as to whether there is an increase in the availability of copper immediately prior to the moult. By stage D_3 of the moult cycle, most of the postecdysial procuticle has been digested from within and reabsorbed (Stevenson, 1985). During this process, significant amounts of copper, previously combined in the exoskeleton matrix, may well be liberated and so become available for uptake in the midgut gland. Although exoskeleton copper concentration is low in crabs (approx. 8 μg g^{-1} dry weight exoskeleton; Bjerregaard &Vislie, 1986), the contribution of exoskeleton to the total dry weight is high (> 80 %; Jennings & Rainbow, 1979). Thus in a 30 g dry weight *C. maenas*, approximately 200 μg of copper is stored in the exoskeleton. The copper content of the less heavily calcified exoskeletons of shrimps and prawns is even higher (230 μg g^{-1} dry weight in *Callianassa*; Ahsanullah et al., 1981). Also, prior to moulting considerable quantities of muscle are catabolised. For example, in *Gecarcinus lateralis*, immediately prior to moult the muscle mass is only 60–70 % of that during the intermoult period (Skinner, 1966). Muscle represents approx. 5 % of the dry weight of *C. maenas* (Jennings & Rainbow, 1979) and has a copper content of approx. 28 μg Cu g^{-1} dry weight (Bjerregaard & Vislie, 1986). Degeneration of muscle is therefore likely to make available a further 15–20 μg of copper.

In summary, potential endogenous sources of copper other than haemocyanin do exist and could account for the observed rises in midgut gland copper. It is not clear why copper levels should rise in some moults but not in others. One possible explanation is that when moulting, decapods may sometimes be forced to endure prolonged starvation which itself is associated with a rise in midgut gland copper (see below).

HAEMOCYANIN CONCENTRATION AND NUTRITIONAL STATE

Haemolymph protein concentration in decapods varies with nutritional state. Both total haemolymph protein and haemocyanin levels decrease during starvation (Stewart et al., 1967a, b; Adiyodi, 1969; Uglow, 1969b; Djangmah, 1970). Starvation effects on protein concentration diminish with decreasing temperature (Stewart et al., 1967a, b).

Uglow (1969a, b) identified two different haemocyanin components in *Carcinus maenas* haemolymph; electrophoretically "fast" and "slow" haemocyanin, as well as apohaemocyanin. He found that "fast" haemocyanin levels remain unchanged during starvation as this component fulfils a respiratory role. "Slow" haemocyanin and apohaemocyanin levels decline indicating that they may be utilised as a nutrient source.

In *Crangon vulgaris*, 30–40 days starvation results in a more than 3-fold reduction in total haemolymph protein, and copper levels fall from 106 $\mu g\ ml^{-1}$ to 16 $\mu g\ ml^{-1}$ after 37 days food deprivation (Djangmah, 1970). If blood volume represents 35 % fresh weight (Hagerman & Weber, 1981; Prosser, 1973), then in a 2 g fresh weight shrimp, 63 µg of copper would be lost from the haemolymph. Djangmah (1970) also observed a progressive accumulation of copper in the midgut gland during starvation which he speculated was derived from the catabolism of haemocyanin in its capacity as a food reserve. Reciprocal changes in blood and midgut gland copper levels during starvation have also been observed in *C. maenas* (Fraser & Grove, unpublished, cited by Djangmah, 1970). These trends are reversible by re-feeding (Djangmah, 1970). The data suggest that the midgut gland acts as a temporary store of copper during periods of food shortage. However, some doubt remains. Smith & Dall (1982) reported that in penaeid shrimps, blood volume varies inversely with total protein concentration of the haemolymph. Furthermore, after 24 days starvation, blood protein concentration decreased by 65 % while blood volume increased by 50 %. This was interpreted as indicating a reduction in tissue mass during starvation with a concomitant increase in fluid volume. Thus, the reduction in haemocyanin concentration may again be a dilution effect rather than the result of catabolism. Using Djangmah's (1970) data for changes in midgut gland copper during starvation, it is possible to calculate the absolute increase in the amount of copper in a 2 g fresh weight *Crangon vulgaris*. Midgut gland copper increased from 82 $\mu g\ g^{-1}$ dry weight to 3177 $\mu g\ g^{-1}$ dry weight. The midgut gland is estimated to represent approx. 8.0 % of the dry weight in shrimps (White & Rainbow, 1984). Thus, in a 2 g fresh weight *C. vulgaris* (0.58 g dry weight, based on a fresh weight/dry weight ratio of 3.478, determined using 30 *Palaemon adspersus*; Depledge, unpublished), the midgut gland copper content would have increased from 3.8 µg to 146.4 µg. This is 2.26 times as much copper as was lost from the haemolymph. If a more recent blood volume estimate for shrimps is used (blood volume = 10 % fresh weight initially, then 15 % fresh weight after starvation; Smith & Dall, 1982) then only 16.4 µg Cu would have been lost from the blood.

This raises the question of where the additional copper in the midgut gland might have come from.

Uglow's (1969) data for *Carcinus maenas* reveal a reduction in haemocyanin concentration from 3.91 g% to 3.11 g% after 28 days starvation. Thus, copper concentration fell from approx. 66 μg Cu ml^{-1} to 52.5 μg Cu ml^{-1}. In a 100 g fresh weight *C. maenas* (approx. 30 g dry weight) this represents a loss of approx. 500 μg of copper from the blood. The midgut gland has been reported to constitute 1.2 % of the dry weight of *C. maenas* (Jennings & Rainbow, 1979), although Bjerregaard (unpublished) has found a higher value, 3.6 %. If the latter value is used, the copper concentration in the midgut gland of the above crab would have increased by approx. 460 μg Cu g^{-1} if all the copper lost from the blood were taken up. This seems an unrealistically large amount when typical midgut gland copper concentrations of crabs starved for a similar period, generally fall in the range 70–90 μg Cu g^{-1} dry weight (Bjerregaard & Vislie, 1986). It may be concluded that, in crabs at least, only a proportion of the copper lost from the blood is taken up in the midgut gland during starvation. Further support for this view can be obtained by performing similar analyses on data for lobsters undergoing starvation (Hagerman, 1983; Bryan, 1968).

HAEMOCYANIN CONCENTRATION AND ENVIRONMENTAL FLUCTUATIONS

A number of environmental factors are thought to influence haemolymph protein and copper (haemocyanin) concentrations. A trend of seasonal variation was reported by Uglow (1969a) which closely followed temperature changes. Crowley (1963) and Andrews (1967) working with *Astacus nigriscens* and *Orconectes limosus* respectively, also found seasonal changes in blood proteins.

A compensatory increase in haemocyanin concentration may occur with prolonged exposure to hypoxia. Butler et al. (1978) were unable to detect significant changes in lobsters exposed to low oxygen tensions for 120 h, but both Senkbeil & Wriston (1981) and Hagerman & Uglow (1985) reported hypoxia-induced haemocyanin synthesis in *Homarus americanus* and *Nephrops norvegicus*. Unfortunately, data are not available to determine whether or not the midgut gland functioned as a copper donor to sustain synthesis of the respiratory pigment. *Nephrops norvegicus* exposed to prolonged hypoxia in situ were found to have lowered levels of haemocyanin in the blood. This was attributed to starvation resulting from reduced feeding at low environmental oxygen tensions (Hagerman & Pihl Baden, 1988).

The most dramatic example of an environmental factor affecting haemolymph protein composition is that reported by Boone & Schoffeniels (1979) for *Carcinus maenas* exposed to hypo-osmotic seawater. In 50 % seawater, haemocyanin concentration almost doubled within 48 h, increasing from 15–20 mg ml^{-1} to 35 mg ml^{-1}. This was associated with an increase in haemolymph copper from 27 μg ml^{-1} to 62 μg ml^{-1},but apparently no significant increase in total body copper. Boone & Schoffeniels (1979) concluded that the copper required for haemocyanin synthesis was from an endogenous store, probably the midgut gland. However, a simple calculation indicates that this could not have taken place. The crabs they used weighed 30 g (10 g dry weight). It is reasonable to assume that haemolymph volume was approx. 30 % fresh weight (Prosser, 1973) and that midgut gland constituted 5 % of the dry weight (probably an over-estimate). When haemocyanin

content doubled, the total amount of copper in the blood must have increased from 243 μg to 558 μg. If the midgut gland had a normal copper content (85 μg Cu g^{-1} d.w.; Rainbow, 1985; Bjerregaard & Vislie, 1986), then even if its entire copper load were removed it would only yield 42.5 μg of copper. This represents 13 % of the copper required for the synthesis of new haemocyanin. It is difficult to envisage where else the copper could have originated from in view of Boone & Schoffeniels (1979) finding that total body copper load was unchanged.

Taylor (1977) investigated the oxygen content of *C. maenas* exposed to different salinities. From his data, it is possible to calculate haemocyanin concentration in the blood of the crabs, (at the oxygen tensions Taylor measured, the haemocyanin was fully saturated; 1mg of haemocyanin binds 0.0003 ml O_2). At salinities from 34 ‰ down to 12 ‰ haemocyanin concentration was in the range of 44 to 46 mg ml^{-1}. This casts further doubt on Boone & Schoffeniels' (1979) findings.

REGULATION OF HAEMOCYANIN CONCENTRATION

From the foregoing account it is clear that haemolymph protein composition and concentration vary widely but that a number of factors produce predictable changes. Furthermore, most of the variation in concentration is associated with changes in haemocyanin levels and blood volume. The absolute copper and haemocyanin contents are much less variable. It is reasonable to infer that control mechanisms exist to regulate haemocyanin concentration, although such mechanisms may not be finely tuned. For control to be effective there must be some means of regulating copper availability from endogenous sources or accumulation of copper from exogenous sources, or both. Also, there must be be some means of monitoring the changes that occur. Little is known of these processes.

The midgut gland has been identified as a likely copper store and metallothioneins as proteins capable of holding copper in a non-toxic form (see review by Rainbow, 1988). Engel (1987) concludes that there is clear evidence supporting the involvement of metallothionein in haemocyanin synthesis as part of the normal physiology of decapods. But, when reviewing his data, it is difficult to envisage how such a mechanism could exert a significant effect on blood copper levels. For a 100 g f. w. *Callinectes sapidus* haemolymph volume is 25 ml (Gleeson & Zubkoff, 1977), and the midgut gland is likely to constitute approx. 8 % of the crab's fresh weight. Using the copper concentrations given by Engel (1987), the total copper content of the midgut gland during the moult cycle would vary between 20 to 122 μg Cu while that of the haemolymph would vary between 635 to 2387 μg Cu (assuming no change in blood volume during the moult cycle). Thus, the change in copper content of the midgut gland represents only 5 % of the change in copper content of the blood. If it is assumed that blood volume increased during the moult (stage A_2, soft crab) by a factor of 3, as is seen in other decapods (see earlier), then Engel's (1987) data indicate a dramatic increase in the total copper content of the blood.

In addition, the release of metallothionein-bound copper to convert apohaemocyanin into haemocyanin remains in doubt (Brouwer et al., 1986). There is evidence which indicates that copper is deposited at the free ends of midgut cells and may be excreted into the alimentary tract and lost (Rainbow, 1988). Ogura (1959) found that such excretion was promoted by removal of the eyestalks. This may indicate that copper storage and

release are under hormonal control. Hormones may also be involved in the regulation of metallothionein synthesis and breakdown, but at present no model exists to explain how copper is moved to cyanocytes, or how affinities of apohaemocyanin and metallothionein for copper are modulated. Metallothionein synthesis is copper-induced by gene activation (Hunziker & Kagi, 1985; Rainbow, 1988), but why copper released during metallothionein breakdown does not re-induce metallothionein synthesis is not clear. In Man, copper exposure induces simultaneous synthesis of metallothionein and apoceruloplasmin (Harrison & Hoare, 1980). It remains to be seen if an analogous response occurs with metallothionein and apohaemocyanin synthesis in crabs.

The important contribution haemocyanin makes to the total haemolymph protein concentration is beyond doubt but, in turn, haemocyanin levels depend on apohaemocyanin concentration and the availablity of copper from endogenous stores. In the final section, copper levels in decapods will be briefly discussed in this context.

COPPER LEVELS IN DECAPODS

Bryan (1968) found that whole body copper levels (including exoskeleton) in decapod crustaceans fall within a narrow range (20–35 $\mu g\ g^{-1}$ fresh weight). It has been inferred from this that decapods are capable of regulating copper levels in their bodies. Less attentation has been paid to the fact that within specific tissues, copper concentrations vary widely. For example, in the blood of lobsters, copper levels can vary from 10 to 150 $\mu g\ ml^{-1}$ (Senkbeil & Wriston, 1981) while in the midgut gland of shrimps a range from 13 to 480 $\mu g\ g^{-1}$ dry weight is not uncommon (Djangmah & Grove, 1970). It is difficult to imagine how the tissue concentrations are adjusted with respect to one another to ensure a steady whole body level.

For a 100g fresh weight (30 g dry weight) *Carcinus maenas* the total body load is estimated to be 2000 to 3500 µg Cu (Bryan, 1968). From data presented earlier the contribution from exoskeleton (80 % dry weight) is approximately 200 µg. Thus, the remaining 20 % dry weight which includes the soft tissues and dehydrated haemolymph, contains 1800 to 3300 µg Cu. This gives tissue concentration in the range 300 to 550 $\mu g\ g^{-1}$ dry weight. White & Rainbow (1985) made theoretical estimates of tissue copper levels and arrived at a value of 84 $\mu g\ g^{-1}$ dry weight (26.3 $\mu g\ g^{-1}$ enzymatic requirement plus 57.4 $\mu g\ g^{-1}$ haemocyanin requirement) which show little agreement with the above values. White & Rainbow's (1985) estimates were reached by making a number of unwarranted assumptions. For example, it is doubtful that their assumption that zinc and copper associated enzymes are present in a constant proportion in all organisms is valid. Consider carbonic anhydrase, a zinc associated enzyme which is found in high concentrations in vertebrate blood (within the erythrocytes) but is almost completely lacking from crustacean blood.

In a 100g fresh weight (30g dry weight) crab, copper concentrations measured in the haemolymph fall within the range 50–70 $\mu g\ g^{-1}$ (Uglow, 1969a; Boone & Schoffeniels, 1979; Bjerregaard & Vislie, 1986). The validity of this range is confirmed by converting measured haemolymph protein concentrations (usually 40–80 $mg\ ml^{-1}$, see earlier) to copper concentration, assuming that 80 % of the protein is haemocyanin. This gives copper concentrations in the range 54–109 $\mu g\ ml^{-1}$. Blood volume in *C. maenas* is estimated to be 30–37 % of fresh weight (Nicol, 1967; Prosser, 1973). Thus, in a 100g fresh

weight specimen there would be at least 30 ml of haemolymph. Therefore, the copper content of the blood would be 1500 to 2100 µg Cu. This represents between 64–83 % of the total copper content of the soft parts. Similarly, Djangmah (1970) reports a haemolymph copper concentration of 106 µg ml^{-1} in the shrimp, *Crangon vulgaris*. With a blood volume of 10 % f. w. (Smith & Dall, 1982), this gives a haemolymph copper content for a 2g fresh weight (0.58g dry weight) specimen of 21 µg Cu. Using Bryan's (1968) upper estimate for whole body copper (27.5 µg Cu g^{-1} fresh weight), the predicted whole body copper load (including haemolymph and all tissues) would be approx. 55 µg Cu. Thus, at least 50 % of the copper content of the soft parts is located in the haemolymph (when exoskeleton copper is excluded). If a larger blood volume estimate is used, the proportion of the whole body copper stored in the blood increases.

An important factor affecting calculations of whole body copper loads from tissue concentrations is that blood cannot be removed from the tissues prior to the analysis and may be counted twice. Nonetheless, it is clear that the haemolymph copper level is the principal factor in determining the copper content of the whole animal.

Using data from Jennings & Rainbow (1979) and Bjerregaard & Vislie (1986), calculations based on a 30g dry weight crab show the copper contents of the tissues to be: exoskeleton 192 µg; muscle 42 µg; gill 53 µg; and midgut gland 28 µg. The rest of the tissues (including dehydrated haemocyanin) constitute 5 % of the crabs' dry weight. If the average copper content of these tissues is approx. 75 µg g^{-1} dry weight (Rainbow, 1985), they contribute a further 113 µg Cu to the body load. The remaining copper is bound in haemocyanin in the dehydrated haemolymph. It was estimated earlier that this would constitute a further 1800 µg of copper. Therefore, in this example, the whole body copper load is 2210 µg, the whole body concentration (including exoskeleton) is 74 µg g^{-1} dry weight, the tissue concentration (excluding exoskeleton) is 336 µg g^{-1} dry weight, the enzymic copper requirement of the tissues (excluding haemocyanin) is 36 µg g^{-1} dry weight and the haemocyanin copper requirement, expressed on a tissue dry weight basis (c.f. White & Rainbow, 1985) is 300 µg g^{-1} dry weight. The copper requirement for haemocyanin is more than 8 times as great as the tissue copper requirement, not twice as great as implied by White & Rainbow (1985).

When measured, copper concentrations of individual tissues (taken from Bjerregaard & Vislie, 1986, and which presumably give an indication of tissue enzymatic requirements) are compared with the average tissue copper content deduced above, the midgut gland, the gills, the heart and the hypodermis have 2 to 3 times the average tissue value while muscle, testes and carapace have somewhat less than the average tissue value. The biochemical significance of these differences awaits investigation.

With regard to copper distribution, it is clear from the above that at least 50–60 % of the copper in decapods is stored in the haemolymph. This contrasts with earlier reports, for example Johnston & Barber (1969), who state that 75 % of the copper in lobsters is stored in the midgut gland. The current analysis suggests that a more accurate estimate for the latter tissue would be < 3 %. Furthermore, it appears that sophisticated mechanisms of copper regulation in decapods need not be envisaged. Whole body copper levels are likely to remain within a narrow range despite large fluctuations in concentrations in tissues such as the midgut gland, providing the total copper content of the blood does not alter much. The data presented in this article indicate that the total copper content of the blood does indeed remain within a narrow range during moulting, and during exposure

to salinity stress and hypoxia. The only circumstances under which whole body copper levels might fall significantly is during starvation. Experiments are currently underway to investigate this possibility.

SUMMARY

The tissue and haemolymph copper concentrations in decapods are far greater than have been estimated theoretically in the past. Great caution should be exercised in assigning a physiological regulatory role to the midgut gland regarding copper levels, although there is clear evidence that the midgut gland is involved in the handling of excess copper prior to elimination from the body. The available evidence suggests that while the midgut gland is capable of releasing copper for haemocyanin synthesis, haemolymph haemocyanin concentration would be affected only over long periods. For such synthesis to occur, copper in the midgut gland would have to be replenished continuously.

Similarly, the role of metallothionein in the storage and release of copper in the normal regulation of blood haemocyanin levels is by no means certain, even though metallothionein does bind excess copper, thereby reducing toxicity.

Identification of the mechanisms by which copper levels are regulated would be of great value in understanding both the normal physiology of decapods and their responses to copper pollution.

Acknowledgements. We are grateful to Professor R. E. Weber and Dr. P. S. Rainbow for valuable discussions during the preparation of this manuscript.

LITERATURE CITED

Adelung, D. & Ponat, A., 1977. Studies to establish an optimal diet for the decapod crab, *Carcinus maenas* under culture conditions. – Mar. Biol. *44*, 287–292.

Adiyodi, R. G., 1969. Protein metabolism in relation to reproduction and moulting in the crab *Paratelphusa hydronomous:* Part III. RNA activity and protein yolk biosynthesis during normal vitellogenesis and under conditions of acute inanition. – Indian J. exp. Biol. *7*, 13–16.

Ahsanullah, M., Negilski, D. S. & Mobley, M. C., 1981. Toxicity of zinc, cadmium and copper to the shrimp *Callianassa australiensis.* III. Accumulation of metals. – Mar. Biol. *64*, 311–316.

Andrews, P., 1967. Seasonal variation in the haemolymph composition in the crayfish *Orconectes limosus.* – Z. vergl. Physiol. *57*, 7–43.

Andrieux, N., Porcheron, P., Berreur-Bonnenfant, J. & Dray, F., 1976. Détermination du taux d'ecdysone au cours du cycle d'intermue chez le crabe *Carcinus maenas;* comparaison entre individus sains et parasités par *Sacculina carcini.* – C. r. hebd. Séanc. Acad. Sci. Paris *283*, 1429–1432.

Arumugam, M. & Ravindranath, M. H., 1986. Significance of variation in haemolymph copper-free and -bound proteins during ageing and the time of day in the crab *Scylla serrata* (Forskal). – Archs int. Physiol. Biochim. *94*, 11–17.

Bang, F. B., 1983. Crustacean disease responses. In: The biology of Crustacea. Ed. by A. J. Provenzano. Acad. Press, New York, *6*, 113–153.

Bauchau, A. G. & Mengeot, J. C., 1978. Structure et fonction des hémocytes chez les Crustacés. – Archs Zool. exp. gén. *119*, 227–248.

Bjerregaard, P. & Vislie, T., 1986. Effect of copper on ion- and osmoregulation in the shore crab *Carcinus maenas.* – Mar. Biol. *91*, 69–76.

Boone, W. R. & Schoffeniels, E., 1979. Hemocyanin synthesis during hypo-osmotic stress in the shore crab *Carcinus maenas* (L.). – Comp. Biochem. Physiol. *63B*, 207–214.

Brouwer, M., Whaling, P. & Engel D. W., 1986. Copper-metallothioneins in the American lobster, *Homarus americanus:* potential role as Cu (I) donors to apohaemocyanin. – Envir. Hlth Perspect. *65,* 93–100.

Bryan, G. W., 1968. Concentrations of zinc and copper in the tissues of decapod crustaceans. – J. mar. biol. Ass. U.K. *48,* 303–321.

Bryan, G. W., 1984. Pollution due to heavy metals and their compounds. In: Marine ecology. Ed. by O. Kinne. Wiley, London, *5,* 1289–1431.

Busselen, P., 1970. Effects of moulting cycle and nutritional conditions on haemolymph proteins in *Carcinus maenas.* – Comp. Biochem. Physiol. *37,* 73–83.

Butler, P. J., Taylor, E. W. & McMahon, B. R., 1978. Respiratory and circulatory changes in the lobster *(Homarus vulgaris)* during long term exposure to moderate hypoxia. – J. exp. Biol. *73,* 131–146.

Cantacuzene, J., 1925. Immunité d'*Eupagurus prideauxii* vis-a-vis des poisons de l'*Adamsia pallatia.* – C. r. Séanc. Soc. Biol. *92,* 1133–1136.

Carlisle, D. B., 1954. On the hormonal inhibition of moulting in decapod Crustacea. – J. mar. biol. Ass. U.K. *33,* 61–63.

Castell, J. D. & Budson, S. D., 1974. Lobster nutrition: The effect on *Homarus americanus* of dietary protein levels. – J. Fish. Res. Bd Can. *31,* 1363–1370.

Ceccaldi, H. J., 1968. Recherches sur la biologie des associations entre protéines et caroténoides chez les Crustacés Decapodes; aspects métaboliques et moléculaires. D. Thesis, Univ. d'Aix-Marseille.

Chaisemartin, R. A., Chaisemartin, C. & Breton, J., 1968. Variations du protéinogramme sérique chez les Astacidae. – C. r. Séanc. Soc. Biol. *162,* 2207–2211.

Claybrook, D. L., 1983. Nitrogen metabolism. In: The biology of Crustacea. Ed. by L. H. Mantel. Acad. Press, New York, *5,* 163–213.

Crowley, G. J., 1963. Studies in arthropod serology. Part 1: Changes in hemolymph composition as related to ecdysal cycle. – Wasmann J. Biol. *22,* 177–191.

Dall, W., 1974. Indices of nutritional state of the western rock lobster, *Panulirus longipes* (Milne Edwards). I. Blood and tissue constituents and water content. – J. exp. mar. Biol. Ecol. *16,* 167–180.

Damboviceanu, A., 1928. Variations des substances protéiques coagulables par la chaleur dans le plasma des *Carcinus maenas* normaux et sacculinés. – C. r. Séanc. Soc. Biol. *98,* 1633–1635.

Depledge, M. H., Chan, R. & Loh, T. T., 1986. Iron distribution and transport in *Scylla serrata* (Forskal). – Asian mar. Biol. *3,* 101–110.

Djangmah, J. S., 1970. The effects of feeding and starvation on copper in the blood and hepatopancreas, and on blood proteins of *Crangon vulgaris* (Fabricius). – Comp. Biochem. Physiol. *32,* 709–731.

Djangmah, J. S. & Grove, D. J., 1970. Blood and hepatopancreas copper in *Crangon vulgaris* (Fabricius). – Comp. Biochem. Physiol. *32,* 733–745.

Drilhon, A., 1936. Quelques constantes chimiques et physicochimiques du milieu intérieur du crabe sacculiné *(Carcinus maenas).* – C. r. hebd. Séanc. Acad. Sci. Paris *202,* 981–982.

Drilhon, A. & Pora, E. A., 1936. Sacculine et crabe. Étude chimique et physico-chimique. – Trav. Stn biol. Roscoff *14,* 111–120.

Durliat, M., 1974. Étude de la coagulation de l'hémolymphe chez les Crustacés-Decapoda. Analyse éléctrophorétique des protéins du sérum et du plasma d'Écrevisse *(Astacus leptodactylus).* – C. r. hebd. Séanc. Acad. Sci. Paris *278,* 473–476.

Engel, D. W., 1987. Metal regulation and moulting in the blue crab, *Callinectes sapidus:* copper, zinc and metallothionein. – Biol. Bull. mar. biol. Lab., Woods Hole *172,* 69–82.

Engel, D. W. & Brouwer, M., 1984. Trace metal binding proteins in marine molluscs and crustaceans. – Mar. envir. Res. *13,* 177–194.

Engel, D. W., Brouwer, M. & Thurburg, F. P., 1985. Comparison of metal metabolism and metal binding proteins in the blue crab and the american lobster. In: Marine pollution and physiology: recent advances. Ed. by F. J. Vernberg, F. P. Thurberg, A. Calabrese & W. Vernberg. Univ. of South Carolina Press, Columbia, 229–245.

Evans, E. E., Painter, B., Evans, M. L., Weinheimer, P. & Acton, R. T., 1968. An induced bactericidin in the spiny lobster, *Panulirus argus.* – Proc. Soc. exp. Biol. Med. *128,* 394.

Frédéricq, L., 1879. Note sur le sang du homard. – Bull. Acad. r. Belg. Cl. Sci. *47,* 409–413.

Ghidalia, W., 1985. Structural and biological aspects of pigments. In: The biology of Crustacea. Ed. by D. H. Bliss & L. H. Mantel. Acad. Press, New York, *9*, 301–394.

Ghidalia, W., Fine, J. M. & Marnaux, M., 1982. On the presence of an iron-binding protein in the serum of a decapod crustacean (*Macropipus puber* (Linné)). – Comp. Biochem. Physiol. *41B*, 349–354.

Ghiretti-Magaldi, A., Milanesi, C. & Tognon, G., 1977. Hemopoeisis in Crustacea Decapoda: Origin and evolution of hemocytes and cyanocytes of *Carcinus maenas*. – Cell Differ. *6*, 167–186.

Gilles, R., 1977. Effects of osmotic stress on the protein concentration and pattern of *Eriocheir sinensis* blood. – Comp. Biochem. Physiol. *56A*, 109–114.

Glavind, J., 1948. Studies on the coagulation of crustacean blood. Bucks, Copenhagen, 137 pp.

Gleeson, R. A. & Zubkoff, P. L., 1977. The determination of hemolymph volume in the blue crab *Callinectes sapidus*, utilising ^{14}C-thiocyanate. – Comp. Biochem. Physiol. *56A*, 411–413.

Glynn, J. P., 1968. Studies on the ionic, protein and phosphate changes associated with the moult cycle of *Homarus vulgaris*. – Comp. Biochem. Physiol. *26*, 937–946.

Guary, J. C. & Negrel, R., 1980. Plutonium and iron association with metal-binding proteins in the crab *Cancer pagurus* (L.). – J. exp. mar. Biol. Ecol. *42*, 87–98.

Hagerman, L., 1983. Haemocyanin concentration of juvenile lobsters *(Homarus gammarus)* in relation to moulting cycle and feeding conditions. – Mar. Biol. *77*, 11–19.

Hagerman, L. & Uglow, R. F., 1985. Effects of hypoxia on the respiratory and circulatory regulation of *Nephrops norvegicus*. – Mar. Biol. *87*, 273–278.

Hagerman, L. & Weber, R. E., 1981. Respiratory rate, haemolymph oxygen tension and haemocyanin level in the shrimp *Palaemon adspersus* Rathke. – J. exp. mar. Biol. Ecol. *54*, 13–20.

Hagerman, L. & Pihl Baden, S., 1988. *Nephrops norvegicus:* field study of effects of oxygen deficiency on haemocyanin concentration. – J. exp. mar. Biol. Ecol. *116*, 135–142.

Hammond, P. B. & Beliles, R. P., 1980. Metals. In: Casarett and Doull's Toxicology. Ed. by J. Doull, C. D. Klaassen & M. O. Amdur. Macmillan, New York, 409–467.

Harrison, P. M. & Hoare, R. J., 1980. Metals in biochemistry. Chapman & Hall, London, 80 pp.

Henke, G. A., 1985. Variations of hemolymph proteins of the common shore crab *Carcinus maenas* after a bacterial infection. – Mar. Biol. *87*, 181–183.

Hildemann, W. H., 1974. Phylogeny of immune responses in invertebrates. – Life Sci. *14*, 605–614.

Hildemann, W. H. & Reddy, A. L., 1973. Phylogeny of immune responsiveness: marine invertebrates. – Fedn Proc. Fedn Am. Socs exp. Biol. *32*, 2188–2194.

Horn, E. C. & Kerr, M. S., 1963. Hemolymph protein and copper concentrations of adult blue crabs (*Callinectes sapidus*, Rathbun). – Biol. Bull. mar. biol. Lab., Woods Hole *125*, 499–507.

Huebers, H. A., Huebers, E., Finch, C. A. & Martin, A. W., 1982. Characterisation of an invertebrate transferrin from the crab, *Cancer magister* (Arthropoda). – J. comp. Physiol. *148*, 101–109.

Hunziker, P. E. & Kagi, J. H. R., 1985. Metallothionein. In: Metalloproteins. Ed. by P. M. Harrison. Macmillan, London, *2*, 149–181.

Jennings, J. R. & Rainbow, P. S., 1979. Studies on the uptake of cadmium by the crab *Carcinus maenas* in the laboratory. I. Accumulation from seawater and a food source. – Mar. Biol. *50*, 131–139.

Johnston, W. & Barber, A. A., 1969. Reconstitution of functional hemocyanin from apohemocyanin: the hepatopancreas as a copper donor. – Comp. Biochem. Physiol. *28*, 1259–1273.

Kerkut, G. A., Moritz, P. M. & Munday, K. A., 1961. Variations of copper concentrations in *Carcinus maenas*. – Cah. Biol. mar. *2*, 399–408.

Kerridge, P. M. T., 1926. The buffering power of the blood of *Maia squinado*. – J. Physiol. *62*, 65–73.

Kleinholz, L. H., 1985. Biochemistry of crustacean hormones. In: The biology of Crustacea. Ed. by D. E. Bliss & L. H. Mantel. Acad. Press, New York, *9*, 464–522.

Leone, C. A., 1953. Preliminary observations on intraspecific variation of the levels of total protein in the sera of some decapod crustacea. – Science, N. Y. *118*, 295–296.

Mangum, C. P., 1983. Oxygen transport in the blood. In: The biology of Crustacea. Ed. by L. H. Mantel. Acad. Press, New York, *5*, 73–429.

Manwell, C. & Baker, C. M. A., 1963. Starch gel electrophoresis of sera from some marine arthropods: Studies on the heterogeneity of haemocyanin and on a "ceruloplasmin-like" protein. – Comp. Biochem. Physiol. *8*, 193–208.

Martin, J.-L. M., 1973. Iron metabolism in *Cancer irroratus* (Crustacea, Decapoda) during the

intermoult cycle with special reference to iron in the gills. – Comp. Biochem. Physiol. *46A*, 123–129.

Miller, V. H., Ballback, R. S., Pauley, G. B. & Krassner, S. M., 1972. A preliminary physicochemical characterisation of an agglutinin found in the hemolymph of the crayfish *Procambarus clarkii*. – J. Invertebr. Pathol. *19*, 83–93.

Nicol, J. A., 1967. The biology of marine animals. Pitman, London, 699 pp.

Nott, J. A. & Martin, L. J., 1986. Adaptation of a quantitative programme for the X-ray analysis of solubilized tissue as microdroplets in the transmission electron microscope: application to the moult cycle of the shrimp *Crangon crangon* (L.) – Histochem. J. *18*, 507–518.

Ogura, K., 1959. Midgut gland cells accumulating iron and copper in the crayfish *Procambarus clarkii*. – Annotnes zool. jap. *32*, 133–142.

Passano, L. M., 1960. Moulting and its control. In: The physiology of Crustacea. Ed. by T. H. Waterman. Acad. Press, New York, *1*, 473–536.

Pauley, G. B., 1974. Comparison of a natural agglutinin in the hemolymph of the blue crab *Callinectes sapidus*, with agglutinins of other invertebrates. – Contemp. Topics Immunobiol. *4*, 421–260.

Pequeux, A., Vallota, A. C. & Gilles, R., 1979. Blood proteins as related to osmoregulation in crustacea. – Comp. Biochem. Physiol. *64A*, 433–435.

Phillips, D. J. H., 1976. The common mussel *Mytilus edulis* as an indicator of pollution by zinc, cadmium, lead and copper. I. Effects of environmental variables on uptake of metals. – Mar. Biol. *38*, 59–69.

Prosser, C. L., 1973. Comparative animal physiology. Saunders, Philadelphia, 996 pp.

Rainbow, P. S., 1985. Accumulation of Zn, Cu and Cd by crabs and barnacles. – Estuar. coast. Shelf Sci. *21*, 669–686.

Rainbow, P. S., 1988. The significance of trace metal concentrations in decapods. – Symp. zool. Soc. Lond. *59*, 291–313.

Robertson, J. D., 1937. Some features of the calcium metabolism of the shore crab (*Carcinus maenas* Pennant). – Proc. R. Soc. (B) *124*, 162–182.

Robertson, J. D., 1960. Osmotic and ionic regulation. In: The physiology of Crustacea. Ed. by T. H. Waterman. Acad. Press, New York, *1*, 317–339.

Rothschild, M. A. & Oratz, M., 1976. Albumin synthesis and degradation. In: Structure and function of plasma proteins. Ed. by A. C. Allison. Plenum Press, New York, *2*, 79–105.

Schoffeniels, E., 1976. Adaptations with respect to salinity. – Biochem. Soc. Symp. *41*, 179–204.

Senkbeil, E. G. & Wriston, J. C., 1981. Hemocyanin synthesis in the american lobster, *Homarus americanus*. – Comp. Biochem. Physiol. *68B*, 163–171.

Skinner, D. M., 1966. Breakdown and reformation of somatic muscle during the molt cycle of the land crab, *Gecarcinus lateralis*., – J. exp. Zool. *163*, 115–124.

Smith, D. M. & Dall, W., 1982. Blood protein, blood volume and extracellular space relationships in two *Penaeus* spp. (Decapoda : Crustacea). – J. exp. mar. Biol. Ecol. *63*, 1–15.

Spindler-Barth, M., 1976. A bacterial infection in the common shore crab *Carcinus maenas* and the fiddler crab *Uca pugilator*. – Mar. Biol. *36*, 1–4.

Stewart, J. E., Dingle, J. R. & Odense, P. H., 1966. Constituents of the hemolymph of the lobster, *Homarus americanus* Milne Edwards. – Can. J. Biochem. *44*, 1447–1459.

Stewart, J. E., Cornick, J. W., Foley, D. M., Li, M. F. & Bishop, C. M., 1967a. Muscle weight relationship to serum proteins, hemocytes and hepatopancreas in the lobster, *Homarus americanus*. – J. Fish. Res. Bd Can. *24*, 2339–2354.

Stewart, J. E., Cornick, J. W. & Dingle, J. R., 1967b. An electronic method for counting lobster (*Homarus americanus* Milne Edwards) hemocytes and the influence of diet on hemocyte numbers and hemolymph proteins. – Can. J. Zool. *45*, 291–304.

Stevenson, J. R., 1985. Dynamics of the integument. – In: The biology of Crustacea. Ed. by D. E. Bliss & L. H. Mantel. Acad. Press, New York, *9*, 2–42.

Taylor, A. C., 1977. The respiratory responses of *Carcinus maenas* (L.) to changes in environmental salinity. – J. exp. mar. Biol. Ecol. *29*, 197–210.

Tyler, A. & Metz, C. B., 1945. Natural heteroagglutinins in the serum of the spiny lobster, *Panulirus interruptus*. – J. exp. Zool. *100*, 387–406.

Uglow, R. F., 1969a. Haemolymph protein concentrations in portunid crabs. 1. Studies on adult *Carcinus maenas*. – Comp. Biochem. Physiol. *30*, 1083–1090.

Uglow, R. F., 1969b. Haemolymph protein concentrations in portunid crabs. 2. The effects of imposed fasting on *Carcinus maenas*. – Comp. Biochem. Physiol. *31*, 959–967.

Uglow, R. F., 1969c. Haemolymph protein concentrations in portunid crabs. 3. The effect of *Sacculina*. – Comp. Biochem. Physiol. *31*, 969–973.

Vendrely, R., Ghidalia, W., Coirault, Y. & Montgomery, C., 1979. Coagulation de l'hémolymphe chez les crustacés. Mise en évidence par éléctrophorése d'un composant plasmatique intervenant dans l'hémostase chez un crustacé du Groupe a *(Cancer pagurus)*. – C. r. hebd. Séanc. Acad. Sci. Paris. *289*, 849–851.

Weatherall, D. J., Ledingham, J. G. G. & Warrell, D. A., 1984. Oxford textbook of medicine. Oxford Medical Publ., Oxford, 27. 7 pp.

White, S. L. & Rainbow, P. S., 1984. Zinc flux in *Palaemon elegans* (Crustacea: Decapoda): moulting, individual variation and tissue distribution. – Mar. Ecol. Prog. Ser. *19*, 153–166.

White, S. L. & Rainbow, P. S., 1985. On the metabolic requirements for copper and zinc in mollucs and crustaceans. – Mar. envir. Res. *16*, 215–229.

White, S. L. & Rainbow, P. S., 1987. Heavy metal concentrations and size effects in the mesopelagic decapod crustacean *Systellaspis debilis*. – Mar. Ecol. Prog. Ser. *37*, 147–151.

Wieser, W., 1965. Electrophoretic studies on blood proteins in an ecological series of isopod and amphipod species. – J. mar. biol. Ass U. K. *45*, 507–523.

Windom, H. L. & Smith, R. G., 1979. Copper concentrations in surface waters off the southeastern Atlantic coast, U. S. A. – Mar. Chem. *7*, 157.

Wong, V. W. T. & Rainbow, P. S., 1986a. Two metallothioneins in the shore crab *Carcinus maenas*. – Comp. Biochem. Physiol. *83A*, 149–156.

Wong, V. W. T. & Rainbow, P. S., 1968b. Apparent and real variability in the presence and metal contents of metallothioneins in the crab *Carcinus maenas* including the effects of the isolation procedure and metal induction., – Comp. Biochem. Physiol. *83A*, 157–177.

Zuckerkandl, E., 1957. La teneur en cuivre de l'hépato-pancreas de *Maia squinado* aux divers stades d'intermue. – C. r. Séanc. Soc. Biol. *151*, 676–679.

Zuckerkandl, E., 1960. Hemocyanin et cuivre chez un Crustacé Decapode dans leurs rapports avec le cycle d'intermue. – Annls Inst. océanogr.Monaco *38*, 1–122.