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Growth of tissues related to haemolymph copper throughout the moult cycle of the lobster *Homarus gammarus*

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ABSTRACT: The relationship between tissue weights and haemolymph copper levels in field-collected European lobsters *Homarus gammarus* was investigated to assess whether haemolymph copper concentration can be used to reliably determine nutritional condition in lobsters. During the moult cycle, the soft tissue weight (mostly muscle) increased concomitantly with a decrease in haemolymph weight while exoskeleton weight increased from postmoult to intermoult. Haemolymph copper concentration gradually increased from postmoult to premoult with a range of 29.7 (Stage B) to 163.5 µg g^{-1} wet weight (ww) (Stage D_0). Our measurements suggest that the main processes that increase haemolymph copper concentration is a reduction in haemolymph space and an increase in haemolymph copper content, indicating synthesis of new haemocyanin for the oxygen supply of growing tissues. During the moult cycle, haemolymph copper content was proportional to soft tissue mass and amounted to 43.6 µg Cu g^{-1} of soft-tissue ww (95% confidence limits = 35.5 and 51.6 µg g^{-1}). Models for estimating soft-tissue and haemolymph wet weights from the haemolymph copper concentration and moult stage are presented. The use of haemolymph copper concentration as an index of nutritional condition reflecting tissue growth and food quantity and quality is validated.

KEY WORDS: European lobster · Exoskeleton · Haemolymph · Muscle · Soft tissues · Haemocyanin · Copper requirement · Condition index

INTRODUCTION

Variations in haemolymph copper, protein and haemocyanin levels during the moult cycle have been investigated in many crustaceans. These 3 parameters decrease to low levels after ecdysis, increase during intermoult and reach their maximum during the premoult period (Zuckerkandl 1960, Busselen 1970, Dall 1974, Truchot 1978, Smith & Dall 1982, Hagerman 1983, Engel & Brouwer 1991, Scott-Fordsmand & Depledge 1997). The postmoult growth of muscle reduces the haemolymph volume, and thus the concentrations of haemolymph protein proportionally increase (Smith & Dall 1982). Accordingly, measurements of these concentrations have been proposed as an index of muscle weight and haemolymph space. For the American lobster *Homarus americanus*, Stewart et al. (1967) and Castell & Budson (1974) proved that muscle weight is correlated to haemolymph protein concentration. Additionally, the haemolymph protein concentration was proposed as an index of haemolymph volume in the western rock lobster *Panulirus longipes* (Dall 1974) and the penaeid prawn *Penaeus esculentus* (Smith & Dall 1982). Haemolymph copper concentration is also a reliable indicator of the haemolymph volume in intermoult shore crabs *Carcinus aestuarii* (reported as *C. mediterraneus*: Devescovi & Lucu 1995).

The body copper concentration in decapod crustaceans is regulated within the range of 20 to 35 µg g^{-1} wet weight (ww) (Bryan 1968). Most of the copper is metabolically active. At least 50 to 60 % of the body copper is stored in the haemolymph, bound to the respiratory pigment haemocyanin, and approximately 30 to 50% is bound to enzymes in soft tissues. Metabolically inactive copper amounts to less than 10% of the

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total and is stored in the exoskeleton (White & Rainbow 1985, Depledge 1989, Depledge & Bjerregaard 1989, Rainbow 1993).

Despite a steady body copper concentration, haemolymph copper concentration shows broad variations in relation to hypoxia, salinity stress and starvation (Depledge & Bjerregaard 1989). Hypoxia-induced haemocyanin synthesis has been reported for the American lobster *Homarus americanus* (Senkbeil & Wriston 1981) and the Norway lobster *Nephrops norvegicus* (Hagerman & Uglow 1985). Thus, an increase in haemolymph copper content can be expected in decapods exposed to low ambient oxygen concentrations. After hypo-osmotic stress, haemolymph copper concentration also increased in the shore crab *Carcinus maenas* (Boone & Schoffeniels 1979) and in the American lobster (Senkbeil & Wriston 1981), but this was due to a transitory decrease in the haemolymph volume (Devescovi & Lucu 1996), not to haemocyanin synthesis (Senkbeil & Wriston 1981, Depledge 1989).

Copper, haemocyanin and protein concentrations in crustaceans decrease during starvation (Stewart et al. 1967, Uglow 1969, Djangmah 1970, Castell & Budson 1974, Smith & Dall 1982, Hagerman 1983), which may be a dilution effect rather than the result of haemocyanin catabolism (Depledge & Bjerregaard 1989). During starvation the extracellular fluid volume increases due to the breakdown of tissues (Smith & Dall 1982).

The aim of the present study was to investigate the potential of haemolymph copper concentrations in estimating both weights of soft tissue and haemolymph for field-collected European lobsters *Homarus gammarus*. Since the moult cycle imposes constraints on haemolymph copper concentrations, lobsters at different moult stages were examined. Using the haemolymph copper concentration as an index of nutritional condition would be preferable in circumstances when lobsters cannot be sacrificed. Nondestructive techniques are valuable particularly in aquaculture and holding facilities during work with small populations, or in the field when an immediate result is required. Additionally, knowledge of the relationship between tissue weight and haemolymph copper levels could be used to assess copper requirements and physiological changes in field-collected and artificially reared lobsters.

MATERIALS AND METHODS

European lobsters *Homarus gammarus* of both sexes, weighing approximately 200 to 400 g, were collected by diving in the north Adriatic off Rovinj (Istria, Croatia) from October to December 1999. The salinity of the bottom water (28 m depth) in this period was 38.04 ± 0.19 ppt, temperature was 17.55 ± 3.65°C and oxygen saturation was 96.50 ± 0.01% (Center for Marine Research data base).

A total of 46 field-collected lobsters were examined, 24 males and 22 females. In the females, ovaries were either not visible or were very small and white, indicating immaturity. Moult stages were determined by external examination of the exoskeleton and by microscopic examination of pleopods (Aiken 1973, 1980). The number of lobsters per moult stage was: Stage A = 2; B = 3; C_1 and C_2 = 4 each; C_3, C_4, D_0, and D_1 = 7 each; D_2 = 3; D_3 = 2. We did not find lobsters in Stages E and D_4 in the field. The duration of these stages is only a few hours in total (Aiken 1980).

Live weight was measured after slightly raising the branchiostegites to allow water surrounding the gills to drain, and drying the lobsters' surface with filter paper. Using a hypodermic syringe, a haemolymph sample was withdrawn from the pericardial region of the cephalothorax of each lobster. Immediately after withdrawal, 0.9 ml of the haemolymph was added to Eppendorf vials, containing 100 µl of 10% potassium oxalate ($K_2C_2O_4 \cdot H_2O$, Sigma) as an anticoagulant, according to Stewart et al. (1966).

After centrifugation at 10 000 rpm for 15 min, the haemolymph copper concentration in the supernatants (diluted 50-fold with distilled water) was measured by flame atomic absorption spectrophotometry (VARIAN, AAS 1250) at 324.7 nm. For the preparation of the calibration curve, a $CuCl_2$ standard was used (Merck, Titrisol). Copper concentration was expressed in µg g^{-1} of haemolymph ww assuming a haemolymph specific weight of 1.0429 g ml^{-1} measured from 10 randomly chosen lobsters (coefficient of variation = 1.1%). The amount of haemolymph specific weight that is due to inorganic salt is close to that of seawater (1.0271 g ml^{-1}), and every 10 mg protein per ml elevates the specific weight by 0.0030 g ml^{-1} (Weast 1974). Thus, for an expected protein concentration ranging from 20 to 100 mg ml^{-1}, the specific weight range would be 1.0331 to 1.0571 g ml^{-1}, suggesting that use of this range would negligibly affect copper concentration.

The ventral ganglion of the lobster was pierced by a needle inserted through the ventral side of the cephalothorax and the carapace was lifted rapidly. Then, gills with adhering seawater and the gut with its contents were dissected. Hepatopancreas, abdominal and chelae muscles, carapace, the exoskeleton covering the abdomen and chelae, antennal glands, gastroliths (when present) and gonads in females were dissected and thoroughly blotted free of serum and coagulate. The tail, walking legs, tegument and adhering muscle internal to the carapace, mandibles,

antennae and other appendages were cut in small fragments and serum and coagulate were absorbed by filter paper. After weighing the dissected parts, the live weight (ww) of each lobster was subdivided into 3 components: haemolymph, exoskeleton and soft tissue.

Haemolymph wet weight. This was estimated (in grams) by subtracting the total weight of the dissected parts from the lobster's live weight. This method was proposed by Scott-Fordsmand & Depledge (1997) for estimating haemolymph volume in *Carcinus maenas.* (In contrast, tracer techniques usually overestimate the haemolymph space where haemocyanin circulates: Smith & Dall 1982.)

Exoskeleton wet weight. This weight (*E*, g) was calculated using an estimate of the weight of the exoskeleton covering the body (*BE*), the weight of the chelae exoskeleton (*CE*) and the weight of the gastroliths (*G*) according to the equation:

$$E = BE + CE + G \quad (1)$$

BE was estimated by multiplying the weight of the exoskeleton covering the abdomen by a factor of 4.5. This factor was obtained using the exuviae of 5 lobsters maintained in captivity. It represents the ratio of the body exuvium weight to the abdomen exuvium weight (coefficient of variation = 8.9%). We assumed that this proportion was close to that for the *BE* in live lobsters. Because of the difficulty in dissecting of the endophragmal skeleton and the cuticle from parts such as the walking legs, pleopods, mandibles etc., *BE* was not determined directly. Gastroliths were included in the exoskeleton weight because they are concretions composed of calcium carbonate, although they form on the lateral walls of the stomach and are not a part of the cuticle.

Soft tissue wet weight. This was estimated (in grams) by subtracting from the lobster's live weight the weights of haemolymph, exoskeleton, gut content and the water adhering to the gills. The gut content was determined by the difference of the gut weight before and after removal of gut contents. The weight of water adhering to gills was assessed in the same manner after pressing the gills between filter paper. Soft tissues included muscle, hepatopancreas, hypodermis, gonads, antennal glands, heart and nerves.

Haemolymph copper content (*Cu cont.*). This was calculated by multiplying the haemolymph weight and haemolymph copper concentration according to the equation:

$$Cu\ cont. = Haem. \times cCu \quad (2)$$

where *Haem.* is the haemolymph weight (g) and *cCu* is the haemolymph copper concentration expressed as µg g^{-1} of ww.

Tissue dry weights were determined by drying at 110°C for 24 h. Whole lobster dry weight was then calculated as a percentage of lobster live weight.

The computer software package SYSTAT (Version 5.01, Systat) was used for all analyses. For multiple regression analysis, grouped moult stages were transformed using dummy values of 0 and 1. Moult stages were grouped as early postmoult (Stages A, B and C_1), later postmoult (Stages C_2 and C_3), intermoult–early premoult (Stages C_4 and D_0) and premoult Stages (D_1, D_2 and D_3). The intermoult–early premoult group was the control (dummy variables of X_1, X_2 and X_3 = 0). Independent variables were selected by the backward elimination procedure (Zar 1999).

RESULTS

Tissue and copper fluctuations

Soft tissues of *Homarus gammarus* were mainly composed of muscle. Hepatopancreas, antennal glands and gonads in females amounted to 8.0 ± 1.3, 0.7 ± 0.2 and 1.0 ± 0.7% of the total soft tissue ww, respectively. Soft tissue weights varied from 35.4 (Stage C_4) to 60.5% of the lobsters' live weight (Stage D_0). During Premoult Stages D_0 to D_3, percentages of soft tissue were generally higher than in Postmoult Stages B to C_3 or in the Intermoult Stage C_4 (Fig. 1A). Haemolymph wet weights ranged from 45.3 (Stage C_2) to 12.1% (Stage D_3) of the lobsters' live weight. In contrast to soft tissue weight. Haemolymph weight decreased from postmoult to premoult (Fig. 1B). Exoskeleton weight expressed as a percentage of the lobsters' live weight varied from 10.2 (Stage B) to 30.1% (Stage C_4) and increased from postmoult to intermoult (Fig. 1C).

Haemolymph copper concentrations generally increased during the moult cycle (Fig. 2A) with a range of 29.7 (Stage B) to 163.5 µg g^{-1} ww (Stage D_0). Large fluctuations in haemolymph copper concentration were observed in Stages C_4 (38.4 to 109.6 µg g^{-1} ww) and D_0 (82.0 to 163.5 µg g^{-1} ww). Haemolymph copper content expressed per gram lobster live weight slightly increased during the moult cycle. However, the copper content in Stage A was close to that in premoult (Fig. 2B). In contrast, the haemolymph copper content expressed per gram dry weight decreased during the moult cycle (Fig. 2C) concomitant with an increase in the dry weight of the lobsters (Fig. 3). This was due to exoskeleton calcification, growth of soft tissues, and decreasing haemolymph weight during the moult cycle.

Prediction of tissue weights

Models for predicting of soft tissue and haemolymph weight were obtained by 2 methods. Multiple regression analysis was used to predict tissue weights from haemolymph copper concentration and moult-stage dummy variables. Subsequently, models were based on a biological constant—the metabolic haemolymph copper requirement.

Method 1

Results of the multiple regression analyses are shown in Table 1. The models obtained are:

$$Soft\ tiss.\% = 29.709 + 0.178 \times cCu + 4.724 \times X_1 + 3.249 \times X_2 \quad (3)$$

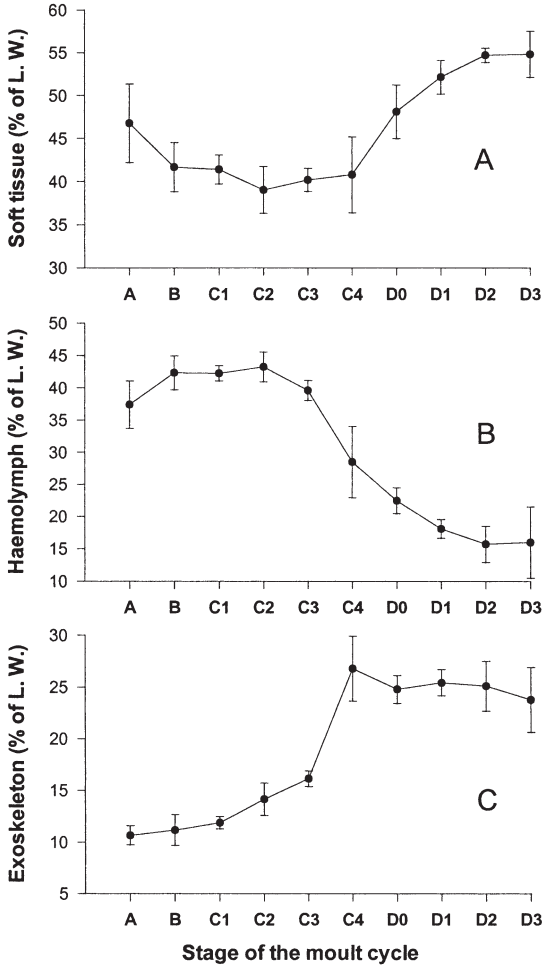

Fig. 1. *Homarus gammarus.* Weight of tissues throughout the moult cycle. Tissue weights expressed as percent of the lobsters' live weight (L.W.). Values are means ± SD. Number of lobsters per moult stage were Stage A = 2; B = 3; C_1 and C_2 = 4, each; C_3, C_4, D_0, D_1 = 7, each; D_2 = 3; D_3 = 2

Table 1. *Homarus gammarus.* Multiple regression analyses testing the dependence of soft tissue and haemolymph weights expressed as percent of the lobsters' live weight (L.W.) on haemolymph copper concentration (cCu) and moult-stage dummy variables X_1, X_2, and X_3; the latter (X_3) was deleted from models using backward selection of independent variables. Grouped moult stages, early postmoult (Stages A, B and C_1), later postmoult (C_2 and C_3), intermoult—early premoult (C_4 and D_0) and premoult (D_1, D_2 and D_3) stages were binary-transformed (0; 1) taking the intermoult—early premoult group as control (X_1, X_2, and X_3 = 0)

Variable	Coefficient	Stand. coeff.	Tolerance	p
Dependent variable: Soft tissue (%)				
cCu	0.178	1.111	0.428	<0.001
X_1	4.724	0.270	0.514	0.002
X_2	3.249	0.200	0.483	0.026
N = 46; multiple R^2 = 0.848; SE of estimate = 2.832; *y*-intercept = 29.709 (p < 0.001); ANOVA: F = 78.058; p < 0.001; df (regression) = 3, df (residual) = 42				
Dependent variable: Haemolymph (%)				
cCu	−0.165	−0.650	0.428	<0.001
X_1	9.251	0.335	0.514	<0.001
X_2	7.783	0.303	0.483	<0.001
N = 46; multiple R^2 = 0.947; SE of estimate: 2.634; *y*-intercept = 39.643 (p < 0.001); ANOVA: F = 250.757; p < 0.001; df (regression) = 3, df (residual) = 42				

$$Haem.\% = 39.643 - 0.165 \times cCu + 9.251 \times X_1 + 7.783 \times X_2 \quad (4)$$

where *Soft tiss.%* is the soft tissue weight expressed as percent lobster live weight; *Haem.%* is the haemolymph weight expressed as percent lobster live weight; *cCu* (µg g^{-1} ww) is the haemolymph copper concentration; and X_1 and X_2 are dummy variables. For lobsters in Moult Stages C_4, D_0, D_1, D_2 and D_3: X_1 and X_2 = 0; for Moult Stages A, B and C_1: X_1 = 1 and X_2 = 0; for Moult Stage C_2 and C_3: X_1 = 0 and X_2 = 1.

Method 2

We propose, additionally, another method of predicting tissue weight. A lobster's haemolymph copper content increases linearly with an increase in the wet weight of the soft tissues (Fig. 4) according to the equation:

$$Cu\ cont. = 43.6 \times soft\ tiss. + 91.7 \quad (5)$$

where *Cu cont.* is the haemolymph copper content (µg) and *soft tiss.* is the ww of the soft tissues (g). Thus, a biological meaning can be given to the slope (k = 43.6 µg Cu g^{-1}), which represents an estimate of the metabolic haemolymph copper requirement expressed per g soft tissue ww. The copper represented by the *y*-intercept (91.7 µg) is negligible in comparison with

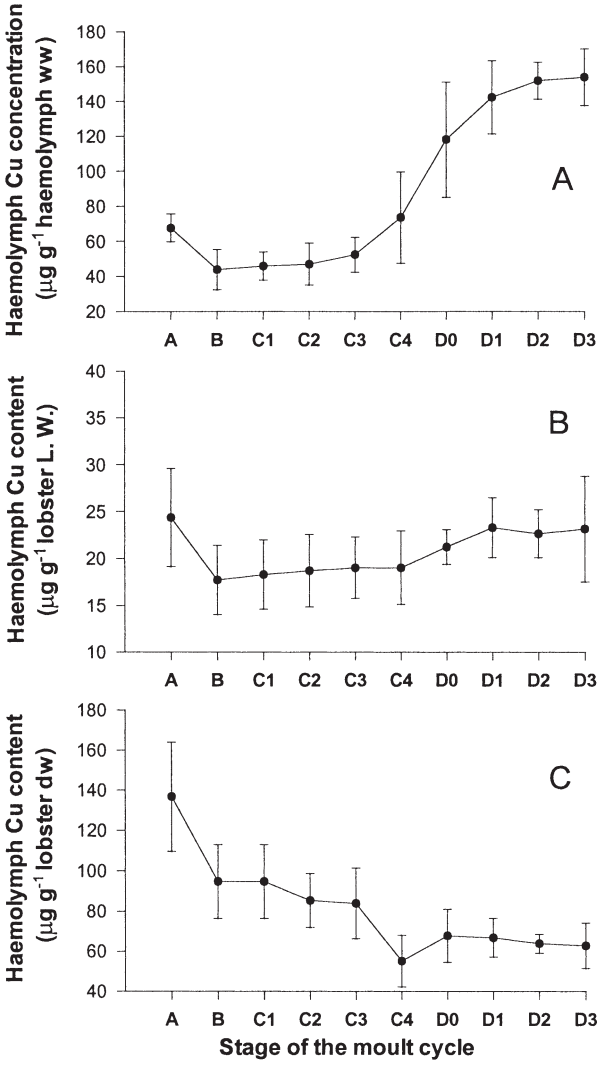

Fig. 2. *Homarus gammarus.* Haemolymph copper levels throughout the moult cycle. Further details as in Fig. 1

the lobster's haemolymph copper content (Fig. 4). Thus, the haemolymph copper content can be approximated by:

$$Haem. \times cCu = 43.6 \times soft\ tiss. \quad (6)$$

where the haemolymph copper content is expressed as the product of the haemolymph weight (*Haem.*) and haemolymph copper concentration (*cCu*) as in Eq. (2).

The proportion of soft tissue and haemolymph contributing to lobster live weight (f%) decreased from postmoult to intermoult. In early postmoult (Stages A, B and C_1), later postmoult (Stages C_2 and C_3) and from intermoult to premoult (Stages C_4 to D_3) total haemolymph and soft tissues averaged 84.0, 81.1 and 70.9% of lobster live weight, respectively (calculated from Fig. 1). Thus, for a lobster of known live weight (L.W.) and moult stage, the following equation can be given:

$$Haem. + soft\ \ tiss. = \frac{f\% \times \text{L.W.}}{100} \quad (7)$$

From Eqs. (6) and (7) one obtains:

$$Soft\ \ tiss.\% = f\% \times \frac{cCu}{k + cCu} \quad (8)$$

$$Haem.\% = f\% \times \frac{k}{k + cCu} \quad (9)$$

in which the symbols are as above. The decrease in the total soft tissue and haemolymph percentages may be attributable to an increase in lobster live weight during the moult cycle, during which the exoskeleton grows and tissues become more dense. In captive lobsters we found an increase in live weight (L.W.) of approximately 15% from Stages A to D_3. Methods 1 and 2 are both valid and give close estimates of soft tissue and haemolymph weight if haemolymph copper concentration and moult stage are known.

DISCUSSION

Because of the rigid exoskeleton in crustaceans, tissue growth is not visible between moults. Consequently, researchers have attempted to estimate tissue weight changes by measuring haemolymph constituents. Previous studies on American lobsters have shown that haemocyte number and haemolymph protein concentration may be used as indices

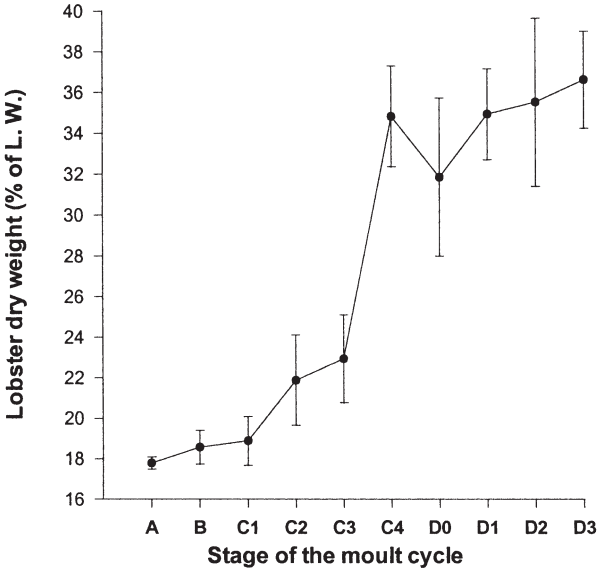

Fig. 3. *Homarus gammarus.* Dry weight throughout the moult cycle. Further details as in Fig. 1

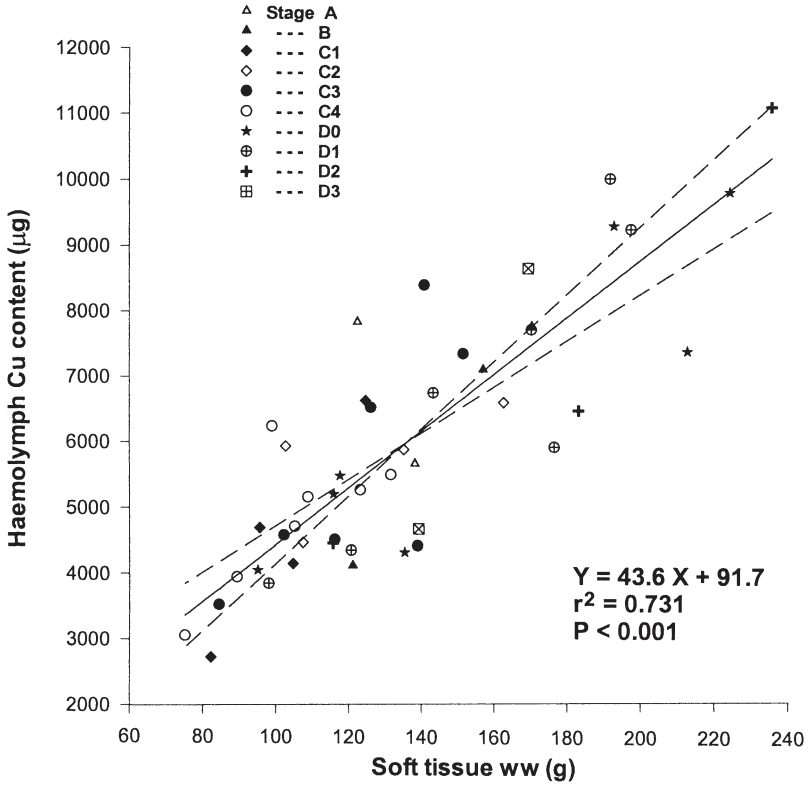

Fig. 4. *Homarus gammarus.* Relationship between haemolymph copper content and soft tissue ww. Data for individual stages are represented by different symbols, as in key. Dotted lines represent the 95% confidence limits of the slope $L_1 = 35.5$, $L_2 = 51.6$

of muscle weight (Stewart et al. 1967, Castell & Budson 1974). Moreover, haemolymph protein or copper concentration were proposed as predictors for the haemolymph space in other crustaceans (Dall 1974, Smith & Dall 1982, Devescovi & Lucu 1995). The results of the present investigation, based on haemolymph copper levels in field-collected European lobsters, confirm these findings. Because the moult cycle constrains copper levels, moult stage was included in our models predicting both soft tissue and haemolymph weight.

We found a significant relationship between soft tissue weight and total haemolymph copper content. About 43.6 µg of copper were present in the lobster haemolymph g^{-1} soft tissue (95% confidence limits = 35.5 and 51.6 µg g^{-1}). This value corresponds to the metabolic haemolymph copper requirement (and therefore the haemocyanin copper requirement) in European lobsters. The haemocyanin metabolic copper requirements of intermoult crustaceans had been theoretically estimated to be in the range of 30 to 60 µg Cu g^{-1} whole crustacean dry weight (White & Rainbow 1985, Depledge 1989, Depledge & Bjerregaard 1989, Rainbow 1993). We estimated that the haemolymph copper content is 55.1 ± 12.9 µg g^{-1} lobster dry weight in intermoult lobsters (Fig. 2C). This value is close to the above-mentioned theoretical estimates, suggesting that the method chosen for the determination of haemolymph weight as a measure of the space wherein haemocyanin is circulating was appropriate. In postmoult lobsters, the haemolymph copper content g^{-1} lobster dry weight was higher due to the lower solids content of lobsters. In certain circumstances the haemocyanin copper can be low. It is possible that the bioavailability of copper in the deep NE Atlantic is so low that the environment is copper-deficient in respect to the requirements of the juvenile mesopelagic decapod crustacean *Systellaspis debilis* (Rainbow & Abdennour 1989).

In spite of the increase in haemolymph copper concentration during the moult cycle (Fig. 2A), the haemolymph copper content expressed g^{-1} lobster live weight remained nearly constant (Fig. 2B). This result is in accordance with the finding that copper content in decapod crustaceans is regulated in a narrow range (Bryan 1968). Small fluctuations in haemolymph copper content could be explained by a compensatory decrease in haemolymph weight (Fig. 1B) or by an increase in lobster live weight during the moult cycle. Such an increase has been observed during the moult cycle of the spiny lobster *Panulirus argus* (Travis 1954) and of the southern rock lobster *Jasus lalandei* (Fielder 1964). A lobster's live weight is augmented by calcification of the exoskeleton and the growth of tissues, which become more dense. Nutritional condition and therefore the growth of tissues may also affect live weight. For example, juvenile rock lobsters *J. edwardsii* fed full rations were 10% heavier than starved lobsters at a comparable carapace length, and sub-satiated lobsters showed intermediate weights (Oliver & MacDiarmid, 2001).

All haemolymph copper in crustaceans from unpolluted areas is essentially bound to haemocyanin (Depledge & Bjerregaard 1989, Rtal & Truchot 1996). Regardless of sex, oxygen binding to haemocyanin does not change significantly during moulting (Mangum 1990). No differences in the monomeric composition of haemocyanin between sexes and stages of the moult cycle have been found (Mangum 1990). Thus, the main processes that cause the haemolymph copper

concentration to increase throughout the moult cycle are the growth of soft tissues, which reduces the haemolymph space concentrating the haemocyanin, and the synthesis of new haemocyanin for the oxygen supply of growing tissues. Other published studies have noted that haemocyanin (Hagerman 1983) and haemolymph protein concentrations (Glynn 1968) increase during the moult cycle in European lobsters. A similar increase in protein concentration was also observed during the moult cycle of the American lobster (Barlow & Ridgway 1969). After ecdysis, haemocyanin and protein concentration dropped to minimum levels due to both ecdysal water uptake and utilization of haemolymph proteins in the synthesis of the new exoskeleton (Glynn 1968, Hagerman 1983).

Natural variations in haemolymph copper concentration in hard-shelled European lobsters range from 34 to 124 µg g^{-1} ww (Bryan 1964). Considering that hard-shelled lobsters are in Intermoult Stage C_4 or in premoult stages, these values are consistent with the findings of our study. In field-collected lobsters, haemolymph copper concentration varied from 38.4 to 109.6 µg g^{-1} ww in Stage C_4 and from 82.0 to 163.5 µg g^{-1} ww in Stage D_0. However, variations in concentration could also be a consequence of nutritional conditions, reflecting food quantity and quality and the growth rate of soft tissue. A deficient diet induced a decrease in haemocyanin concentration and moulting frequency in European lobsters (Hagerman 1983). Additionally, natural variations in the concentration of haemocyanin of intermoult Norway lobsters *Nephrops norvegicus* and swimming crabs *Liocarcinus depurator* off the west coast of Sweden could be related to the amount of food consumed (Spicer & Baden 2000).

Our results show that rapid, nondestructive estimates of soft tissue and haemolymph weights can be obtained by the measurement of haemolymph copper concentration accompanied by determination of moult stage in adult European lobsters. The proposed methods should be useful for evaluating the effects of environmental factors on the growth of tissues, and are simple and feasible in the field and laboratory, augmenting tag and recapture experiments.

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