EFFECT OF COPPER ON THE GILL STRUCTURE OF A EURYHALINE CRAB, *CARCINUS MAENAS* (CRUSTACEA:DECAPODA)

by

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Effect of copper on the gill structure of a euryhaline crab, *Carcinus maenas* (Crustacea:Decapoda)

Sarah Louise Lawson

ABSTRACT

A posterior gill of the common shore crab *Carcinus maenas* (Crustacea:Decapoda) was used to study the effects of sublethal copper concentrations on gill morphometry, and gill cell ultrastructure and distribution. Primarily, the ultrastructure and distribution of gill cells in the untreated gill of crabs acclimated to 35‰ seawater were characterised. Gill ultrastructure was subsequently shown to vary markedly at 10‰ compared to 35‰ seawater. Ultrastructural and distributional, rather than gross morphological, change occurred in the gill following exposure to copper at each salinity. Ultrastructural studies showed that as the length of copper exposure increased the gill epithelial layer became highly vacuolated, the number of plasma membrane infoldings and mitochondria became reduced, the microtubular network became disrupted, the endoplasmic reticulum became swollen and the cell cytoplasm contained many free ribosomes. Copper exposure also resulted in an increase in the number of haemocytes in the gill, some of which became attached to the basal epithelial cell surface or actually infiltrated into the epithelial cell cytoplasm. Ultrastructural change was more extensive when copper was administered at 35‰ rather than 10‰ seawater. These effects are described and correlated with previously documented biochemical and physiological responses to heavy metals in crustaceans. In addition, the elemental composition of the gill granular haemocytes suggested they play an important role in the immobilisation and removal of copper from the gill.This study has shown the way forward in creating a better understanding of the mechanisms behind heavy metal toxicity in marine organisms is the use of environmentally realistic concentrations of heavy metal administered in flow-through seawater systems.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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A programme of advanced study was undertaken, which included the postgraduate course "Practical Introduction to Cryo Electron Microscopy and X-ray Microanalysis" held at the University of Plymouth, July 1991.

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Publications (or presentations of other forms of creative work):

Lawson, S.L.; Jones, M.B. & Moate, R.M. (in press) Structural variability and distribution of cells in a posterior gill of *Carcinus maenas* (Decapoda:Brachyura). *Journal of the Marine Biological Association of the United Kingdom*.

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Presentations and Conferences Attended:

1st European Crustacean Conference, Paris, August 1992.

Society for Experimental Biology meeting, Canterbury, March 1993, oral presentation entitled "The Effect of Selected Heavy Metals on Crustacean Gill Structure".

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28th European Marine Biology Symposium, Crete, September 1993, poster presentation entitled "Structural Observations of the Posterior Gill Filament in the Common Shore Crab *Carcinus maenas* Following Exposure to Sublethal Concentrations of Copper in Seawater".

Society for Experimental Biology meeting, Swansea, April 1994.

3rd International Conference on Trace Metals in the Aquatic Environment, Denmark, May 1994, oral presentation entitled "Effects of Copper on the Ultrastructure of the Gill Epithelium of *Carcinus maenas* (Decapoda:Brachyura)".

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Date 19/8/94

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

In recent years, the effects of heavy metals on marine organisms have received considerable attention. The uptake and accumulation of heavy metals, and the inhibition of biochemical and physiological processes by heavy metals, have been productive areas of research (see, for example, Wright, 1978; Spicer & Weber, 1991; Hansen *et al.*, 1992a,b; Viarengo, 1985). Although extensive efforts have been made to study heavy metal toxicity, a clear understanding of the mechanisms by which heavy metals exert their effect on biochemical and physiological processes has yet to be achieved.

The first studies of the effects of heavy metals on marine organisms measured acute toxicity either as a median lethal concentration (LC_{50}) at an agreed time limit, usually 96 hours, or as a median lethal time (LT_{50}) at a specified concentration. These approaches are used still as an extreme measurement of the toxicity of a compound, however, such lethal acute toxicity studies have limitations which have to be taken into consideration when trying to extrapolate from laboratory data to the field situation (Abel & Axiak, 1991). Also, although acute episodes of heavy metal contamination may occur periodically in the estuarine and coastal environment, these may be accompanied by a much greater incidence of lower, sublethal concentrations of heavy metal. Many heavy metals may induce marked changes in physiology, morphology or behaviour at concentrations well below their lethal level. Therefore, the measurement of physiological change, morphological alteration or the inhibition of some bodily function is equally as important as observed mortality. It has become increasingly desirable to seek sublethal criteria as a better measurement of the toxicity of a substance (McLusky *et al.*, 1986) and to determine whether levels of pollution recorded regularly in estuaries, and along the coast, exert any effect on marine organisms.

This study investigates the effects of sublethal concentrations of one heavy metal, copper, on the gill structure of the common shore crab *Carcinus maenas* (L.)

(Crustacea:Decapoda).

1.2 Copper

1.2.1 Chemical forms

In seawater, copper can exist in a variety of states, ranging from free ions, through inorganic and organic complexes, to dispersed colloids and copper adsorbed onto particulate material (for reviews on the chemistry of copper in seawater see Zirino & Yamamoto, 1972; Dyrssen & Wedborg, 1974; Whitfield, 1975; Mantoura *et al.*, 1978). Even in solution there are many different chemical species of copper whose proportions are altered by environmental variables such as pH and salinity (Fig. 1.1). For example, a decrease in salinity results in an increased proportion of the free uncomplexed ion in solution; this ion being the most bioavailable species of metal for uptake by aquatic biota (Sunda *et al.*, 1978; Engel & Fowler, 1979; Zamuda & Sunda, 1982; Borgman, 1983; Florence, 1983; Campbell & Stokes, 1985; Wangersky, 1986; Nugegoda & Rainbow, 1988; Luoma, 1989). Indeed, decreased salinities have been associated with enhanced metal toxicity in marine crustaceans (Bryan, 1976; Chen & Slinn, 1980; McLusky *et al.*, 1986; McLusky & Hagerman, 1987). Therefore, it is important to consider chemical speciation changes when assessing the effect of heavy metals on gill structure. The combined effect of exposure to copper and low salinity on the gill ultrastructure of *Carcinus maenas* is included in Chapter 7 of this study.

1.2.2 Concentrations

In unpolluted waters, copper levels can reach concentrations of 0.5-20μgl^{-1} (Baker, 1969; Bjerregaard & Vislie, 1986), although copper concentrations of up to 0.6mgl^{-1} have been reported in some estuarine and coastal areas (Bryan, 1976). Due to natural geological or anthropogenic input, the concentration of copper in estuarine and coastal

areas can be orders of magnitude greater than in open waters (Preston, 1973). Copper enters the environment naturally by the erosional action of water on soils and mineral deposits (Spear & Pierce, 1979). However, the anthropogenic input of copper in urban and industrialised areas may be at least equal to the terrestrial input (Bruland et al., 1974). Of man's activities, mining probably makes the most significant contribution to elevated levels of copper witnessed in coastal and estuarine regions, where mineral rich washings are discharged directly into the sea (Scott & Major, 1972). Copper compounds enter the environment also via power stations, desalination plants, dredging operations and through by-products of industrial processes, including oil refining. Algicides, fungicides, pesticides and anti-fouling preparations containing copper compounds also ultimately pass into the sea (Ray, 1959; Hubschamnn, 1966). The effects of environmentally realistic concentrations of heavy metal on the gill structure of marine crustaceans have been investigated seldomly. This study employs sublethal copper concentrations to facilitate a better understanding of the effects of environmentally realistic concentrations of copper on *Carcinus maenas* gill structure.

1.2.3 Toxicity

In crustaceans, copper is an essential trace element for growth and is required for the metabolic functioning of the oxygen-binding haemolymph pigment, haemocyanin (White & Rainbow, 1982; Spaargaren, 1983; Rainbow, 1985). Decapods can regulate body copper levels over a wide range of metal bioavailability (White & Rainbow, 1982; Rainbow, 1985). Above a threshold concentration, however, regulation may break down and copper may be accumulated. Despite the fact that copper is known to be an essential trace metal, it has been shown to adversely affect behavioural, physiological and biochemical processes in several crustacean species.

1.3 The common shore crab, *Carcinus maenas*

Carcinus maenas, a euryhaline decapod crustacean, is a common intertidal crab found predominantly along coastlines in the Eastern Atlantic Ocean and warm, temperate seas in the Northern Hemisphere (Coleman, 1991). The extensive body of published work on *C. maenas* ranges from superficial statements in general accounts of the ecology of the seashore to specialised physiological, biochemical and ultrastructural studies. Since crustacean gills are the first target of water-borne pollutants and are involved in respiratory gas exchange, ionic regulation, osmoregulation, acid and base regulation and ammonia excretion (for a review see Taylor & Taylor, 1992) they have received considerable attention in heavy metal toxicity studies in recent years.

1.3.1 Gill structure

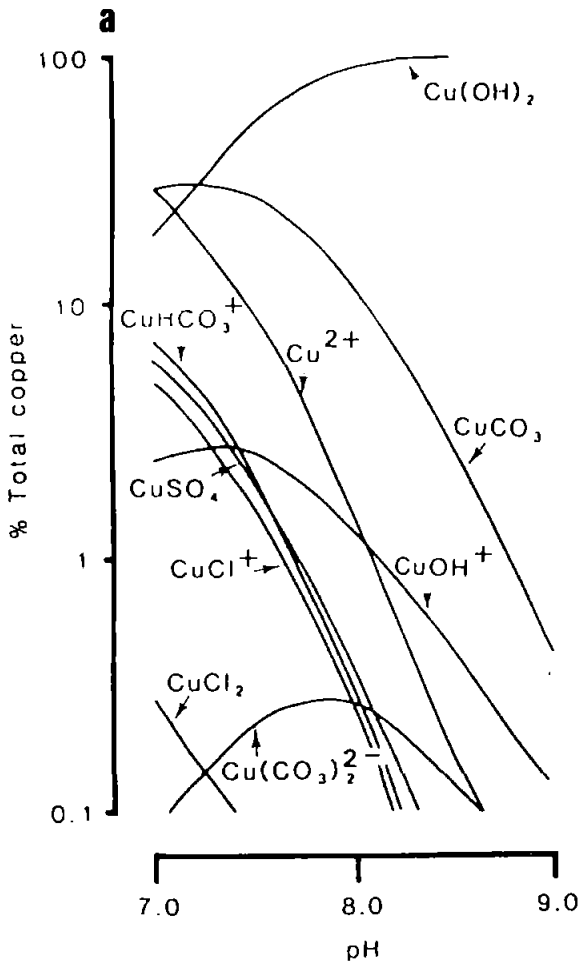
There are nine gills in each of the two branchial chambers of *Carcinus maenas* (Fig. 1.2). Except for the podobranch of the second maxilliped, which is directed horizontally backward, each gill curves inwards and upwards from its attachment point, so that the tapering tips converge along the dorso-median wall of the branchial chamber (Fretter & Graham, 1976). The gills are phyllobranchiate, consisting of a flattened central raphe, bearing regularly spaced lamellae on each side. The lamellae are sack-like structures covered by a cuticle. In cross-section, the central raphe is dumbbell shaped with the afferent and efferent vessels at each end (Fig. 1.3). The afferent and efferent vessels deliver and remove venous haemolymph to and from the gill respectively. A pair of branchial arteries, medial to either branchial vessel, is also present in the central raphe (Fig. 1.3). Branches from these arteries radiate to all parts of the lamellae [demonstrated in *C. maenas* by Ali (1966), using Indian ink]. Gill number 8, a pleurobranch gill corresponding to the first walking leg (Scammel & Hughes, 1982), was used throughout this study since it contains both respiratory and osmoregulatory epithelia (Towle &

Kays, 1986). Thus, by the removal of a single gill the effect of copper on the cells involved in the physiological processes of respiratory gas exchange, ionic and osmoregulation can be studied simultaneously.

1.4 Aims

This study documents the changes in the gill structure of *Carcinus maenas* following exposure to sublethal concentrations of copper at high and low salinities with the aim of gaining a better understanding of the mechanisms by which the physiological processes of ionic and osmoregulation and respiratory gas exchange are affected by heavy metals. The effect of copper on various gill parameters using image analysis techniques is presented in Chapter 2. The first ultrastructural investigations of the gills of crustaceans were completed many years ago (for example, Drach, 1930); however, a detailed description of the ultrastructure of posterior gill number 8 of *C. maenas* has yet to be fully developed. Therefore, there remains the question as to whether there are specialised gill areas for the physiological processes of ionic regulation, osmoregulation and respiratory gas exchange. Chapter 3 details the general materials and methods used for all the electron microscopy work. The ultrastructure and distribution of the gill cells of *C. maenas* acclimated to 35‰ seawater is documented in Chapter 4 and the effect of decreased salinity (10‰ seawater) on gill cell ultrastructure and distribution is discussed in Chapter 5. The effects of sublethal copper concentrations, administered at both 35‰ and 10‰ seawater, on *C. maenas* gill ultrastructure are documented in Chapters 6 and 7 respectively. An X-ray microanalytical study of the granular haemocytes, found circulating in the gill following exposure to copper, is described in Chapter 8 and the potential for using X-ray microanalytical techniques in heavy metal studies highlighted. Finally, a general discussion of these findings, together with ideas for future ecotoxicological studies, is outlined in Chapter 9.

Figure 1.1. Calculated distribution of the chemical species of copper in seawater as a function of **(a)** pH (after Zirino & Yamamoto, 1972), and **(b)** salinity (after Mantoura *et al.*, 1978).


a
100
10
1
0.1
% Total copper
Cu(OH)₂
CuHCO₃⁺
Cu²⁺
CuCO₃
CuSO₄
CuCl⁺
CuOH⁺
CuCl₂
Cu(CO₃)₂²⁻
7.0
8.0
9.0
pH

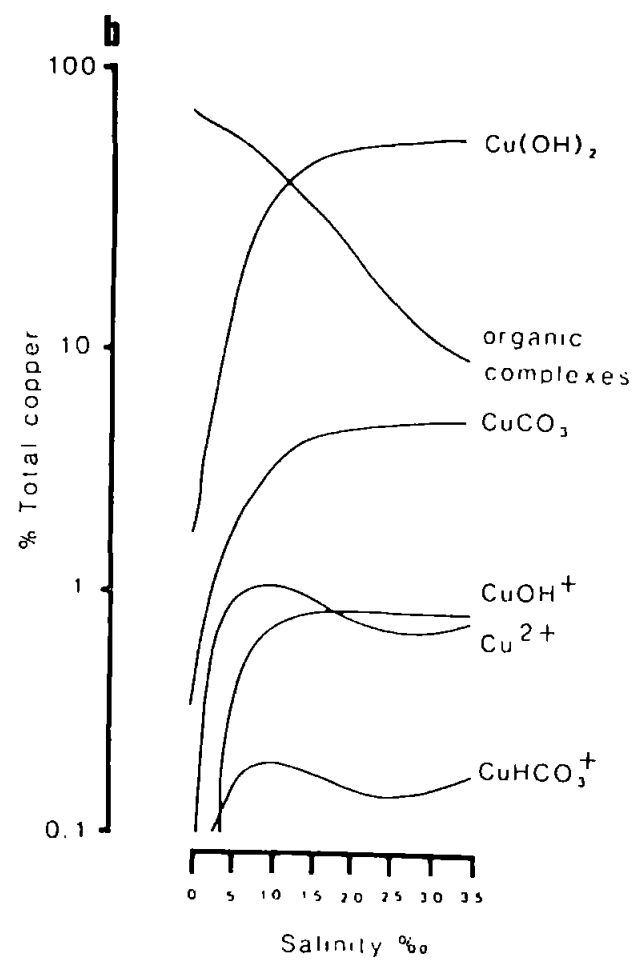
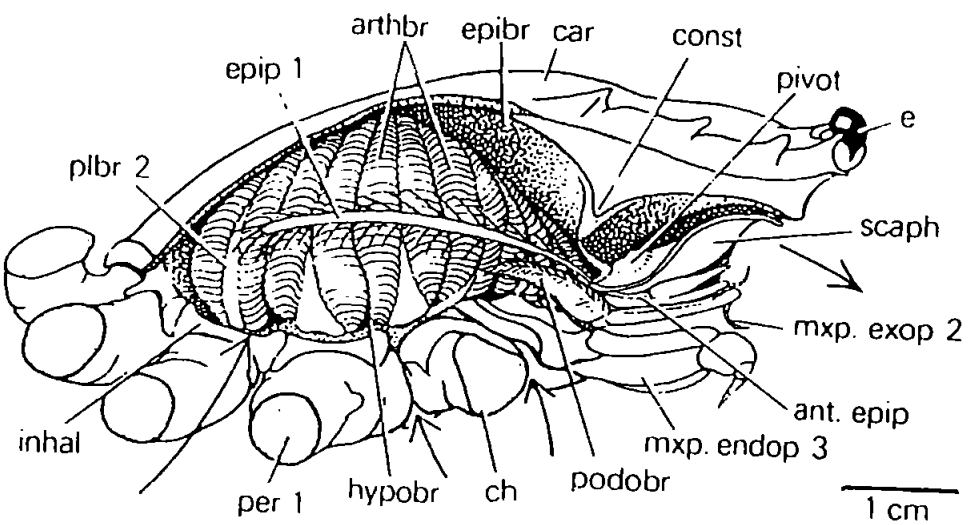

b
100
10
1
0.1
% Total copper
Cu(OH)₂
organic
complexes
CuCO₃
CuOH⁺
Cu²⁺
CuHCO₃⁺
0 5 10 15 20 25 30 35
Salinity ‰

Figure 1.2. Lateral dissection of *Carcinus maenas* to show the branchial chamber (after Fretter & Graham, 1976) (ant.epip: anterior part of epipodite of 1st maxilliped; arthbr: arthrobranchs of cheliped; car: carapace; ch: cheliped; const: constriction; e: eye; epibr: epibranchial space; hypobr: passage to hypobranchial space; inhal: ventral lip of inhalent opening; mxp.endop: endopodite of maxilliped; mxp.exop: exopodite of maxilliped; per 1: base of 1st pereiopod; pivot: pivot of scaphognathite; plbr 2: pleurobranch of 2nd pereiopod; podobr: podobranch of 2nd maxilliped; scaph: scaphognathite).


arthbr
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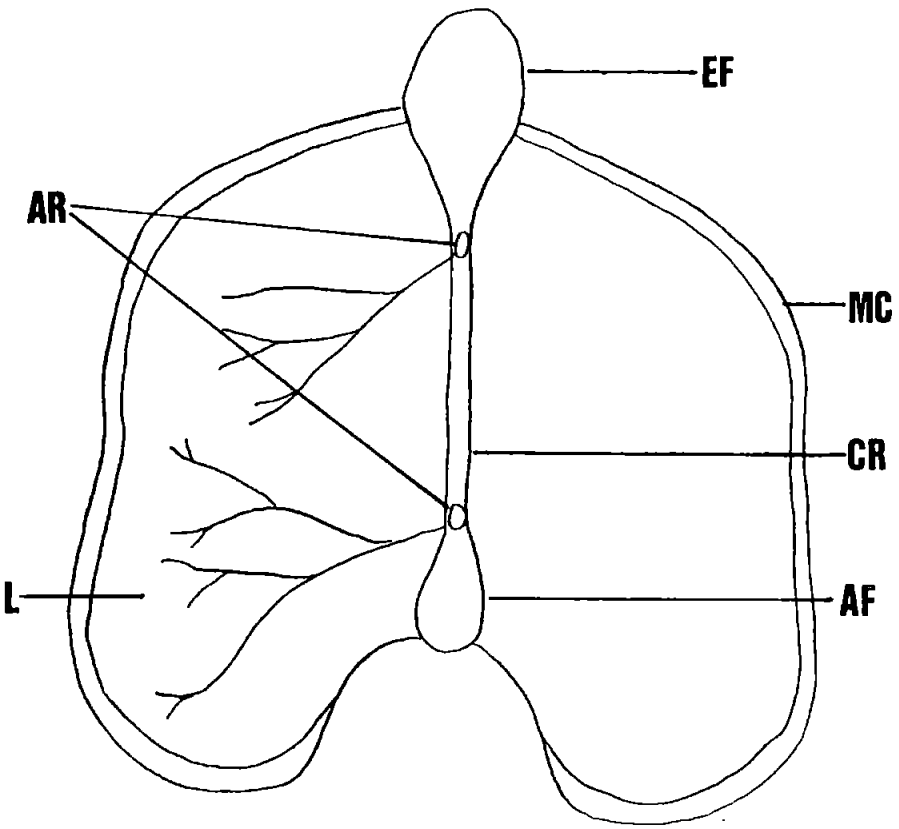
1 cm

Figure 1.3. Diagrammatic representation of a transverse section of gill lamellae of *Carcinus maenas* (AF: afferent vessel; AR: branchial arteries; CR: central raphe; EF: efferent vessel; L: lamella; MC: marginal canal).


EF
AR
MC
CR
L
AF

CHAPTER 2

THE EFFECT OF COPPER ON THE GROSS GILL MORPHOMETRY OF *CARCINUS MAENAS*

2.1 Introduction

Gill area measurements have been made previously on various crustaceans (Gray, 1957; Ambore & Venkatachari, 1978; Cameron, 1981; Hawkins & Jones, 1982; Hughes, 1983; Moore & Taylor, 1984; Spicer & Taylor, 1986; Burd, 1988; Johnson & Rees, 1988); however, these studies employed laborious area measurement techniques and focused mainly on comparative natural variabilities in whole gill size between and within species. The effect of heavy metals on gross crustacean gill morphometry remains to be studied. As previous authors have correlated gill size with habitat variability and metabolic activity (Gray, 1957; Hughes, 1983; Spicer & Taylor, 1986), it may be anticipated that exposure to copper, potentially a physiologically and structurally disruptive heavy metal, may also result in changes in gill morphometry. The effect of a sublethal copper concentration on the morphometry of the gill of *Carcinus maenas* (L.) is reported here, using image analysis techniques. Image analysis has been applied recently to many areas of biological research (for a review see Vecht-Lifshitz & Ison, 1992). Image analysis is an indispensable tool for microscopists who need to obtain accurate quantitative information from their samples; however, present applications are mainly in the field of biotechnology, with very few studies in marine biological research.

This chapter examines the area, perimeter and width of the whole gill and sections from the respective gill regions of untreated and copper-exposed *Carcinus maenas* kept in 35‰ seawater. Any changes in these gill parameters following copper exposure were recorded and quantified using image analysis techniques. Area and perimeter measurements of the afferent and efferent gill vessels were taken to assess whether exposure to copper induced changes in the size of the gill haemolymph vessels. It is known that the gill is an important organ of respiratory gas exchange and ionic and osmoregulation (for a review see Taylor and Taylor, 1992) and that these physiological

processes are affected by exposure to copper (discussed in Chapter 6). Thus, the monitoring of gill morphometrical changes following copper exposure may aid the understanding of the mechanisms underlying the gill physiological disruption in the presence of this heavy metal.

2.2 Materials and methods

2.2.1 Collection of animals

In April and June 1992, intermoult *Carcinus maenas* (15-20g wet weight; carapace width 30-45mm) were collected from Portwrinkle, Cornwall, U.K. (4°18'W, 50°21'N); these sizes were selected because the majority of crabs found were within this range. Only males were collected as females were ovigerous at this time. Crabs were transported to the University of Plymouth in aerated seawater (35‰) obtained from the collection site.

2.2.2 Experimental system

Crabs were maintained individually in four covered, plastic tanks (each of 25l volume) supplied with recirculating seawater (35‰, 12 ± 1°C); eight crabs were maintained in each tank. Crabs, acclimated to these experimental conditions for three weeks, were fed every three days with chopped coley [*Pollachius virens* (L.)] until three days prior to copper exposure. Following the acclimation period, crabs in two of the tanks were exposed to a nominal copper [$Cu(NO_3)_2.3H_2O$] concentration of $0.5mgl^{-1}$ for 20 days. Atomic absorption spectrophotometry (Varian-975) showed that the actual initial concentration in the seawater was $0.2mgl^{-1}$; this difference from the nominal concentration could be due to loss of copper via aeration, complexation or absorption to the container. Atomic absorption spectrophotometry also confirmed that the initial actual concentration was not significantly depleted over the exposure period. Crabs in

the remaining two tanks were not exposed to copper and served as controls. After 20 days, the gills from each crab were dissected (see 2.2.3 "Preparation of specimens"). Thirty crabs each from the control and copper-exposed tanks were dissected (thus, n = 30 for each parameter measured and each treatment). Two collections of crabs (one in April, the other in June) were necessary as sixty crabs could not be accommodated in the experimental system at the same time.

2.2.3 Preparation of specimens

For each crab, the carapace was removed and the gills flooded *in situ* with a primary fixative (1% glutaraldehyde buffered at pH7.2 with 0.2M sodium cacodylate). Gill number 8 was removed from the right branchial chamber by cutting the basal attachment with dissection scissors. Although only gills from the right branchial chambers were used, atomic absorption spectrophotometric measurements have shown that the uptake of heavy metals in *Carcinus maenas* does not vary significantly between gills from the right and left branchial chambers (Clausen *et al.*, 1993). Each excised gill was transferred to a vial of primary fixative at 4°C for 1h, washed in cacodylate buffer (pH7.2), placed in a petri dish and examined under a stereoscopic dissecting microscope connected to a Sony 3CCD colour camera. Images were relayed to the computer by the camera and measurements of the area, perimeter and width of the whole gill (Fig. 2.1a) were recorded using the Quantimet-570 image analyzer following the programme (Programme 1) outlined in Appendix I.

The gills were then divided, using a scalpel, into proximal, mid and distal gill regions (Fig. 2.1b); vernier callipers were used to divide the gill into three equal parts. Further sections, each consisting of five lamellae, were then removed from the middle of each gill region (vernier callipers were again used for measuring purposes). These latter sections were stained in Gomori's solution for 20min and rinsed thoroughly with

distilled water. Gill sections were placed in a petri dish and examined individually under a stereoscopic dissecting microscope, a Sony 3CCD colour camera relayed images to the computer. Measurements of the area, perimeter and width of the section (Fig. 2.2a), and the area and perimeter of the afferent and efferent gill vessels (Fig. 2.2b) were recorded using the Quantimet-570 image analysis system following the programme (Programme 2) outlined in Appendix II.

The Quantimet-570 image analyzer (marketed by Leica of Cambridge) is capable of processing any two-dimensional image, that is, any image that can be acquired by a still or video camera. The system can measure a wide range of features, including area, height, length, width, perimeter and breadth. Specific programmes incorporating the desired measurements from this extensive list can be developed for specific uses ensuring reproducibility of techniques. Image analysis involves the digitization of images into grids of points or picture elements (pixels) and measurement of the intensity of light at each point. Black and white images may be recorded by a single measurement of intensity (grey level). There are several stages involved in the image analysis of a sample including image acquisition, feature detection, measurement, data processing and image storage. Nevertheless these are all completed in a very short time and allow an accurate and rapid quantification of biological analysis.

2.3 Results

Much experimentation with the presentation and analyses of the image analysis data occurred before the final versions, as shown here, were selected. Initially, simple graphical plots of carapace width against gill parameter, with each data point represented as "U" (untreated) or "T" (copper-exposed) were produced and best fit regression lines were plotted through the data. The plots yielded little useful information, and any differences between untreated and copper-exposed crab gills were

difficult to isolate. Also, the correlation (r^2) values of the regression lines were very low, indicating that this method of presentation and analysis was not particularly helpful in isolating differences between treatments. The data were represented finally in the form of multiple boxplots, the mean and quartile range were compared between treatments, and one-way analyses of variance (ANOVA) were used to isolate significant effects. Clearly, it is meaningless to compare gill measurements between untreated and copper-exposed crabs without any consideration of the sizes of the crabs used. Analysis of co-variance (ANCOVA) could have been used on the image analysis data to test the dependent variables (gill area, perimeter etc.) for homogeneity between group means, having adjusted for the group differences in the independent variable (carapace width, in this case). However, one-way ANOVA showed that the carapace width did not vary significantly between untreated and copper-exposed crabs (P=0.911). Figure 2.3 confirms that the mean, upper and lower quartiles, and maximum and minimum carapace width values were almost identical in untreated and copper-exposed crabs. Thus, a one-way ANOVA between untreated and copper-exposed gill parameters, excluding carapace width as a variable, was used to analyze for statistical differences, and a multiple boxplot was used to illustrate each data set.

2.3.1 Whole gill

The area, perimeter and width of the whole gill did not differ significantly between untreated and copper-exposed crabs (Fig. 2.4).

2.3.2 Proximal gill region

The area, perimeter and width of the proximal gill sections did not vary significantly between untreated and copper-exposed crabs (Fig. 2.5). However, the area, perimeter and width data points of the proximal gill sections were dispersed more evenly along

the full range of measurements in copper-exposed than in untreated crabs, making the quartile range wider in copper-exposed compared to untreated crabs. Similarly, the area and perimeter of the afferent and efferent gill vessels in the proximal gill region did not vary significantly between treatments (Figs 2.6 & 2.7), and there were no clear trends between the two treatments.

2.3.3 Mid gill region

Although the area, perimeter and width of mid gill sections did not vary significantly between treatments, these measurements were consistently lower in copper-exposed than in untreated crabs (Fig. 2.8). Similarly, the area and perimeter of each gill vessel did not differ significantly between untreated and copper-exposed crabs, however, many outlying data points were present in the gill vessel data, particularly in the untreated crabs (Figs 2.9 & 2.10). Such variation hindered any clear trends and may have accounted for the lack of significant differences.

2.3.4 Distal gill region

There were no significant differences and no observable trends in area, perimeter and width of the distal gill sections between untreated and copper-exposed crabs (Fig. 2.11). In addition, the afferent vessel area was not significantly different between untreated and copper-exposed crabs (Fig. 2.12a), however, the mean afferent vessel area was higher, and the quartile range wider, in the gills of copper-exposed compared to untreated crabs. Similarly, there was no significant difference between the afferent gill vessel perimeter between untreated and copper-exposed crabs, but the mean afferent vessel perimeter was higher, and the quartile range wider, in copper-exposed compared to untreated crab gills (Fig. 2.12b).

No significant difference in the efferent gill vessel area between treatments was found,

however, the quartile range in copper-exposed crabs, unlike the majority of measurements, was much narrower than in untreated crabs, although the full range of data was approximately equal in each treatment (Fig. 2.13a). Conversely, the efferent gill vessel perimeter did vary significantly between copper-exposed and untreated crabs; the mean efferent vessel perimeter was greater and the quartile range narrower in copper-exposed than in untreated crab gills (Fig. 2.13b). A test for homogeneity of variance (Bartlett's test) showed that there was no significant difference in homogeneity between the untreated and copper-exposed efferent vessel perimeter data distributions (P=0.11).

Although this study did not concentrate on any variation within a treatment between proximal, mid and distal gill regions, a few general trends were noticeable and worth mentioning. The relative size of the gill section decreased in the order proximal > mid > distal gill region (c.f. Figs 2.5, 2.8 & 2.11). This same trend was apparent for the area and perimeter of the afferent (c.f. Figs 2.6, 2.9 & 2.12) and efferent (c.f. Figs 2.7, 2.10 & 2.13) gill vessels. In each gill region, the area and perimeter of the afferent vessel was lower than the efferent vessel.

2.4 Discussion

The entire gill area, gill perimeter and gill width measurements of *Carcinus maenas* were unaffected following copper exposure. These data suggest that exposure to a sublethal copper concentration does not induce large morphometric gill changes in the crab, that is, changes in the number of lamellae, or size of individual lamellae already present in the gill. Comparative gill area studies, using different techniques to image analysis, have been made previously on crustaceans (Gray, 1957; Ambore & Venkatachari, 1978; Cameron, 1981; Hawkins & Jones, 1982; Hughes, 1983; Moore & Taylor, 1984; Spicer & Taylor, 1986; Burd, 1988; Johnson & Rees, 1988). In general,

whole gill area is correlated with habitat variability (mainly the amount of oxygen in the habitat) and metabolic activity (Gray, 1957; Hughes, 1983; Spicer & Taylor, 1986). In the former case, quantitative measurements of the relationship between gill surface area and oxygen consumption are lacking. Johnson & Rees (1988) found no direct relationship between gill area and respiration rate in four crab species, and suggested that such a relationship may only emerge under conditions of respiratory stress, when the gill area is a limiting factor to the rate of respiration. Ambore & Venkatachari (1978) proposed that gill surface area alone is not responsible for oxygen consumption; thus, monitoring the size of additional organs, as well as the gill, may be of some use in future heavy metal studies. As stated previously, there have been no comparative morphometrical studies of crustacean gills from clean and polluted sites. It is known that the gill is an important organ of respiratory gas exchange and ionic regulation (for a review see Taylor & Taylor, 1992), and that these physiological processes may be affected by exposure to copper (Chapters 6 & 7). Therefore, it may be anticipated that the morphometry of the gill may also be affected by exposure to copper. Lack of any significant gill change recorded here may be related to the length of the exposure period and/or the concentration of copper in the seawater; each may not have been sufficient to induce changes. Alternatively, gross lamellar changes may not occur in the gill after copper exposure and monitoring of the size of the whole organ may not be an adequate method of assessing change.

Gill area, gill perimeter and gill width measurements of standard sections (five lamellae) from three gill regions (proximal, mid and distal) were taken in this study. In each region, statistical analyses showed no significant differences in these parameters between untreated and copper-exposed crabs. It was evident from the plotted data, however, that some localised changes may have occurred in the gill following copper exposure. For example, the area, perimeter and width measurements of gill sections

from the proximal gill region showed much greater variability in copper-exposed crabs compared to untreated crabs. The measurements were more evenly dispersed throughout the full range in copper-exposed individuals and not concentrated within a narrow quartile range as seen in the untreated specimens. This difference could not be explained by crab size differences in the two treatments as ANOVA tests confirmed that crab carapace width did not vary significantly between treatments. It could, therefore, be suggested that the morphometry of the proximal gill region was altered, following exposure to copper, in some crabs more than others. In addition, area, perimeter and width measurements from mid gill sections were lower (although not significantly) in copper-exposed compared to untreated crabs. A decrease in the size of individual lamellae would equate to a decrease in the surface area for respiratory gas exchange and ionic regulation. A decrease in the area, perimeter or width of the lamellae may be a result of lamellae collapse or cell shrinkage, suggesting that either the mechanisms of structural support (and the cells involved in this operation) or the processes of ionic regulation and osmoregulation (resulting in a change in the amount of water entering into the gill) were affected adversely by exposure to copper. Alternatively, a decrease in the area, perimeter or width of the lamellae may be a structural adaptation to decrease the rate of entry of copper into the gill.

The effect of heavy metals on the diameter of the gill vessels in crustaceans has not been studied previously, possibly indicating a general view that gill vessels are not capable of morphological change. The lack of research in this area is a surprising omission from ecotoxicological studies, as haemolymph flow through the gill during heavy metal exposure may play an important role in the removal of the toxicant away from the gill. A simple mechanism of changing the rate of haemolymph flow would be to alter the diameter of the haemolymph vessels. If the proportion of total haemolymph flow shunted through the lamellae could be controlled, it would provide a homeostatic

system to suit the changing respiratory and ionic requirements of the crab under different environmental conditions. Unfortunately, little is known about the routes and dynamics of haemolymph flow through crustacean gills. Burger & Smythe (1953) suggested that body movements and skeletal muscle contraction provide an additional means of haemolymph circulation to heart-driven flow in the lobster, *Homarus*. Alternatively, Taylor & Taylor (1986) suggested that the valve-like structures located in the gill vessels of *Carcinus maenas* exerted differential control over haemolymph flow. Furthermore, these latter authors suggested that there was a potential for the valve cells to actively change their shape under differing physiological conditions; these cells house numerous mitochondria and microtubules, supporting an active role in configuration changes (Taylor & Taylor, 1986).

From present results it is interesting to note the inconsistencies between area and perimeter measurements for each gill vessel. It might be expected that as the area of the gill vessel increases the perimeter of the gill vessel increases simultaneously; however, independent changes in area or perimeter (as indicated here) imply that the actual shape of the vessel altered following exposure to copper. In addition, only significant differences in gill vessel size between untreated and copper-exposed crabs were observed in the distal gill region. Although, when mid gill vessel measurements were plotted it was noticeable that area and perimeter values of both afferent and efferent gill vessels were more variable in copper-exposed compared to untreated crab gills, implying that an alteration in gill vessel size may have resulted following copper exposure in some, but not all, crabs.

Some trends in the area of the afferent vessel were evident between untreated and copper-exposed crabs. Generally, the area of the afferent vessel was greater in copper-exposed than in untreated crab gills. This increased area suggests that haemolymph flow through this vessel was greater in copper-exposed than in untreated crabs. The perimeter

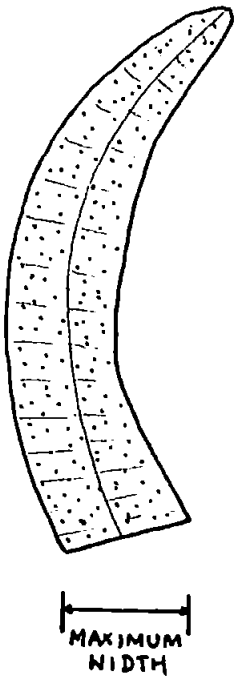
of the afferent vessel also showed a similar trend, with the same implication. Conversely, there were no obvious differences in the efferent vessel area between untreated and copper-exposed crabs, suggesting that either different responses to copper exposure or different methods of diameter control occur in each individual gill vessel. The latter interpretation is supported by Taylor & Taylor (1986) who found valve-like cells only in the efferent gill vessels of *Carcinus maenas*; in contrast, the afferent vessel was smooth and only perforated by rows of apertures with no valves present. Analysis of the efferent vessel perimeter data in the present study indicated that there was a significant difference in the perimeter of the efferent vessel between treatments. Similarly, multiple boxplots of the efferent vessel perimeter data showed that the vessel perimeter was larger (means and upper and lower quartiles were higher) in copper-exposed compared to untreated gills. Since the area and perimeter of the efferent vessel did not show simultaneous changes following copper exposure, it may seem likely (as only the perimeter of the vessel was significantly different between treatments) that the actual shape of the vessel was altered not necessarily with an equal alteration to the gill vessel area. In essence, the vessel became less rounded in copper-exposed compared to untreated gills. Future image analysis studies should measure the roundness ($perimeter^2$ x 1000/4 x π x area) and aspect ratio (length/breadth) of the vessels in the gill as a means of more fully understanding the effect of copper on the flow of haemolymph through the gill.

The results of the present study suggest that measuring specific areas of the gill and gill vessels may provide a better model for assessing the effects of copper on gill morphometry than measuring the size of the entire organ. In the distal gill region, gill vessels undergo the greatest morphometric changes, generally with higher vessel areas and perimeters being observed in copper-exposed compared to untreated crabs; although, only the efferent vessel perimeter in the gill changed significantly following copper

exposure. These data, therefore, suggest that there is potential for the gill vessel diameter, and thus, the rate of haemolymph flow in and out of the gill, to be altered under certain physiological conditions (details of the possible mechanisms of change are not included in this study). In future image analysis studies, more specimens would enable an assessment of the effect of crab size on gill and gill vessel alteration, allowing an examination of whether copper affects small individuals to a greater extent than larger individuals. Image analysis techniques have been used widely in the field of biological research but have not been applied previously to assessments of the effects of metal pollutants on crustacean gills. This study has shown that although significant gill changes were not always evident following copper exposure, the technique has good potential use in future ecotoxicological studies as a simple means of monitoring morphometrical changes in organs following pollutant exposure.

Figure 2.1. Diagrammatic representation of **(a)** whole gill parameters measured using the image analyzer and **(b)** the three regions of the gill of *Carcinus maenas* (stippled area = whole gill area; line enclosing stipple = whole gill perimeter; maximum width = whole gill width).

a



b

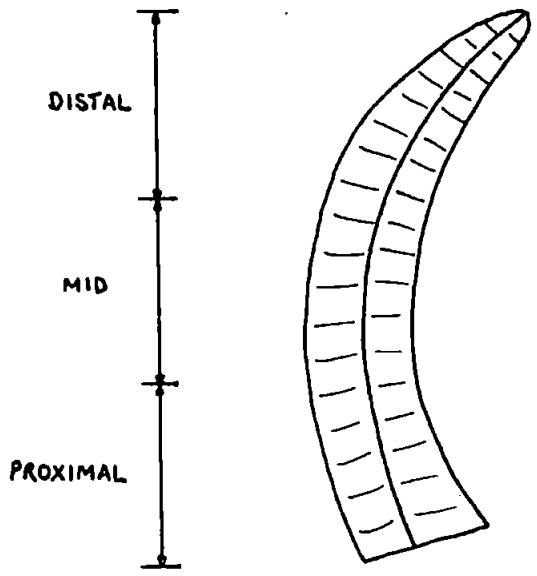
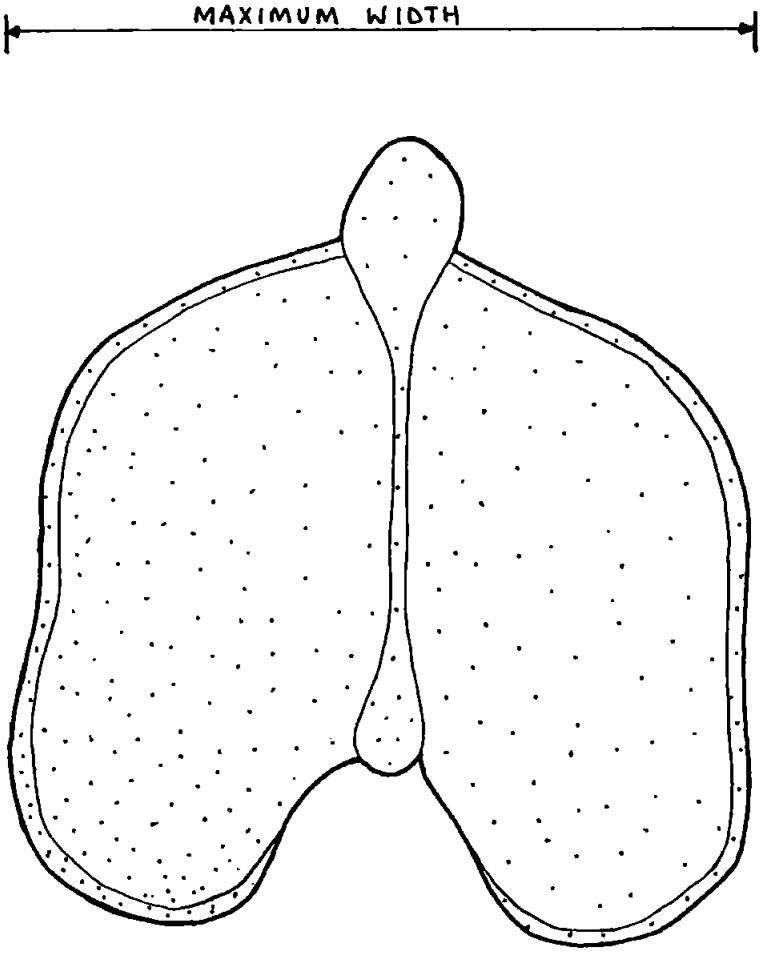


Figure 2.2. Diagrammatic representation of **(a)** gill section and **(b)** gill vessel parameters measured using the image analyzer (stippled area = gill section area; line enclosing stipple = gill section perimeter; maximum width = gill section width; shaded area = efferent vessel area; line enclosing shading = efferent vessel perimeter; hatched area = afferent vessel area; line enclosing hatch = afferent vessel perimeter).

a



b

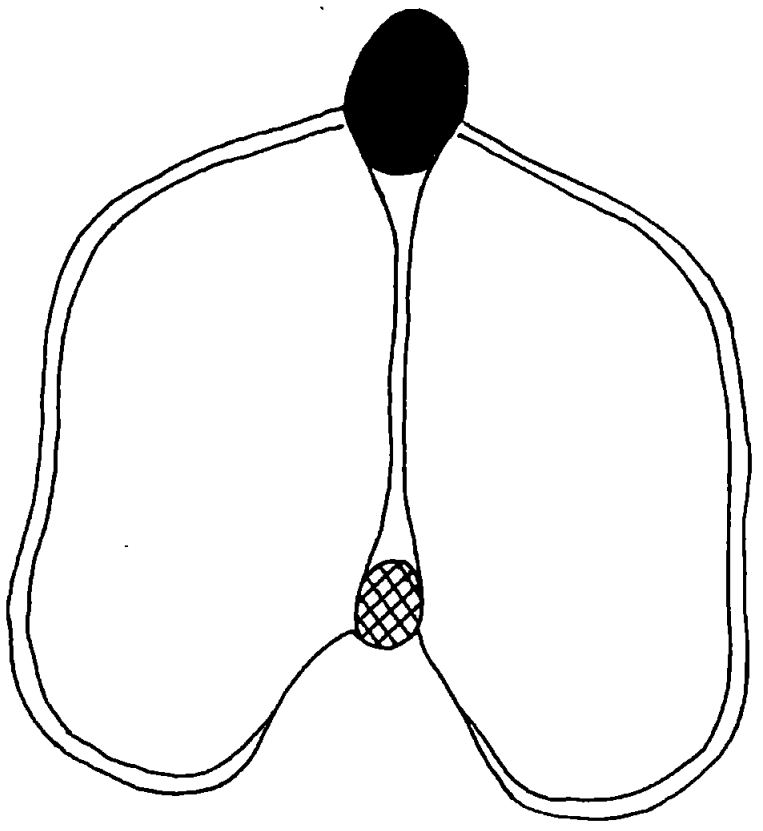


Figure 2.3. Multiple boxplot comparing the carapace width between untreated and copper-exposed crabs. The degree of significance (ANOVA) is included in the top right corner of the graph.

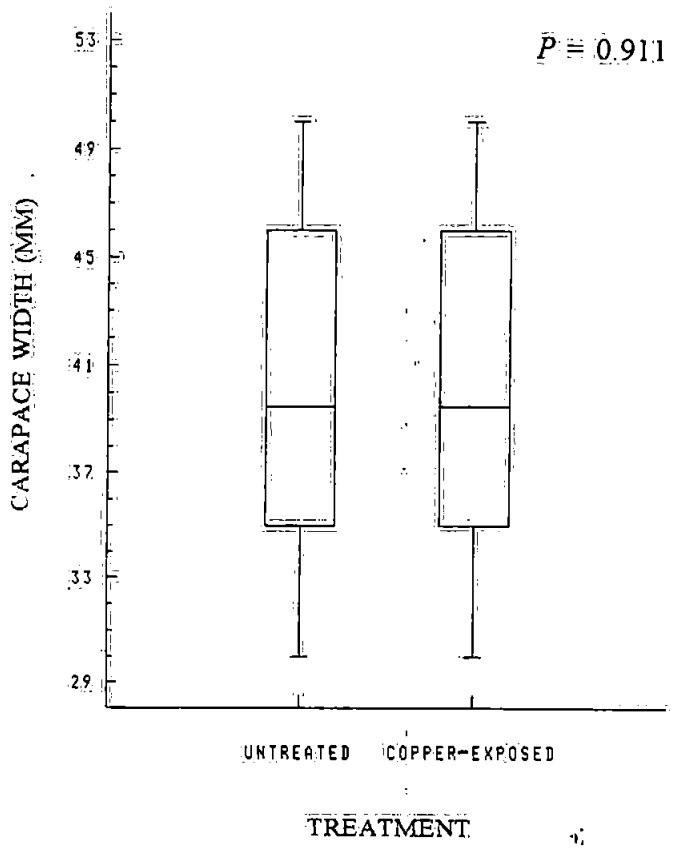
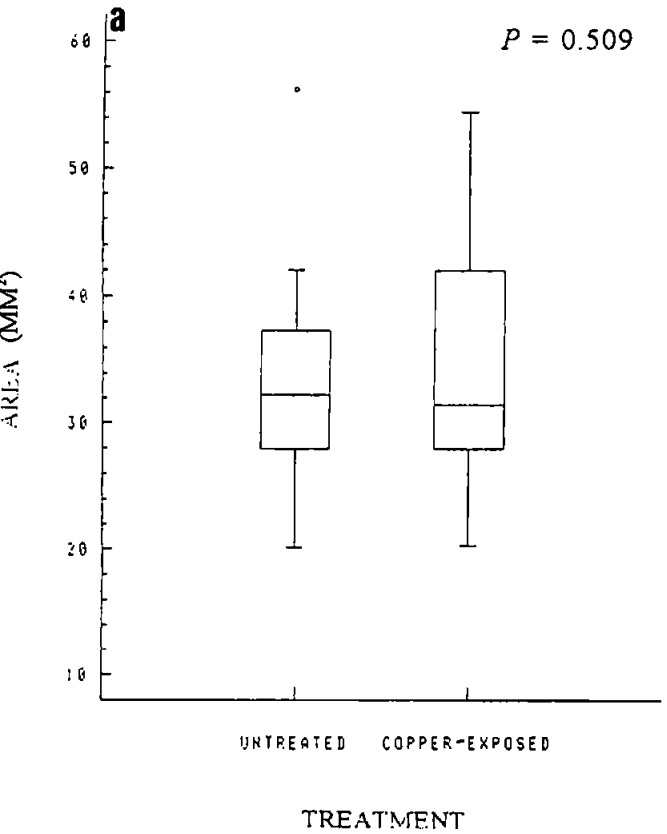
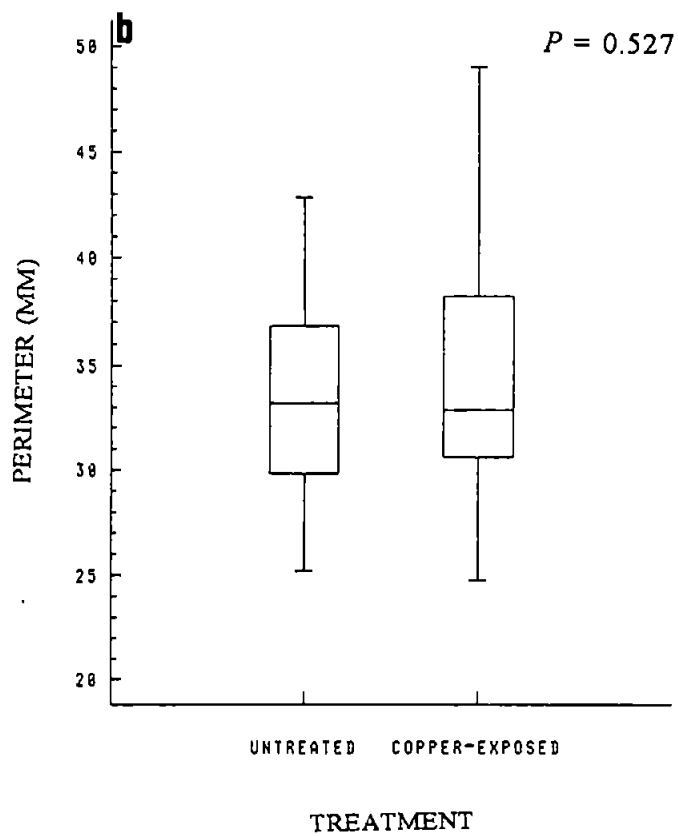

P = 0.911
CARAPACE WIDTH (MM)
53
49
45
41
37
33
29
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.4. Multiple boxplots comparing **(a)** area **(b)** perimeter and **(c)** width of the whole gill of untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.


a
P = 0.509
AREA (MM²)
60
50
40
30
20
10
UNTREATED
COPPER-EXPOSED
TREATMENT


b
P = 0.527
PERIMETER (MM)
50
45
40
35
30
25
20
UNTREATED
COPPER-EXPOSED
TREATMENT

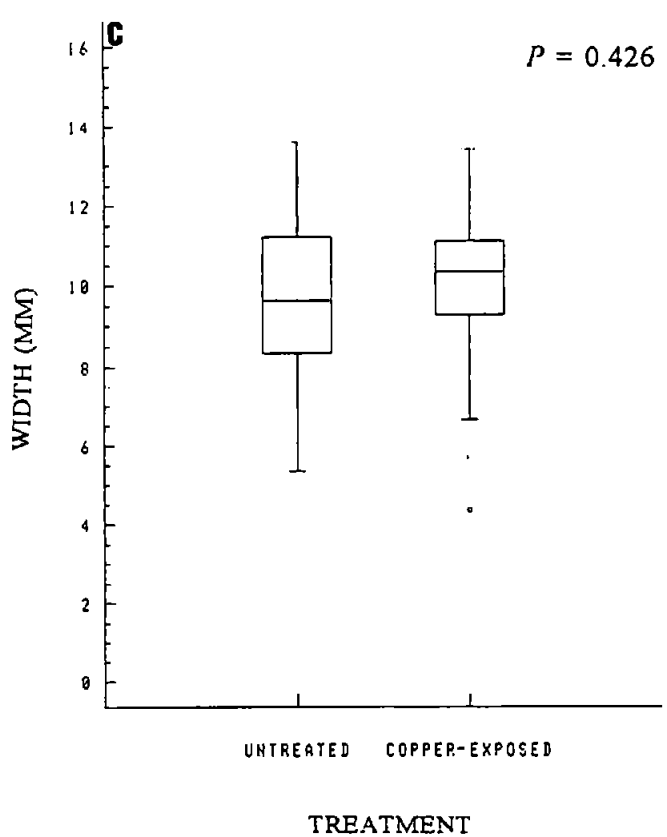

c
P = 0.426
WIDTH (MM)
16
14
12
10
8
6
4
2
0
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.5. Multiple boxplots comparing **(a)** area **(b)** perimeter and **(c)** width of the proximal gill section between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.

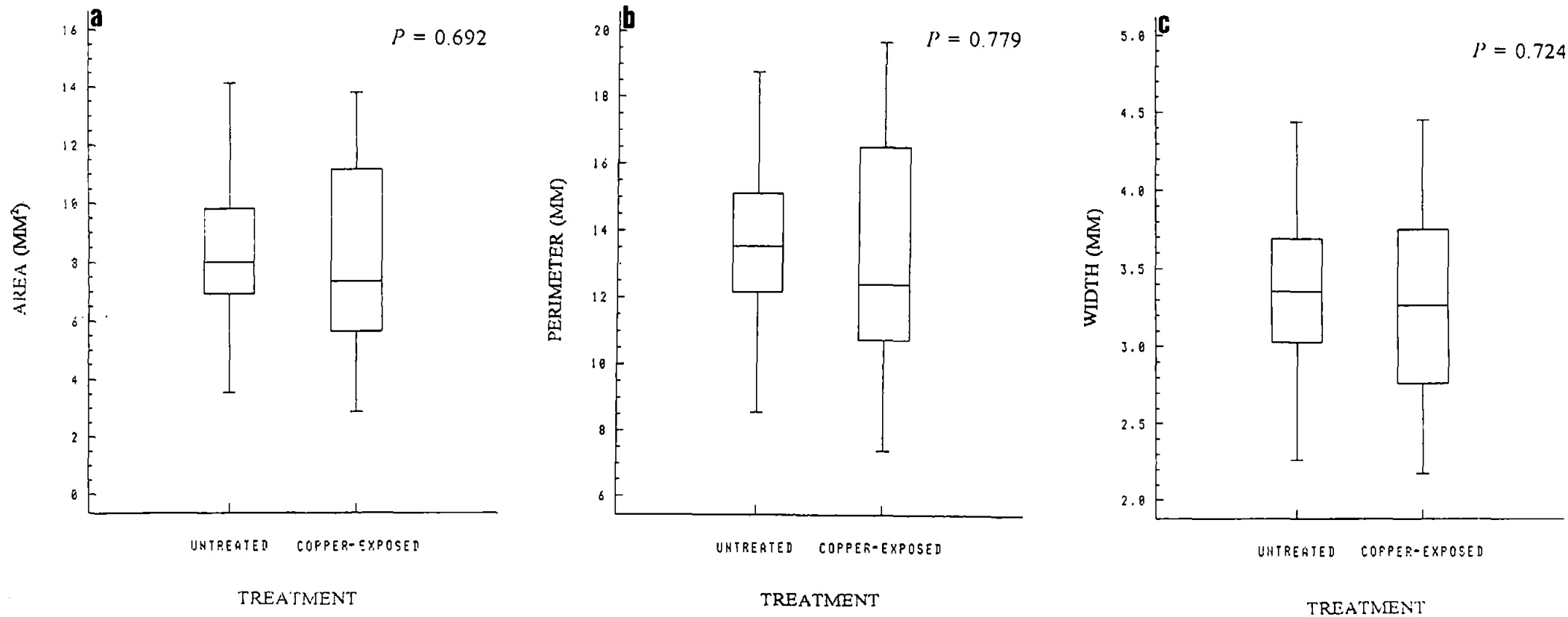

a
P = 0.692
AREA (MM²)
16
14
12
10
8
6
4
2
0
UNTREATED
COPPER-EXPOSED
TREATMENT
b
P = 0.779
PERIMETER (MM)
20
18
16
14
12
10
8
6
UNTREATED
COPPER-EXPOSED
TREATMENT
c
P = 0.724
WIDTH (MM)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.6. Multiple boxplots comparing **(a)** area and **(b)** perimeter of the afferent vessel in the proximal gill region between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.

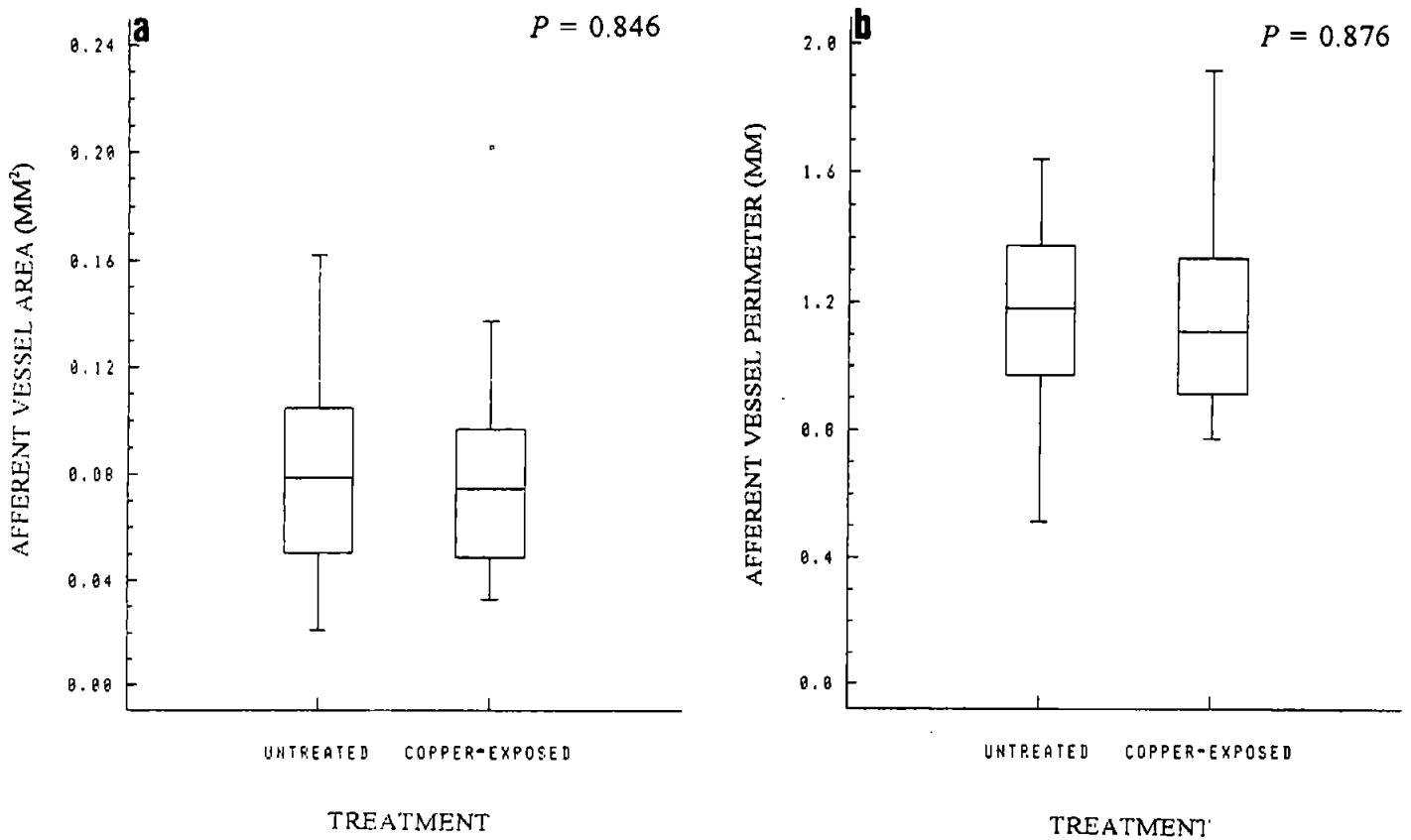

a
P = 0.846
AFFERENT VESSEL AREA (MM²)
0.24
0.20
0.16
0.12
0.08
0.04
0.00
UNTREATED
COPPER-EXPOSED
TREATMENT
b
P = 0.876
AFFERENT VESSEL PERIMETER (MM)
2.0
1.6
1.2
0.8
0.4
0.0
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.7. Multiple boxplots comparing **(a)** area and **(b)** perimeter of the efferent vessel in the proximal gill region between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.

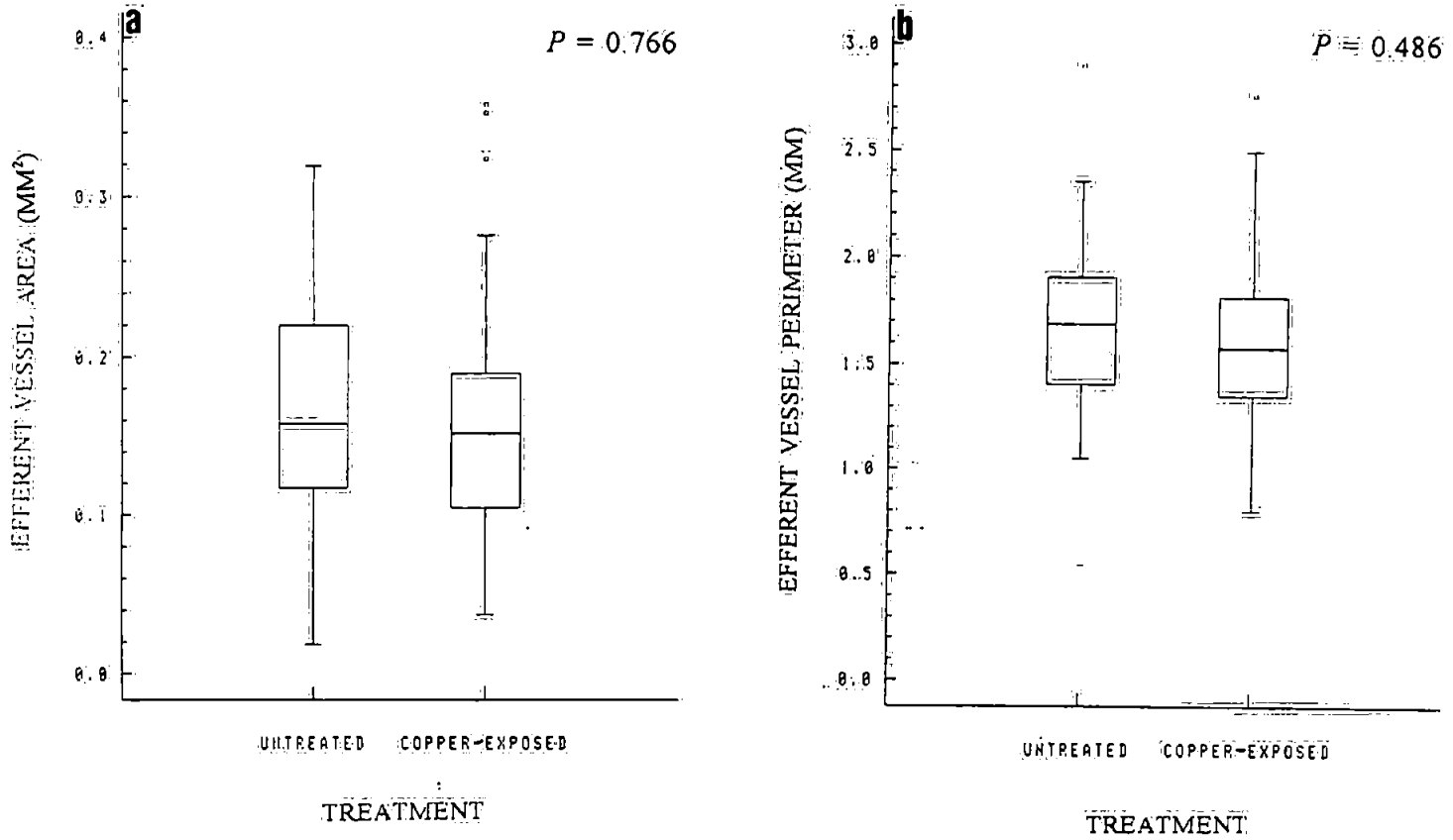

a
P = 0.766
EFFERENT VESSEL AREA (MM²)
0.4
0.3
0.2
0.1
0.0
UNTREATED
COPPER-EXPOSED
TREATMENT
b
P = 0.486
EFFERENT VESSEL PERIMETER (MM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.8. Multiple boxplots comparing (a) area (b) perimeter and (c) width of the mid gill section between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.

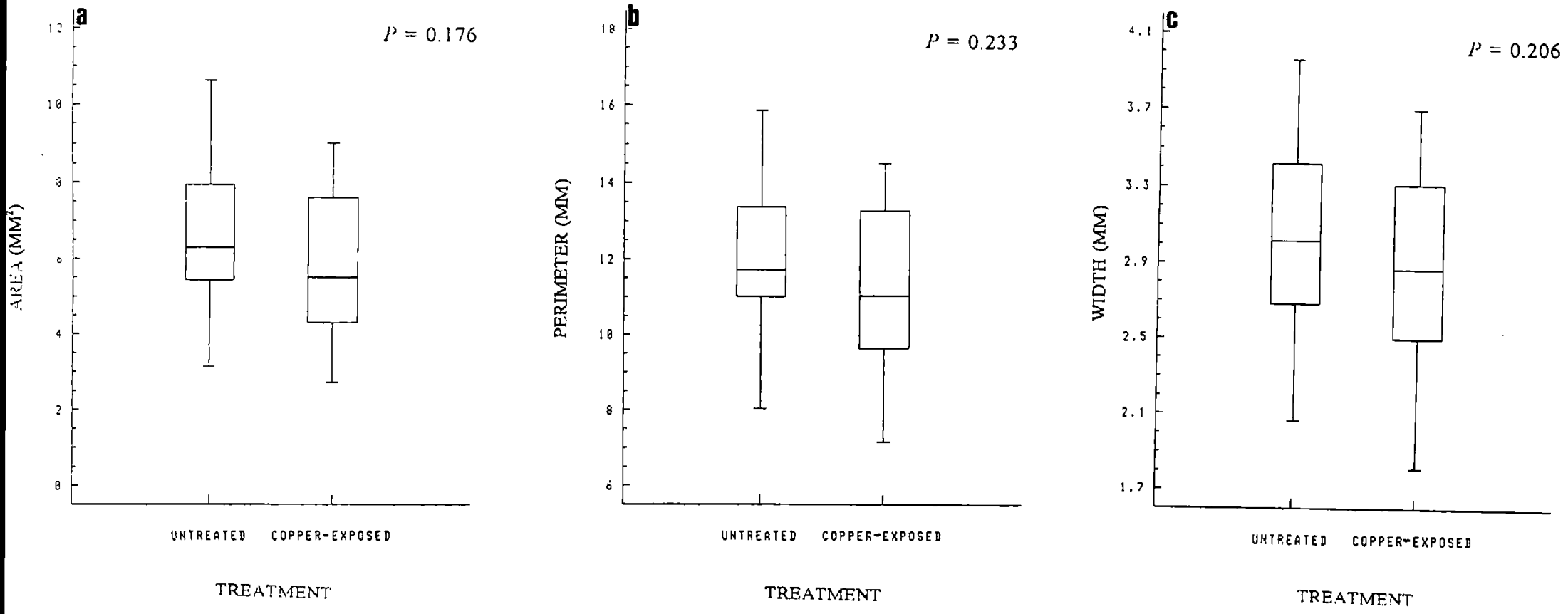
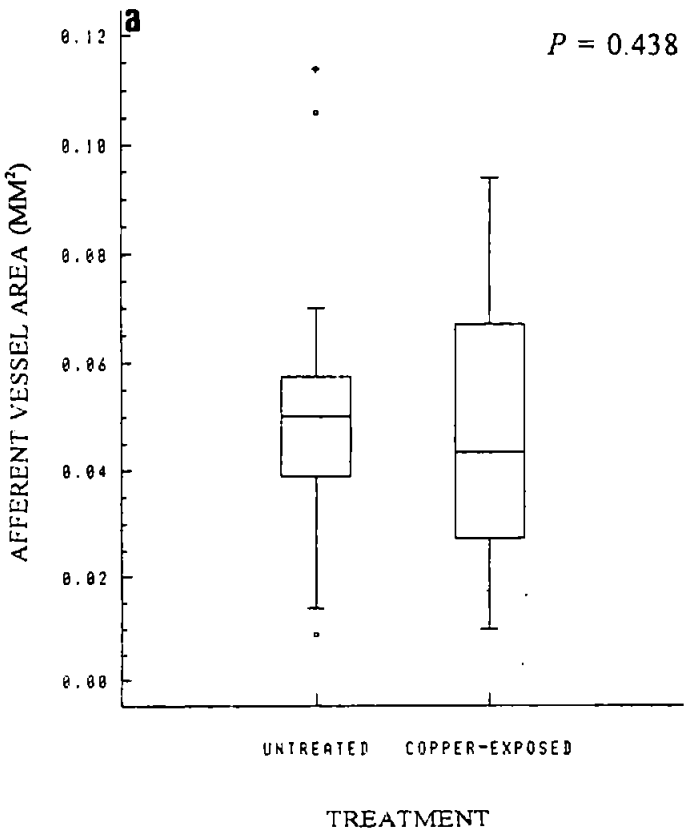

a
P = 0.176
AREA (MM²)
12
10
8
6
4
2
0
UNTREATED
COPPER-EXPOSED
TREATMENT
b
P = 0.233
PERIMETER (MM)
18
16
14
12
10
8
6
UNTREATED
COPPER-EXPOSED
TREATMENT
c
P = 0.206
WIDTH (MM)
4.1
3.7
3.3
2.9
2.5
2.1
1.7
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.9. Multiple boxplots comparing (**a**) area and (**b**) perimeter of the afferent vessel in the mid gill region between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.


a
P = 0.438
AFFERENT VESSEL AREA (MM²)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
UNTREATED
COPPER-EXPOSED
TREATMENT

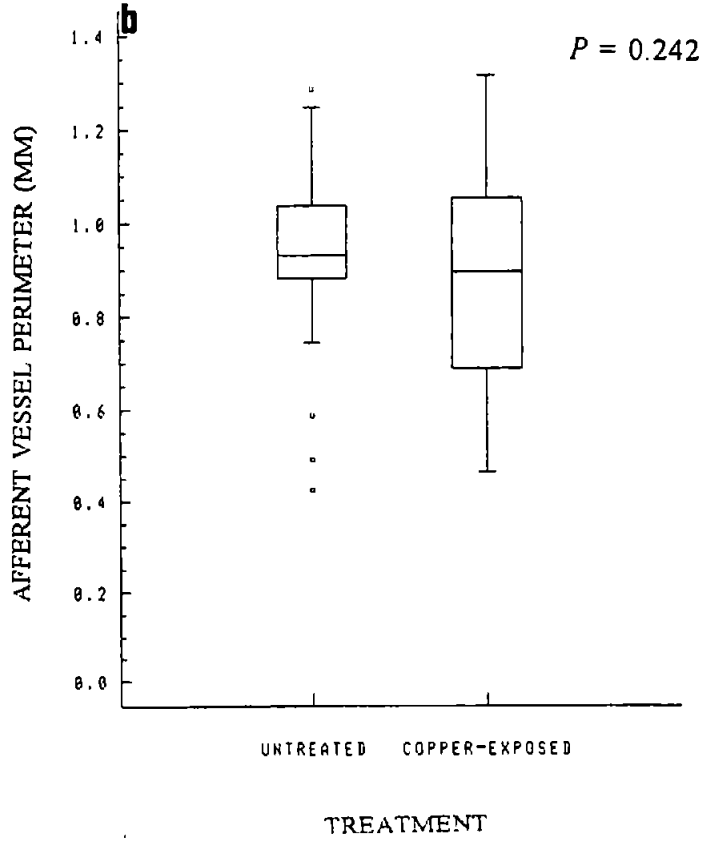

b
P = 0.242
AFFERENT VESSEL PERIMETER (MM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.10. Multiple boxplots comparing **(a)** area and **(b)** perimeter of the efferent vessel in the mid gill region between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.

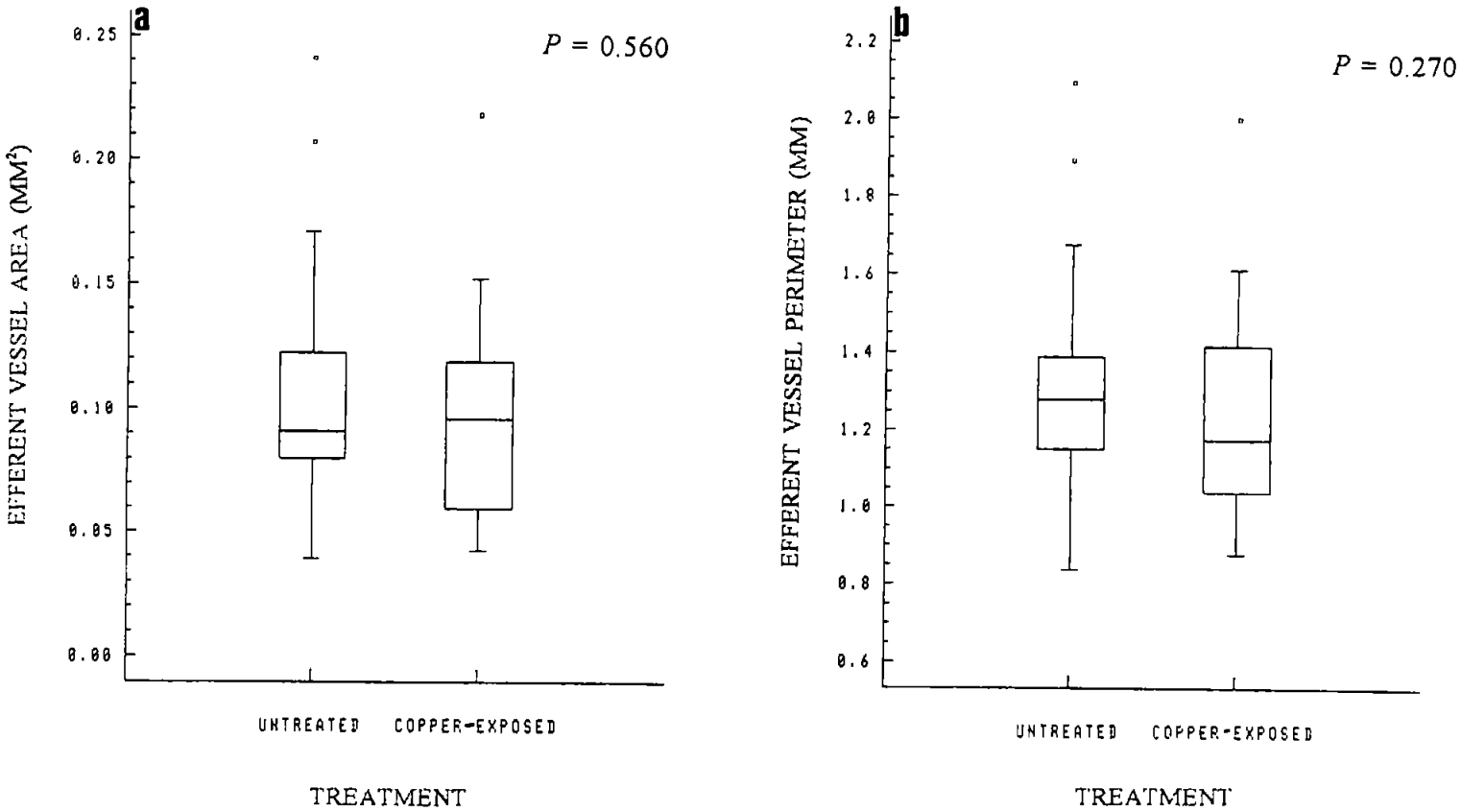
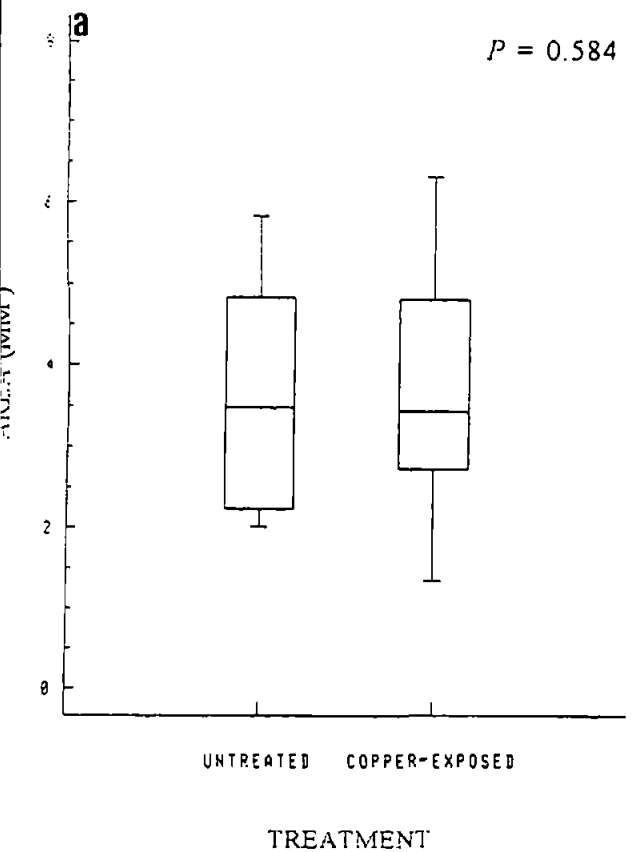
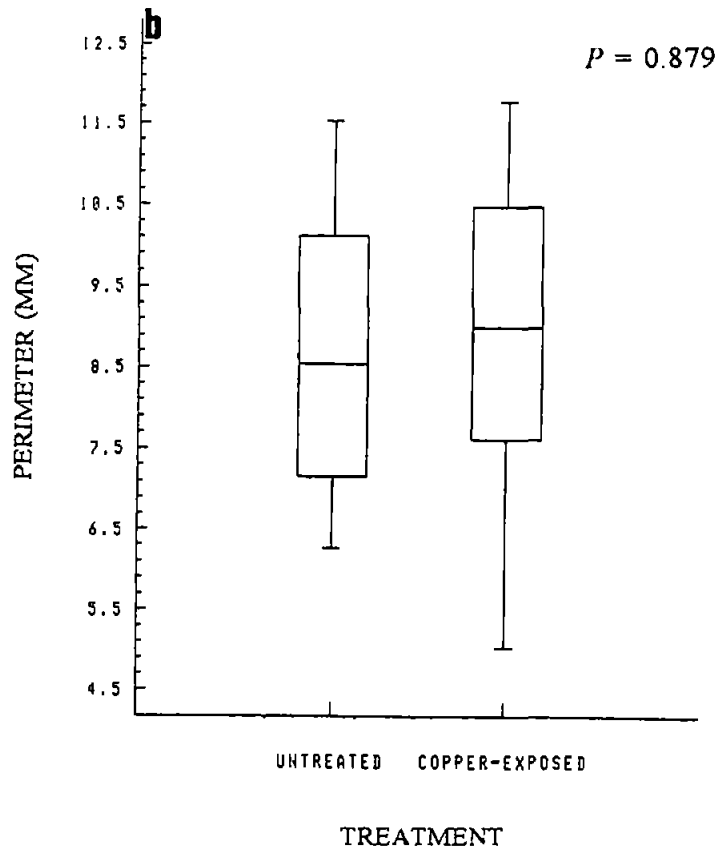

a
P = 0.560
EFFERENT VESSEL AREA (MM²)
0.25
0.20
0.15
0.10
0.05
0.00
UNTREATED
COPPER-EXPOSED
TREATMENT
b
P = 0.270
EFFERENT VESSEL PERIMETER (MM)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.11. Multiple boxplots comparing (a) area (b) perimeter and (c) width of the distal gill section between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.


a
P = 0.584
2
0
UNTREATED
COPPER-EXPOSED
TREATMENT


b
P = 0.879
PERIMETER (MM)
12.5
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
UNTREATED
COPPER-EXPOSED
TREATMENT

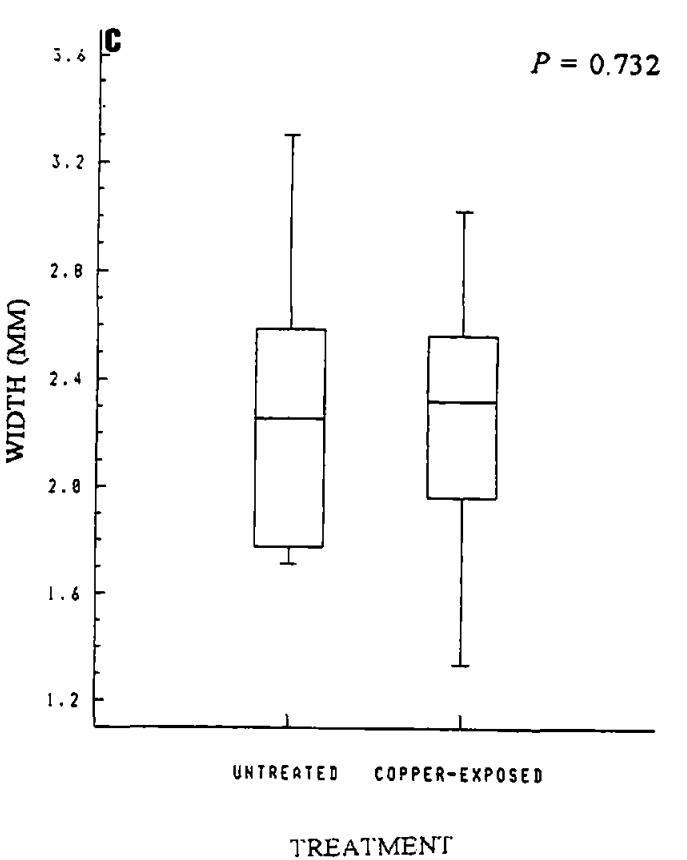
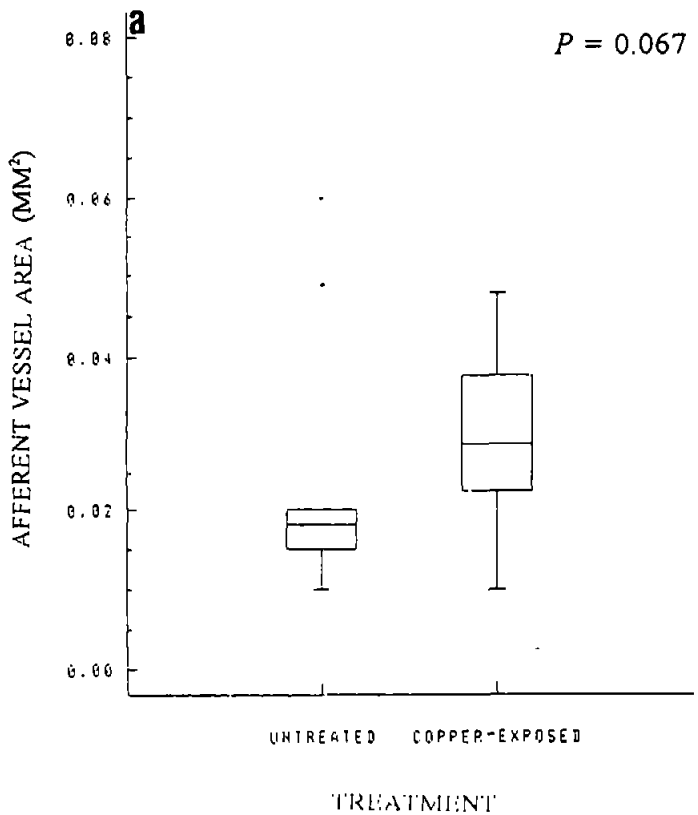

c
P = 0.732
WIDTH (MM)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.12. Multiple boxplots comparing (a) area and (b) perimeter of the afferent vessel in the distal gill region between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.


a
P = 0.067
AFFERENT VESSEL AREA (MM²)
0.08
0.06
0.04
0.02
0.00
UNTREATED
COPPER-EXPOSED
TREATMENT

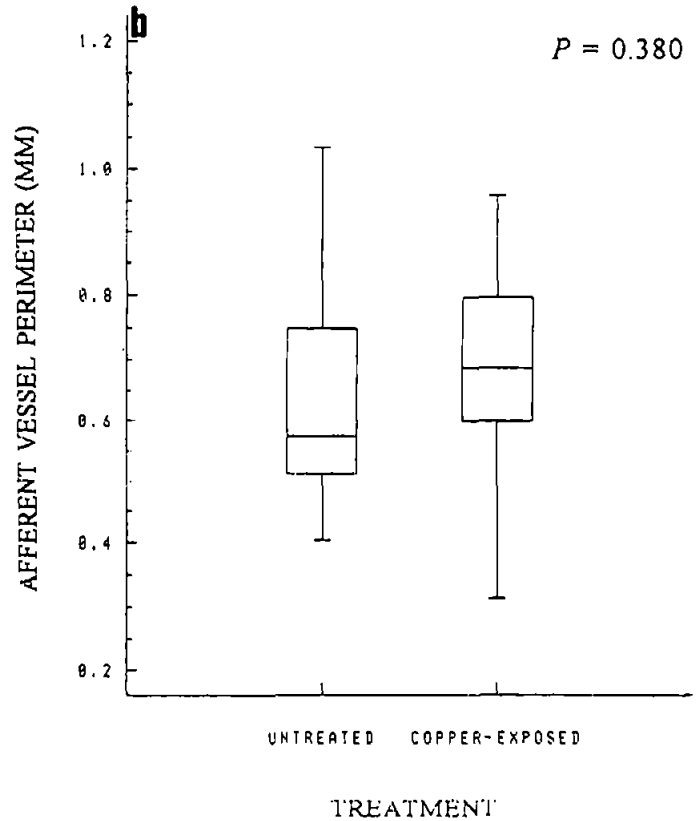
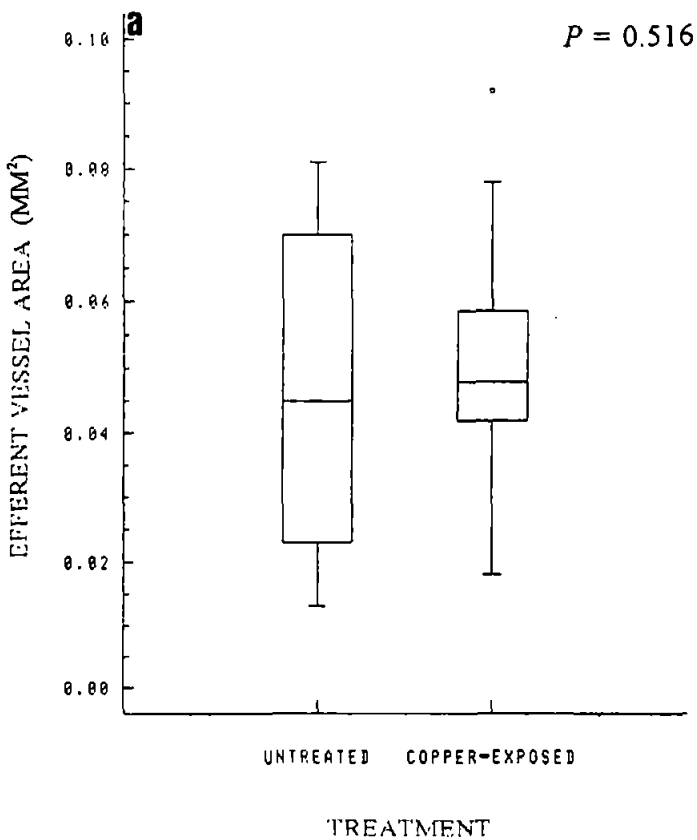
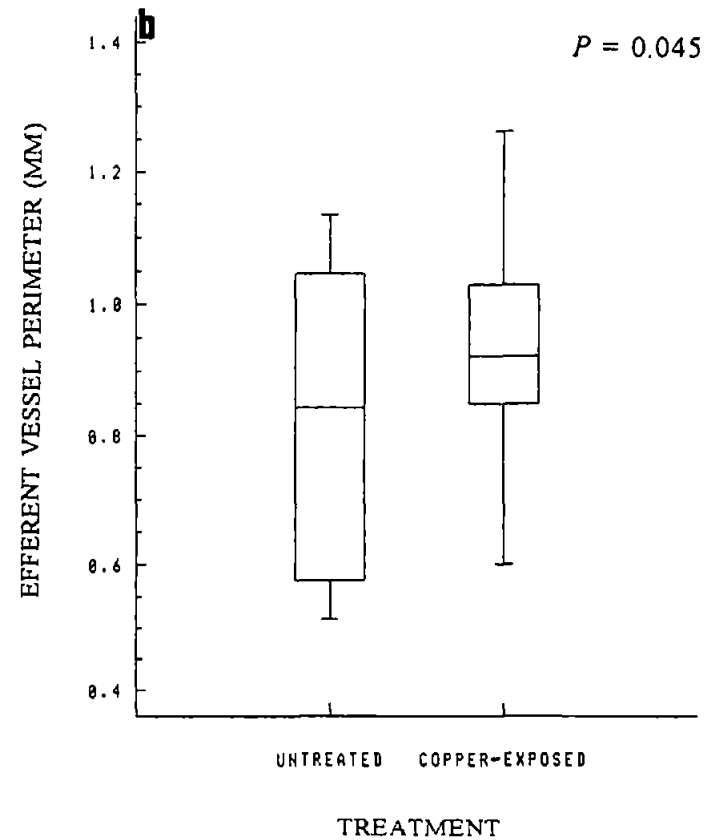

b
P = 0.380
AFFERENT VESSEL PERIMETER (MM)
1.2
1.0
0.8
0.6
0.4
0.2
UNTREATED
COPPER-EXPOSED
TREATMENT

Figure 2.13. Multiple boxplot comparing **(a)** area and **(b)** perimeter of the efferent vessel in the distal gill region between untreated and copper-exposed crabs. Degrees of significance (ANOVA) are included in the top right corner of each graph.


a
P = 0.516
EFFERENT VESSEL AREA (MM²)
0.10
0.08
0.06
0.04
0.02
0.00
UNTREATED
COPPER-EXPOSED
TREATMENT


b
P = 0.045
EFFERENT VESSEL PERIMETER (MM)
1.4
1.2
1.0
0.8
0.6
0.4
UNTREATED
COPPER-EXPOSED
TREATMENT

CHAPTER 3

GENERAL METHODS FOR ELECTRON MICROSCOPY STUDY

3.1 Collection of animals

In June 1992, intermoult *Carcinus maenas* (15-20g wet weight; carapace width 40-42mm) were collected from Portwrinkle, Cornwall, U.K. (4°18'W, 50°21'N); only males were collected as females were ovigerous at this time. Crabs were transported to the Brixham Environmental Laboratory (ZENECA Limited), Brixham, Devon in aerated seawater (35‰) obtained from the collection site.

3.2 Experimental system

Crabs were maintained individually in four covered, plastic tanks (each of 12l volume) supplied with flow-through seawater (35‰ or 10‰) at a rate of 500mlmin^{-1}; eight crabs were held in each tank. The tanks were maintained in a constant temperature room (12 ± 1°C) under 8h light and 16h dark. Crabs were acclimated to these experimental conditions for three weeks, during which they were fed every three days with chopped coley [*Pollachius virens* (L.)] until three days prior to copper exposure. Following the acclimation period, two tanks were exposed to 50μgCul^{-1} from a stock solution of $Cu(NO_3)_2.3H_2O$ prepared in distilled water and delivered using a Watson Marlowe Mhre 22 flow inducer with 1.6mm bore silicon tubing set to deliver 1mlmin^{-1}. The concentration of the stock solution was calculated, taking into account dilution factors, to ensure a constant copper concentration of 50μgl^{-1} in the tanks at all times. Atomic absorption spectrophotometry (Varian-975) confirmed the initial exposure concentration was not depleted over the 10 days of the experiment. The remaining two tanks were not exposed to copper and served as controls. Two crabs, from each of the four tanks, were dissected at 4, 7 and 10 days from the start of the copper exposure period.

3.3 Transmission electron microscopy

For each crab, the carapace was removed and the gills flooded *in situ* with a primary

fixative (2.5% glutaraldehyde buffered at pH7.2 with 0.2M sodium cacodylate). The osmolality of the fixative was adjusted (by the addition of sodium chloride solution) to conform to the haemolymph osmolality of each crab (measured with a Wescor Model 5100C Vapour Pressure Osmometer). Gill number 8 was removed by cutting the basal attachment with dissection scissors. Each excised gill was transferred to a vial of primary fixative at 4°C for 1h, washed in cacodylate buffer (pH7.2), and small pieces (approx. 1mm diameter) from the proximal, mid, and distal regions were cut with a scalpel. Gill pieces were postfixed in osmium tetroxide at 4°C for 1h, re-washed in buffer and dehydrated (20min in each mixture) in a graded alcohol series (30%, 50%, 70%, 90%, 100%), followed by propylene oxide at ambient temperature. Owing to the chitinous cuticle, it was difficult initially to infiltrate the gill pieces with resin. This problem was overcome by using a sequence of graded mixtures of propylene oxide and resin (3:1, 1:1, 1:3); gill pieces were left (in a rotating mixer) for 24h in each mixture, followed by 24h in pure, pre-warmed resin (Spurr, 1969). Gill pieces were transferred to pure resin in rubber moulds, carefully orientated such that lamellae could be transversely sectioned and placed in an oven at 70°C for 8h. Ultrathin sections (0.1μm), cut with a glass knife on a Reichert ultramicrotome, were collected on copper grids, and stained with an aqueous, saturated solution of uranyl acetate and Reynolds lead citrate. Sections were examined with a Jeol 1200 transmission electron microscope operated at 80kV.

CHAPTER 4

STRUCTURAL VARIABILITY AND DISTRIBUTION OF CELLS IN THE GILL OF *CARCINUS MAENAS* KEPT IN 35‰ SEAWATER

[Aspects of this chapter were presented at the Society for Experimental Biology meeting, Canterbury, April, 1993 and at the 3rd International Conference on Trace Metals in the Aquatic Environment, Aarhus, May, 1994. This chapter is also included in Lawson *et al.*, "Structural variability and distribution of cells in a posterior gill of *Carcinus maenas* (Decapoda: Brachyura)". *Journal of the Marine Biological Association of the United Kingdom*, in press.]

4.1 Introduction

Although crustacean gills are known to play a prominent role in respiratory gas exchange, osmoregulation, ionic regulation, acid and base regulation, and ammonia excretion (for review see Taylor & Taylor, 1992), there have been relatively few attempts to relate gill structure to physiological functioning for individual species. The exception is the shore crab *Carcinus maenas* (L.), a moderate osmoregulator (Zanders, 1981), which uses its posterior gills (gills 7, 8 and 9) for both respiration and osmoregulation (Towle & Kays, 1986). Six major cell types have been identified, and related to function, within the posterior gills of *C. maenas* (Table 1). Even so, the distribution of each of these cell types within the gill has received little detailed study (an exception being Taylor & Taylor, 1992) and there remains the question as to whether there are specialised gill areas for each of the physiological functions outlined previously.

This chapter describes the structural variability and distribution of the six major cell types from posterior gill 8 of *Carcinus maenas* acclimated for three weeks to 35‰ seawater. Such details allowed a comprehensive discussion of gill form and functioning, and emphasise the need to establish the detailed structural and distributional variability of gill cells before an assessment of the effect of environmental variables, such as salinity variation and heavy metal pollution, on the gill is made.

4.2. Materials and methods

The materials and methods used in this chapter followed those outlined in Chapter 3. At Brixham, crabs were maintained in 35‰ seawater.

4.3 Results

The cuticle of *Carcinus maenas* was made up of three main layers (Fig. 4.1a). The

outermost layer, the epicuticle, was smooth, contributed relatively little to the total cuticle thickness and was often covered by a film of darkly-stained (probably detrital) material. In addition, a single layer of (probably) symbiotic algal cells (Fig. 4.1b) and/or aggregations of bacterial cells of various shapes and sizes (Fig. 4.1c) were associated with the epicuticle. Bacteria and algal cells were not found near any particular epithelial cell type nor in specific regions of the lamellae. Below the epicuticle, the exocuticle and endocuticle had striations running parallel to the lamellae, the striations being more pronounced in the exocuticle (Fig. 4.1a). The exocuticle made up over 50%, and the endocuticle constituted between 20-30%, of the total cuticle width. The absolute cuticle thickness and the relative proportions of the three layers, however, depended on gill position. The cuticle was thinnest in and around the marginal canal (illustrated in Fig. 1.3) of individual gill lamellae. Underlying the cuticle was a continuous epithelial layer made up of striated, chief and pillar cells. Between the epithelial layers, the haemolymph space was divided by an intralamellar septum (Fig. 4.2). In gill areas where the epithelial layer was particularly thick, the haemolymph space was minimal and the septum was not visible (Fig. 4.3). In general, the septum occupied areas towards the marginal canal of each lamella and extended frequently into the marginal canal itself. The remaining gill cells (nephrocytes, glycocytes and haemocytes) were attached to the septum or were free in the haemolymph space. The ultrastructure of the gill cells did not alter over the 10 day sampling period and there was no ultrastructural difference between cells taken from crabs after 4 and 10 days.

Striated cells (Fig. 4.4a) had an irregular shape, varied in size (8-14μm apical to basal depth) depending on gill position (the largest striated cells were found adjacent to the central raphe, shown in Fig. 1.3) and were packed with mitochondria, which were rounded and contained cristae showing varying degrees of development. The basal membrane of the striated cell was thrown into an extensive network of folds

(mitochondria were sited within these folds) which extended towards the apical side of the cell, the basal lamina remained straight. Striated cells had an extensive network of apical folds, although mitochondria were absent from within the folds. In some areas, the base of the apical infoldings gave rise to small, pinocytotic vesicles.

Chief cells (Fig. 4.4b) were squamous, smaller (2-5μm apical to basal depth) and had a less extensive network of apical folds than striated cells. Chief cells lacked a basal infolding system but did contain mitochondria and some small, irregularly-shaped vacuoles. In all gill regions, the epithelial layer was comprised of a mixture of striated and chief cells; however, the relative proportions of these cells varied with gill region. In the proximal gill region, the epithelium consisted almost exclusively of striated cells which were evenly abundant along the entire length of each individual lamella. In the mid gill region, there was a more even mix of striated and chief cells, while the distal region had the greatest proportion of chief cells. Striated cells were concentrated towards the central raphe and chief cells towards the marginal canal of each lamella (these areas are illustrated in Fig. 1.3).

Pillar cells (Fig. 4.4c) had some ultrastructural characteristics of the two other types of epithelial cell (a dense apical infolding network and packed with mitochondria). They were columnar, often multinucleate and frequently converged and fused, forming a zig-zag junctional complex in the middle of the haemolymph space (Fig. 4.4d). Pillar cells contained bundles of microtubules, arising from the apical surface of the cell and running perpendicular to the cuticle. Rods of electron-dense material stretched along the apical edge of each pillar cell and apparently anchored the microtubules to the cuticle. The pillar cell shape varied depending on gill position. Towards the marginal canal, pillar cells were long and thin (Fig. 4.2), whereas near the central raphe they were hour-glass shaped, with concave sides (Fig. 4.3). The ultrastructure of the pillar cells did not vary with cell shape. In the distal gill region, thin pillar cells were more abundant than

hour-glass cells, however, both cell types were present throughout the whole length of the gill. Small clusters of darkly-stained material, with an electron microscopical characteristic appearance of glycogen, were seen in some of the epithelial cells (Fig. 4.4).

Ladder-like, intercellular junctional complexes occurred throughout the epithelial layer (Fig. 4.4a,b). These had an electron-dense adherence area (*zonula adherens*) near the apical side of the cell which was connected to a band of septate junctions, of varying lengths, that traversed the epithelial cells towards their basal side. Intercellular junctional complexes crossed the whole length of the cell and attached to apical and basal cell surfaces.

Nephrocytes were spherical or irregular in shape, were of variable size (10-24μm diameter) and had a range of cell contents due to sectioning in different planes (Fig. 4.5). Generally, each nephrocyte housed a large central vacuole, the contents of which ranged from needle-like microcrystals to granular electron-dense ovoid structures. Satellite vacuoles and mitochondria were scattered around the periphery of the nephrocyte, and there were a few areas of irregularly-orientated membranes, which may be identified as endoplasmic reticulum. The ovoid nucleus, not often visible due to oblique sectioning, was smaller than the central vacuole and resided in the outskirts of the cell. Another conspicuous cell characteristic was the rim of regularly-spaced processes, or pedicels, beneath the basal lamina; narrow gaps between the pedicels were traversed by a thin diaphragm (Fig. 4.5c). Small, coated vesicles were present in the cell cytoplasm. Nephrocytes were rarely sited in the distal gill region and were most abundant in the proximal and mid gill regions. They occurred, either singularly or in groups of up to 9 or 10, in gill areas where a lamellar septum was present (that is, from mid to marginal canal end of each lamella) and were found in the haemolymph space, often closely associated with the septum (Fig. 4.2).

Glycogen, as well as being located in the epithelial cell cytoplasm as shown by Figure 4.4, also resided in specialised gill cells, glycocytes. These were irregularly-shaped cells (7-9μm diameter) with a central nucleus and cytoplasm packed with glycogen granules (Fig. 4.6a). The perinuclear cytoplasm, devoid of granules, appeared as a pale zone. Typically, glycocytes occurred along the whole gill length, wherever an intralamellar septum was present, and were housed within the septal tissue. The intralamellar septum also housed glandular cells (Fig. 4.6b) and gill capillaries (Fig. 4.6c). Glandular cells and capillaries were, however, relatively infrequently sited in the gill and their distribution was not dependent on gill position.

Haemocytes (6-12μm diameter) were found in the haemolymph space and could be classified into three categories. Agranular haemocytes (Fig. 4.7a) were ovoid, lacked granules and had a large, elongated nucleus which filled much of the cell. The cell cytoplasm of agranular haemocytes formed a relatively thin layer around the nucleus, and contained endoplasmic reticulum and mitochondria. Small-granule haemocytes (Fig. 4.7b), unlike the agranular cells, were readily identifiable by the presence of a varying number of small, electron-dense granules in the cell cytoplasm. They were wider than the agranular cells, had a more oval nucleus and contained more mitochondria. Large-granule haemocytes (Fig. 4.7c), although fewer in number, were also found in the gill. These cells were more spherical and contained much larger, electron-dense granules than the other haemocyte types (approximately 1μm diameter compared to 0.2μm diameter in small-granule haemocytes). Also, the granules of large-granule haemocytes were ovoid or irregular in shape. The nucleus of the large-granule haemocytes was reduced in size and often not visible, and the cytoplasmic content, the amount of endoplasmic reticulum and the number of mitochondria were less than in agranular and small-granule haemocytes.

4.4 Discussion

The basic ultrastructure of *Carcinus maenas* gill 8, as detailed here, was similar to that described for other decapod gills (Copeland, 1963a,b, 1964, 1968; Wright, 1964; Copeland & Fitzjarrell, 1968; Strangeways-Dixon & Smith, 1970; Fisher, 1972; Foster & Howse, 1978; Taylor & Greenaway, 1979; Johnson, 1980; Compere *et al.*, 1989; Goodman & Cavey, 1990; Maina, 1990; Farrelly & Greenaway, 1992). In each decapod, the gill epithelial layer was made up of chief, striated and pillar cells, and nephrocytes, glycocytes and haemocytes resided in the haemolymph space or attached to the intralamellar septum of the gill lamellae. These six major cell types have been identified previously and related to function as shown in Table 1.

Striated cells of *Carcinus maenas* conformed to previous descriptions (Copeland, 1963a,b, 1964, 1968; Copeland & Fitzjarrell, 1968; Johnson, 1980; Compere *et al.*, 1989; Goodman & Cavey, 1990; Farrelly & Greenaway, 1992; Taylor & Taylor, 1992), except that Taylor & Taylor (1992) reported longer and thinner mitochondria, and a less dense apical infolding network compared with present descriptions. These differences may reflect differences associated with adaptations to different environments. Unfortunately, Taylor & Taylor (1992) did not report the source or holding salinity of their *C. maenas*. The high numbers of mitochondria associated with the basal plasma membrane, previously described as "mitochondrial pumps" (Copeland & Fitzjarrell, 1968), indicate that striated cells have a high metabolic activity with special adaptations for water and ion uptake (Copeland, 1968; Copeland & Fitzjarrell, 1968; Berridge & Oschman, 1972; Talbot *et al.*, 1972; Gilles & Pequeux, 1981; Barra *et al.*, 1983). This suggestion has been supported further by the discovery of a Na^+/K^+ exchange mechanism, sited predominantly on the basal side of crustacean gill epithelia, involved in the active transport of Na^+ ions across the gill epithelium (Pequeux & Gilles, 1981; Gilles & Pequeux, 1985). If striated cells are the only sites of ionic regulation and

osmoregulation in the gill, it may well be that certain gill regions are specialised for these functions. Based on the distribution of striated cells, the most likely areas for these physiological processes are in the proximal gill region and towards the central raphe (shown in Fig. 1.3) of each lamella.

The large surface area of chief cells implicated an involvement in gaseous exchange (Copeland, 1968; Copeland & Fitzjarrell, 1968; Johnson, 1980; Compere *et al.*, 1989; Goodman & Cavey, 1990; Farrelly & Greenaway, 1992; Taylor & Taylor, 1992), and their distribution implicated the distal gill region and the marginal canal of each lamella as the most favourable areas for this process. The cuticle width in *Carcinus maenas* has also been shown to be minimal within the marginal canal, supporting further the conclusion that this area was favourable for gaseous exchange (Taylor & Taylor, 1992). The lack of a basal infolding network with many associated mitochondria suggested that these cells have a less prominent role in ionic regulation than the striated cells.

The shape of pillar cells varied along the length of an individual lamella and this plasticity has been reported previously (Taylor & Taylor, 1986; Compere *et al.*, 1989). There are, however, different interpretations for such variation in cell form. Pillar cells may simply reflect the ultrastructural characteristics of the epithelial cells surrounding them (for example, towards the central raphe they resembled striated cells which were most frequently observed in this region; towards the marginal canal, they resembled chief cells which dominated this region), and may be involved in ionic regulation and osmoregulation or respiratory gas exchange, depending on gill position. A second interpretation of pillar cell variability is based on the presence of a well developed microtubular system, suggesting a cytoskeletal role (Fisher, 1972). Therefore, changes in pillar cell shape may result from different gill areas experiencing different hydrodynamic forces and requiring different degrees of structural support. Present results showed a clear relationship between the development of an intralamellar septum

and the shape of the pillar cells. Thin pillar cells were correlated with the presence of a well developed septum. Away from the central raphe, thin pillar cells were anchored laterally by the intralamellar septum, aiding in the overall structural support of the lamella. Near the central raphe, however, the intralamellar septum was much less evident and, to compensate for this lack of support, pillar cells may have had to be more rounded. A final interpretation by some authors (Copeland, 1968; Copeland & Fitzjarrell, 1968; Johnson, 1980; Farrelly & Greenaway, 1992) is that the location of pillar cells determines the flow of haemolymph through the lamellae. Hence, the association between the septum and the shape of the pillar cells may be dictated by the pattern of haemolymph flow rather than the necessity to have lamellar support. Each of these theories may singularly, or in combination, cause the different pillar cell shapes observed in the gill at 35‰ seawater.

The presence of septate junctions in the epithelial layer of arthropods has been well documented (Copeland & Fitzjarrell, 1968; Bubel, 1976; Noiret-Timothee & Noiret, 1980; Green & Bergquist, 1982; Finol & Croghan, 1983; Lane *et al.*, 1984; Martelo & Zanders, 1986; Compere *et al.*, 1989; Goodman & Cavey, 1990), although their role is still open to debate. Septate junctions have been implicated in cellular adhesion and in the restriction of intercellular permeability (Loewenstein & Kano, 1964; Bubel, 1976). The structure and location of the septate junctions, observed in this study, supports both types of involvement.

The ultrastructural characteristics of *Carcinus maenas* nephrocytes were identical to those reported previously for other crustaceans (Drach, 1930; Wright, 1964; Morse *et al.*, 1970; Strangeways-Dixon & Smith, 1970; Doughtie & Rao, 1981; Foster & Howse, 1978; Johnson, 1980; Goodman & Cavey, 1990; Taylor & Taylor, 1992). In *C. maenas*, nephrocytes occurred in the haemolymph space or attached to the intralamellar septum, precluding their involvement in respiratory gas exchange, ionic regulation and

osmoregulation. More likely, nephrocytes form part of the gill defence mechanism (Strangeways-Dixon & Smith, 1970). As the gill forms an interface between the organism and external environment, it is reasonable to assume that gills provide a direct route for the entry of toxic materials and infectious particles. Nephrocytes may endocytose foreign substances from the haemolymph, rendering them unavailable to the rest of the organism. Taylor & Taylor (1992) highlighted similarities between renal podocytes and nephrocytes, and suggested that nephrocytes also function as a molecular filter. Following filtration from the haemolymph, foreign substances may be contained within the coated vesicles of the cell cytoplasm. It has been suggested that the giant vacuoles within the nephrocyte are large lysosomes that may enzymatically digest micro-organisms (Maina, 1990). The central vacuole often contained electron dense material suggesting it functions in the breakdown of cellular debris released into the haemolymph space. In this study, nephrocytes were observed most frequently in the proximal gill region where the largest ventilatory currents occur, making them ideally situated to form a first line of defence against toxic substances and potentially infectious micro-organisms. Nephrocytes were also common towards the marginal canal and, in some cases, were sited within the marginal canal, in agreement with the work by Drach (1930) and Taylor & Taylor (1992) on *Carcinus maenas*. If nephrocytes are involved in host defence mechanisms, the lower abundance of these cells in the distal gill region suggests either defensive mechanisms are not as efficient, or a different line of defensive measures are employed in this area. Differences in size and morphology of nephrocytes within the gill may be explained partly by differences in planes of sectioning and by the possibility that they represent different developmental stages. Drach (1930) and Johnson (1980) discovered that small crustacean nephrocytes with clear vacuoles were gradually transformed, during intermoult, into mature nephrocytes with large vacuoles containing granular material. The present work, however, provided

no evidence for localised gill areas of nephrocyte development.

Although an intralamellar septum appears to be a regular occurrence in the gill lamellae of decapods (and crustaceans in general), its function remains speculative. In general, the intralamellar septum divides the haemolymph space, and may aid in the passage of haemolymph through the lamellae (Barra *et al.*, 1983) and in the overall structural support of the lamellae. In these contexts, the relevance of the decreased extensiveness of the septum in areas near the lamellar raphe and in the distal gill region observed in this study is not understood. In addition, the septum appeared to be involved in glycogen storage (glycocytes were found within the septal tissue). The presence of these storage cells may implicate an involvement of the gill in blood sugar level regulation (Finol & Croghan, 1983), in which case, their distribution would imply blood sugar regulation occurs in specific areas of the gill.

Following the classification scheme for shrimp haemocytes, based on the presence, absence and relative size of granules within the cell (Martin & Graves, 1985), *Carcinus maenas* gill haemocytes consist of agranular, small-granule and large-granule types. Each has characteristics similar to those described for other crustaceans (Toney, 1958; Hearing & Vernick, 1967; Rabin, 1970; Sawyer *et al.*, 1970; Sindermann, 1971; Bauchau & De Brouwer, 1972; Johnston *et al.*, 1973; Bubel, 1976; Bubel & Jones, 1974; Bodammer, 1978; Cornick & Stewart, 1978; Ratcliffe & Rowley, 1979). Previously, *C. maenas* haemocytes have been implicated in carbohydrate transport and metabolism (Johnston & Davies, 1972; Johnston *et al.*, 1973; Williams & Lutz, 1975), as a reservoir of free amino-acids (Evans, 1972), to assist in the relocation of lipoprotein in the moult (Sewell, 1955), and to be actively involved in host defence mechanisms (Lochead & Lochead, 1941; Sewell, 1955; Stutman & Dolliver, 1968; Wood *et al.*, 1971; Fontaine & Lightner, 1973a,b, 1975; Johnston *et al.*, 1973; Paterson & Stewart, 1974; Ghiretti-Magaldi *et al.*, 1977; Smith & Ratcliffe, 1978). The differing haemocyte

forms of *C. maenas* were not localised in specific gill areas, however, small-granule haemocytes were the most and large-granule haemocytes the least prevalent type in the gill at 35‰ seawater.

Table 1. Major gill cell types from *Carcinus maenas* and their proposed function. Cell terminology follows Goodman and Cavey (1990).

CELL TYPE	PREVIOUS TERMINOLOGY	PROPOSED FUNCTION	REFERENCES
STRIATED CELL	SALT-ABSORBING CELLS SALT-TRANSPORTING CELLS	ION REGULATION OSMOREGULATION	COPELAND, 1968; COPELAND AND FITZJARRELL, 1968; BERRIDGE AND OSCHMAN, 1972; TALBOT *et al.*, 1972; GILLES AND PEQUEUX, 1981; BARRA *et al.*, 1983; COMPERE *et al.*, 1989; GOODMAN AND CAVEY, 1990; FARRELLY AND GREENAWAY, 1992; TAYLOR AND TAYLOR, 1992.
CHIEF CELL	THIN CELLS	RESPIRATION	COPELAND, 1968; COPELAND AND FITZJARRELL, 1968; JOHNSON, 1980; COMPERE *et al.*, 1989; GOODMAN AND CAVEY, 1990; FARRELLY AND GREENAWAY, 1992; TAYLOR AND TAYLOR, 1992.
PILLAR CELL	PILLASTER CELLS TRABECULAR CELLS	SUPPORT HAEMOLYMPH FLOW	FISHER, 1972. COPELAND, 1968; COPELAND AND FITZJARRELL, 1968; JOHNSON, 1980; FARRELLY AND GREENAWAY, 1992.
NEPHROCYTE	PODOCYTES	ENDOCYTOSIS	CUENOT, 1893; DRACH, 1930; WRIGHT, 1964; MORSE *et al.*, 1970; STRANGEWAYS-DIXON AND SMITH, 1970; DOUGHTIE AND RAO, 1981; FOSTER AND HOWSE, 1978; JOHNSON, 1980; GOODMAN AND CAVEY, 1990; TAYLOR AND TAYLOR, 1992.
GLYCOCYTE		GLYCOGEN STORAGE	FINOL AND CROGHAN, 1983; GOODMAN AND CAVEY, 1990.
HAEMOCYTE	BLOOD CELLS	CARBOHYDRATE TRANSPORT AND METABOLISM AMINO-ACID STRORAGE RELOCATION OF LIPOPROTEIN IN THE MOULT DEFENCE MECHANISMS	JOHNSTON AND DAVIES, 1972; JOHNSTON *et al.*, 1973; WILLIAMS AND LUTZ, 1975. EVANS, 1972. SEWELL, 1955. LOCHEAD AND LOCHEAD, 1941; SEWELL, 1955; STUTMAN AND DOLLIVER, 1968; WOOD *et al.*, 1971; FONTAINE AND LIGHTNER, 1973a,b, 1975; JOHNSTON *et al.*, 1973; PATERSON AND STEWART, 1974; GHIRETTI-MAGALDI *et al.*, 1977; SMITH AND RATCLIFFE, 1978.

Figure 4.1. Transmission electron micrographs of the cuticle from the untreated gill of *Carcinus maenas* kept in 35‰ seawater for three weeks. **(a)** The three main layers of the cuticle. **(b)** Algal cells often found on the outside of the cuticle. **(c)** Bacterial cells also often observed on the outer cuticular layer or on the outside of the algal layer (A: apical infoldings; AL: algal layer; BA: bacterial cells; DE: detritus; EN: endocuticle; EP: epicuticle; EX: exocuticle).

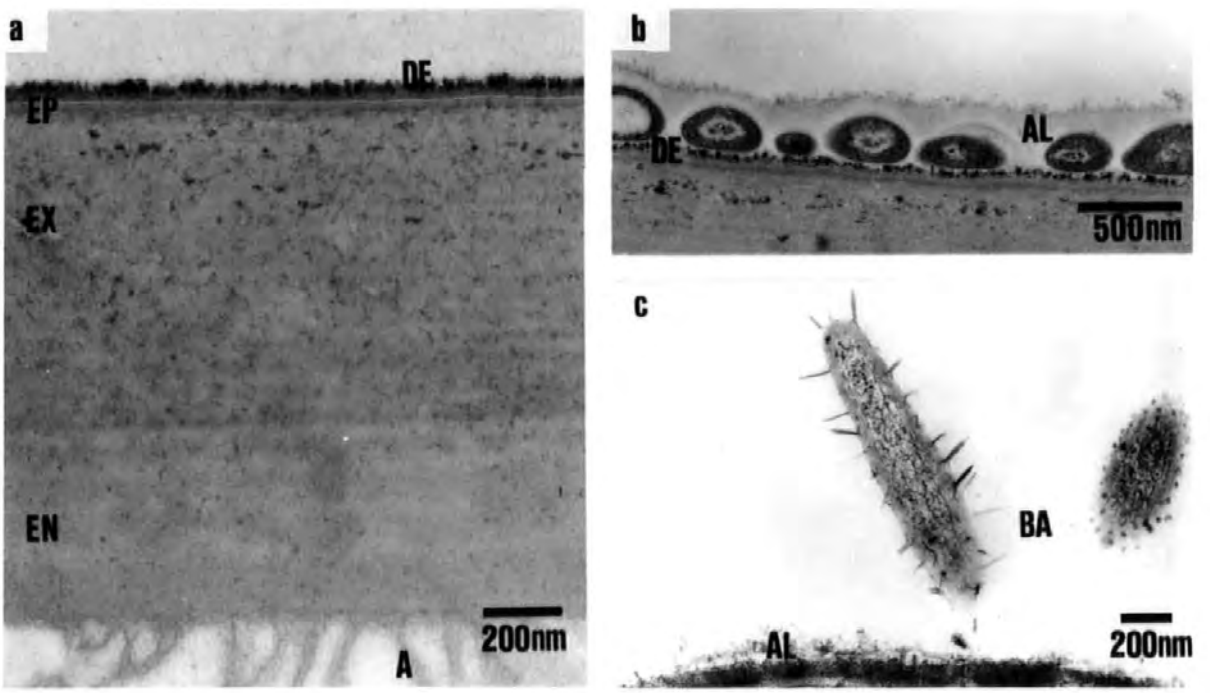

a
DE
EP
EX
EN
200nm
A
b
DE
AL
500nm
c
BA
AL
200nm

Figure 4.2. Semi-thin methylene blue stained section of gill lamellae taken from the proximal region (near the marginal canal) of the untreated gill of *Carcinus maenas* kept in 35‰ seawater for three weeks (C: cuticle; EL: epithelial layer; H: haemocyte; NE: nephrocyte; P: pillar cell; S: intralamellar septum; Z: zig-zag border).

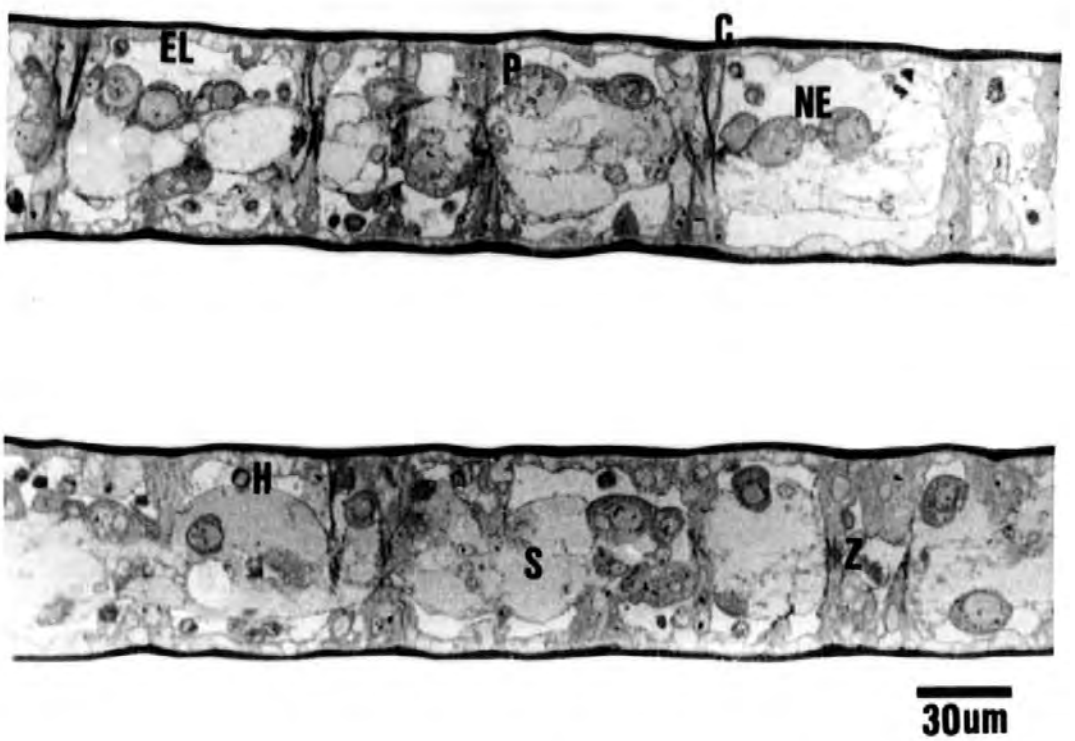

EL
P
C
NE
OH
S
Z
30um

Figure 4.3 Semi-thin methylene blue stained section of a gill lamella taken from the proximal region (near the central raphe) of the untreated gill of *Carcinus maenas* kept in 35‰ seawater for three weeks (C: cuticle; EL: epithelial layer; H: haemocyte; P: pillar cell; Z: zig-zag border).

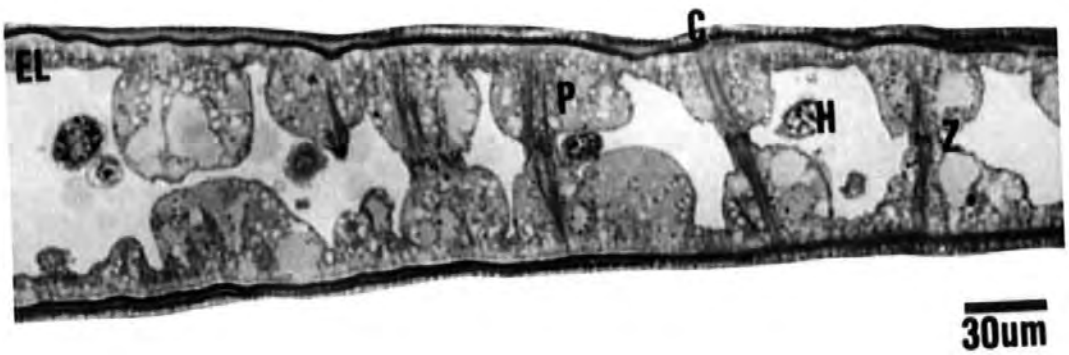

EL
G
P
H
Z
30um

Figure 4.4. Transmission electron micrographs of the epithelial cells of the untreated gill of *Carcinus maenas* kept in 35‰ seawater for three weeks. **(a)** Striated cell taken from the proximal gill region near the marginal canal. **(b)** Chief cell taken from the mid gill region in the middle of the lamella. **(c)** Hour-glass pillar cell taken from the proximal gill region near the central raphe. **(d)** Zig-zag border which is formed where two pillar cells meet (A: apical infoldings; B: basal infoldings; G: glycogen; H: haemocyte; I: intercellular junction; M: mitochondria; N: nucleus; R: anchoring rods; T: microtubules; V: vacuole).

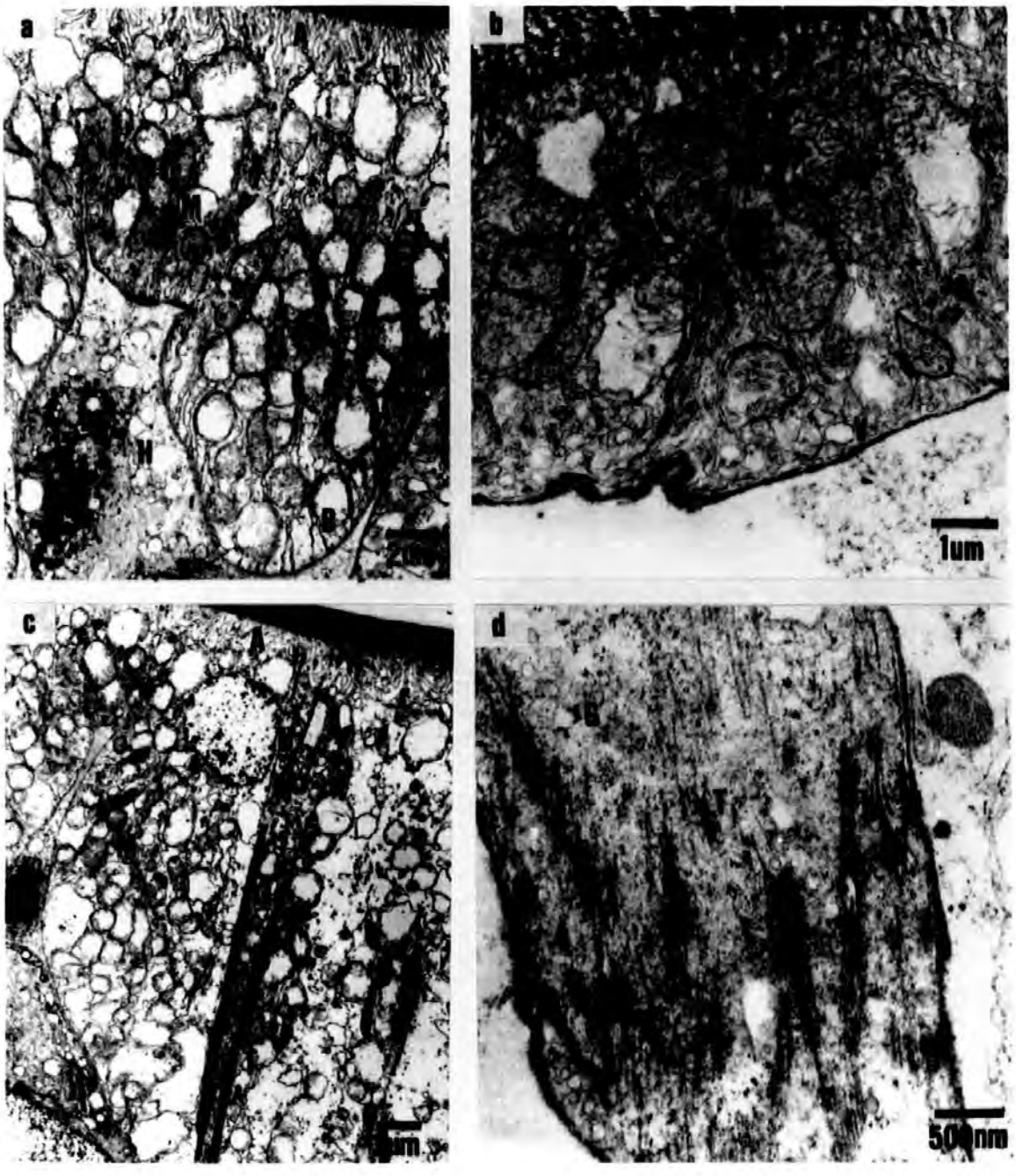

a
b
1um
c
d
500nm

Figure 4.5. Transmission electron micrographs showing the variation in nephrocyte form in the untreated gill of *Carcinus maenas* kept in 35‰ seawater for three weeks. **(a)** Two large nephrocytes taken from the proximal gill region, lodged between two pillar cells. **(b)** A smaller nephrocyte taken from the proximal gill region, surrounded by a chief cell (top) and two larger nephrocytes. **(c)** Basal membrane of a nephrocyte (CV: central vacuole; DP: diaphragm; M: mitochondria; MC: microcrystals; N: nucleus; O: ovoid elements; P: pillar cell; PE: pedicels; SV: satellite vacuoles; VE: coated vesicle).

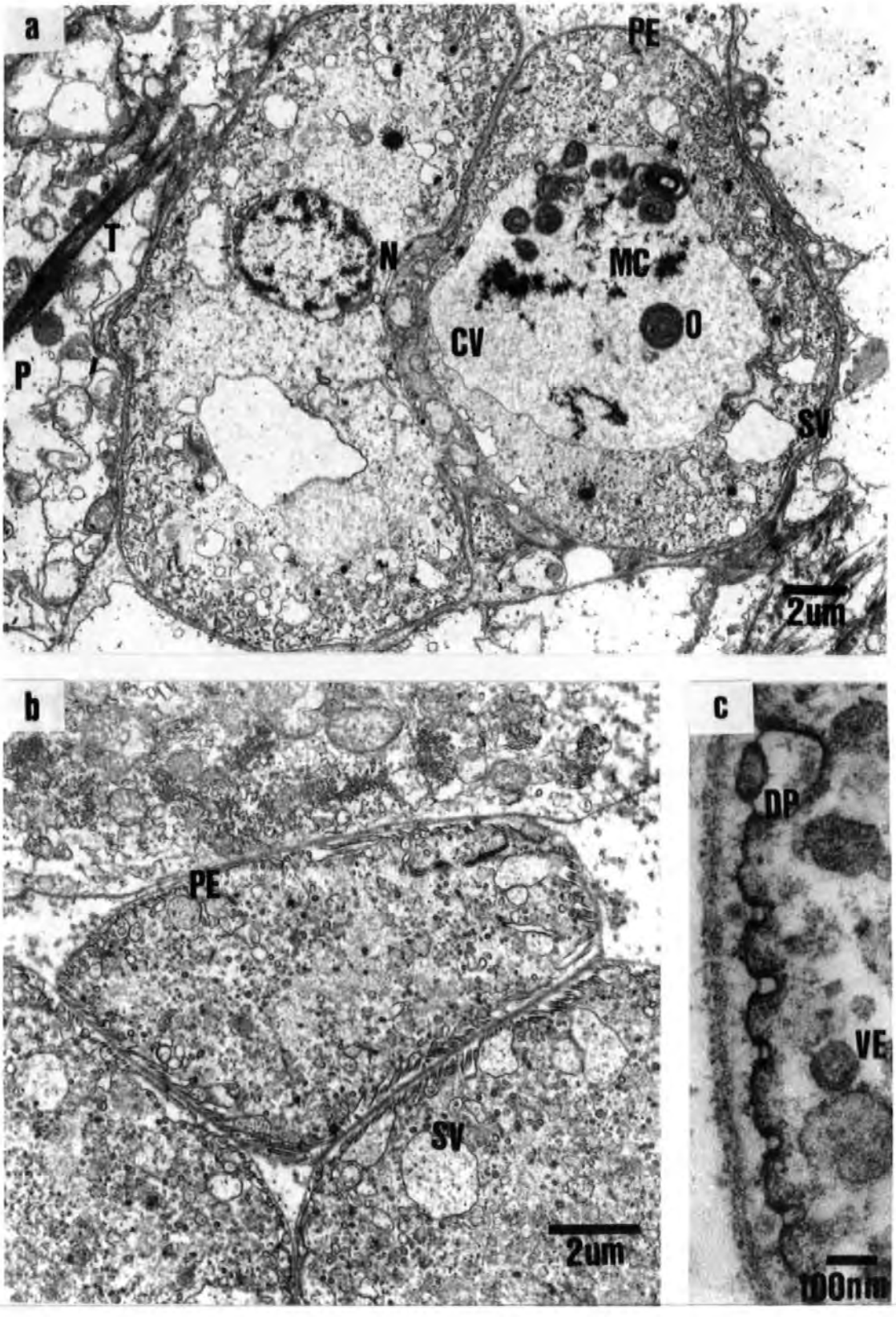

a
PE
T
N
MC
O
CV
P
SV
2um
b
PE
SV
2um
c
DP
VE
100nm

Figure 4.6. Transmission electron micrographs of cells found in the intralamellar septum of the untreated gill of *Carcinus maenas* kept in 35‰ seawater for three weeks. **(a)** Glycocyte. **(b)** Glandular cells. **(c)** Gill capillary (CP: capillary; G: glycogen; GL: glandular cell; M: mitochondria; N: nucleus; PC: perinuclear cytoplasm; S: intralamellar septum).

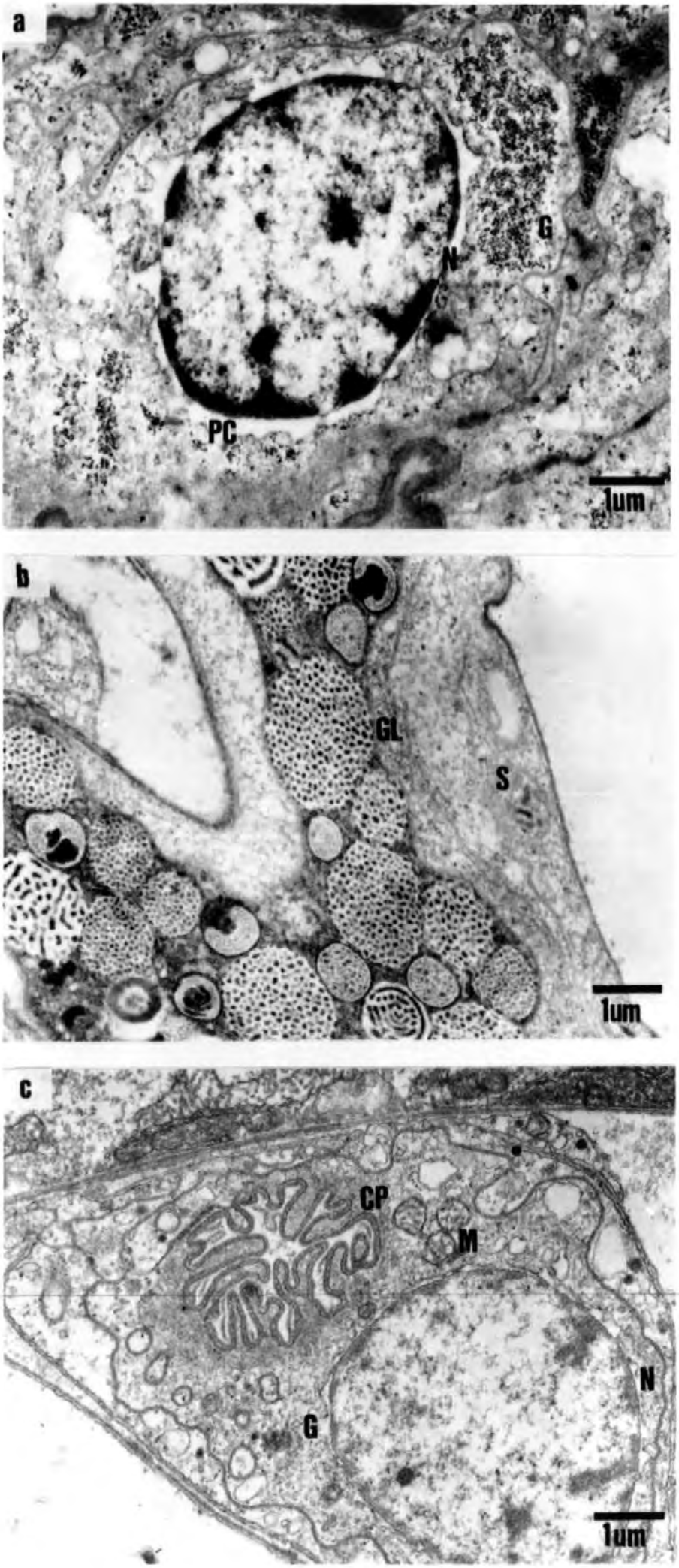
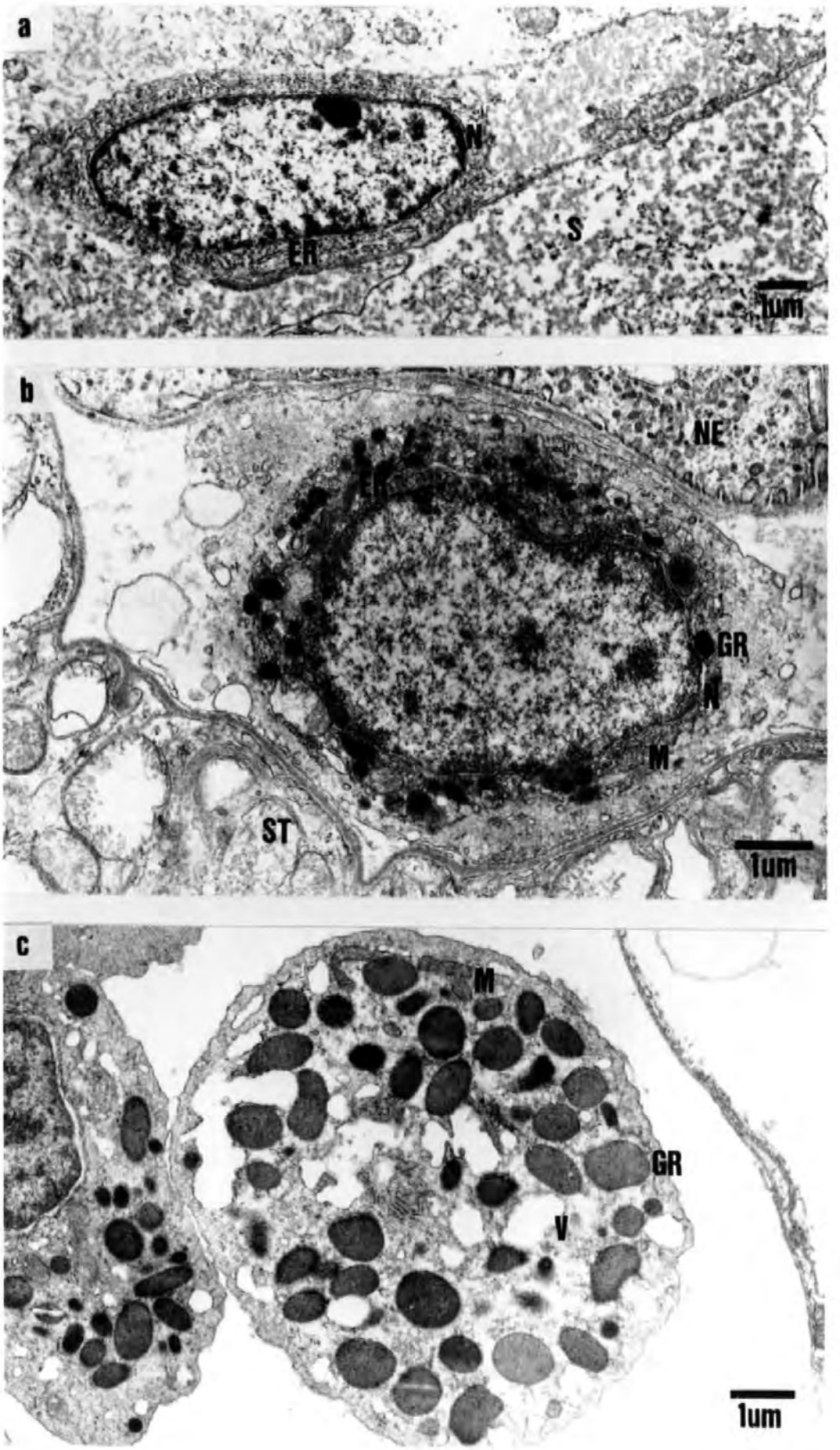

a
N
G
PC
1um
b
GL
S
1um
c
CP
M
N
G
1um

Figure 4.7. Transmission electron micrographs of the haemocytes found in the untreated gill of *Carcinus maenas* kept in 35‰ seawater for three weeks. **(a)** Agranular haemocyte. **(b)** Small-granule haemocyte. **(c)** Large-granule haemocyte (ER: endoplasmic reticulum; GR: granule; M: mitochondria; N: nucleus; NE: nephrocyte; S: intralamellar septum; ST: striated cell; V: vacuole).


a
N
S
ER
1um
b
NE
GR
N
M
ST
1um
c
M
GR
V
1um

CHAPTER 5

STRUCTURAL VARIABILITY AND DISTRIBUTION OF CELLS IN THE GILL OF *CARCINUS MAENAS* KEPT IN 10‰ SEAWATER

[Aspects of this chapter were presented at the 3rd International Conference on Trace Metals in the Aquatic Environment, Denmark, May, 1994 and are included in Lawson *et al.* "Effect of copper on the ultrastructure of the gill epithelium of *Carcinus maenas* (Decapoda: Brachyura)". *Marine Pollution Bulletin*, in press.]

5.1 Introduction

The shore crab *Carcinus maenas* (L.) is found frequently in estuarine and coastal conditions where it is likely to be subjected to fluctuations in salinity (Crothers, 1967). *Carcinus maenas* is a hyperosmoregulator (Compere *et al.*, 1989) and can be regarded as a transition form between truly marine species (basically osmoconformers, *Cancer spp.* and *Maia spp.)* and strong regulators succeeding even in freshwater (*Eriocheir spp.*) or in semi-terrestrial *(Gecarcinus spp., Uca spp., Ocypode spp.)* habitats (Zanders, 1981). Thus in low salinity, the ionic concentration of the haemolymph of *C. maenas* falls and hyperosmoregulation is achieved, in part, by the active uptake of salts at the gills (for a review see Gilles & Pequeux, 1986). The active role played by the gills in ionic regulation and osmoregulation has been discussed and the cells thought to be involved predominantly in salt and water balance have been described in Chapter 4. Since *C. maenas* is a hyperosmoregulator, during times of low salinity, the gill epithelium must represent a balance between the conflicting requirements for maximum oxygen permeability and the provision of adequate space for ionic uptake machinery and its energy supply (Taylor & Greenaway, 1979). The effects of a reduction in salinity on the biochemical and physiological processes in the crustacean gill have been well documented. Levels of enzymes thought to be involved in ionic regulation and respiratory gas exchange have been shown to increase in gills subjected to a decrease in salinity; examples include Na^+K^+ATPase, the enzyme required for the Na^+/K^+ exchange system (Mantel & Olson, 1976; Pequeux & Gilles, 1977, 1978; Towle *et al.*, 1976; Mantel & Landesman, 1977; Spencer *et al.*, 1979; Neufeld *et al.*, 1980; Siebers *et al.*, 1982, 1983, 1985; Pequeux *et al.*, 1984, 1988; Holliday, 1985; Wanson *et al.*, 1984) and carbonic anhydrase, the zinc-containing enzyme that catalyses the hydration of carbon dioxide to carbonic acid (Böttcher *et al.*, 1990a,b; Böttcher & Siebers, 1990). The extensive literature on the effects of low salinity on the physiological process of

respiratory gas exchange in crustaceans, however, is conflicting. Many authors have found that respiration rates and oxygen consumption increase under conditions of osmotic stress (Schlieper, 1929; Schwabe, 1933; Flemister & Flemister, 1951; Lofts, 1956; Rao, 1958; King, 1965; Siebers *et al.*, 1972; Dimock & Groves, 1975; Engel *et al.*, 1975; Taylor, 1977; Shumway & Jones, 1981) whilst others have reported that respiration rate is unaffected by decreasing salinity (Eltringham, 1965; McLusky, 1969; Jones, 1974).

This chapter describes the structural variability and distribution of the six major gill cell types from *Carcinus maenas* acclimated for three weeks to 10‰ seawater. Such details allow a greater understanding of how hyperosmoregulation is attained in the crab gill at low salinities.

5.2 Materials and Methods

The materials and methods used in this chapter followed those outlined in Chapter 3. Crabs were maintained in 10‰ seawater.

5.3 Results

The cuticle of posterior gill 8 of *Carcinus maenas* acclimated to 10‰ seawater for three weeks was the same size and had an identical ultrastructure to the gill cuticle of crabs acclimated to 35‰ seawater for the same time (Chapter 4). The epicuticle was covered frequently with algal and/or bacterial cells, and the abundance and distribution did not vary between 10‰ and 35‰ seawater. The gill epithelial layer was made up of striated, chief and pillar cells, as observed in the 35‰ seawater gill, however, the ultrastructure and distribution of these cells differed markedly between gills at 10‰ and 35‰ seawater. At 10‰ seawater, the ultrastructure of the epithelial layer did not change over the experimental period of 10 days.

Striated cells (Fig. 5.1a) had the same irregular shape and were within the same size range as observed at 35‰ seawater (8-14μm apical to basal depth); however, more larger cells were observed in the gill at 10‰ compared with 35‰ seawater. Very few striated cells less than 10μm in size were sited in the gill at 10‰ seawater; the largest cells occurred towards the central raphe as seen at the higher salinity. The striated cells of *Carcinus maenas* gills acclimated to 10‰ seawater had a denser basal infolding network, with the basal folds extending further towards the apical side of the cell, than in striated cells from crabs acclimated to the higher salinity. In some cases at 10‰ seawater, the basal infoldings were almost in direct contact with the apical infolding network. Each basal fold contained a relatively greater number of mitochondria than observed in the basal folds of striated cells at 35‰ seawater (c.f. Figs 4.4a & 5.1a). At 10‰ seawater, mitochondria were also more electron-dense and longer and thinner in appearance than at 35‰ seawater. The apical infolding network of striated cells at 10‰ seawater was also highly developed and the apical folds extended a considerable way towards the basal side of the cell (Fig. 5.1a). The small pinocytotic vacuoles observed near the apical cell surface in the gill at 35‰ seawater developed into small sub-cuticular vacuoles at the lower salinity (Fig. 5.1a). In each of the three gill regions (proximal, mid and distal), striated cells were more prevalent in the gill epithelial layer at 10‰ compared with 35‰ seawater. The distribution of striated cells from the proximal to the distal gill region followed the same trends as documented for 35‰ seawater, with the distal gill region containing the least, and the proximal gill region the greatest, proportion of this epithelial cell type.

Chief cells were observed more infrequently in the gill epithelial layer of crabs acclimated to 10‰ compared to 35‰ seawater. Chief cells (Fig. 5.1b) were of approximately the same size as chief cells observed at the higher salinity, but their ultrastructure was markedly different. At 10‰ seawater, the basal membrane of the

majority of chief cells developed infoldings (Fig. 5.1b), not seen previously in chief cells at 35‰ seawater. Chief cells had a poorly developed apical infolding network and contained only a few mitochondria at 10‰ seawater.

At 10‰ seawater, pillar cells exhibited the same variation in form as seen at 35‰ seawater. Towards the central raphe, pillar cells were hour-glass shaped with concave sides (Fig. 5.1c); near the marginal canal, they were long and thin (Fig. 5.1d). Thin pillar cells were more prevalent in tells were more prevalent in the distal gill region than the h

Pillar cell ultrastructure also varied with shape at 10‰ seawater. The hour-glass pillar cells were more bulbous and filled more of the haemolymph space at 10‰ than at 35‰ seawater. The basal membrane of hour-glass pillar cells became highly infolded at 10‰ seawater and the cell cytoplasm contained many long and thin mitochondria (Fig. 5.1c). At 10‰ seawater, hour-glass pillar cells had a highly developed apical infolding network, although some sub-cuticular vacuoles were observed in the cell cytoplasm. The microtubular network of hour-glass pillar cells was very poorly developed at 10‰ seawater and microtubules were only present in discrete patches in the cytoplasm of the majority of cells. In addition, very few (in some cases none at all) electron-dense rods were seen at the apical cell surface. At 10‰ seawater, thin pillar cells were similar in ultrastructure to pillar cells observed at 35‰ seawater.

The structure of the intercellular junctions in the gill epithelial layer of crabs acclimated to 10‰ seawater was disrupted compared with 35‰ seawater and loose microtubular material was often seen in the cell cytoplasm (Fig. 5.2). The band of septate junctions was frequently longer at 10‰ than observed at 35‰ seawater and often branched (Fig. 5.2b). In addition, septate junctions did not always attach to the apical and basal cell surfaces at 10‰ seawater; in areas where sub-cuticular vacuoles formed the adherence zones of the junctions pulled away from the cuticle edge.

The haemolymph space was minimal in most areas of the gill at 10‰ seawater due to

the abundance of large striated cells in the epithelial layer (Fig. 5.3). Consequently, the size of the intralamellar septum was reduced compared with 35‰ seawater and, in some areas, was observed only in the form of patchy areas of tissue. The general ultrastructure of the intralamellar septum was the same as observed at 35‰ seawater (c.f. Figs 4.2 & 5.4). Glycocytes were found frequently within the septum, their ultrastructure did not change with a decrease in salinity (c.f. Figs 4.6a & 5.5a). Nephrocytes, the other cell type often found associated with the septum, were also ultrastructurally identical at 10‰ and 35‰ seawater (c.f. Figs 4.5 & 5.5b) but were more abundant in the distal gill region at 10‰ compared to 35‰ seawater. In addition, at the lower salinity there was a greater proportion of small nephrocytes in all gill regions than observed at the higher salinity.

Haemocytes were observed more frequently circulating in the haemolymph space of the gill at 10‰ than at 35‰ seawater. Agranular, small-granule (Fig. 5.6a) and large-granule (Fig. 5.6b) haemocytes were observed, however, the relative proportion of large-granule haemocytes sited was greater in the gill at 10‰ than at 35‰ seawater.

5.4 Discussion

The ultrastructure of the gill epithelial layer of *Carcinus maenas* at 10‰ seawater was markedly different from that seen at 35‰ seawater. As there were no changes over the 10 day experimental period, it was clear that all the ultrastructural alterations occurred during the three week acclimation period. At the lower salinity, the plasma membrane infolding networks became more extensive, with the exception of the chief cell apical infolding system, and the number of mitochondria within the cell cytoplasm increased. All epithelial cell types were not uniformly affected by a decrease in salinity. Striated cells (thought to be predominantly responsible for ionic regulation and osmoregulation; Table 1) contained the most extensive apical and basal membrane infolding networks

and the greatest number of mitochondria of all the epithelial cells. This ultrastructural observation, along with the increased abundance of striated cells in the gill at 10‰ seawater, supported the suggestion that the striated cell was predominantly an ionic regulatory and osmoregulatory cell.

Chief cells (thought to have a predominantly respiratory role; Table 1) developed a basal membrane infolding network at 10‰ seawater which was not observed at 35‰ seawater. Such a system has not been documented in any previous gill ultrastructural study. Development of a basal membrane infolding network may imply that chief cells also play a role in ionic regulation and osmoregulation in certain environmental conditions, such as at decreased salinities. Indeed, it has been suggested previously that chief cells have some involvement in ionic regulation and osmoregulation. For instance, the gill epithelial layer of *Holthuisana transversa* (Taylor & Greenaway, 1979) and *Cancer pagarus* (Pequeux *et al.*, 1988) has been shown to consist solely of chief cells, implying this cell type must be involved in ionic regulation, osmoregulation and respiration. In addition, the hour-glass pillar cells, unlike the thin pillar cells found near the marginal canal, also developed a basal membrane infolding network with many associated mitochondria. This development supports the suggestion, made in Chapter 4, that pillar cells assume similar ultrastructural characteristics and ionic regulatory or respiratory capabilities as the surrounding epithelial cell type.

The majority of investigators agree that Na^+ ions are moved across the gill epithelium towards the haemolymph by a two-step process, an apical membrane located Na^+/H^+ antiporter and a basal membrane located Na^+/K^+ pump (reviewed in Pequeux *et al.*, 1988; Lucu, 1990; Towle, 1990). The increased density of the apical folds in striated and pillar cells, following acclimation to 10‰ seawater, may be necessary to provide a greater surface area for the location of Na^+/H^+ exchange sites. Free amino-acids and nitrogenous organic solutes, as well as Na^+ and K^+ ions, are also important intracellular

osmotic effectors in the gills of aquatic invertebrates (for a review see Gilles, 1979), and the metabolism and transport of these are altered greatly during acclimation to low salinities, in an attempt to control cell volume (Presley & Graves, 1983). One mechanism of ammonium excretion in crustaceans is the Na^+/NH_4^+ exchange system sited on the apical membrane of the gill epithelium (Presley & Graves, 1983). An increased efflux of NH_4^+ could facilitate the absorption of Na^+ ions, easing the osmotic stress on the gill. Thus, an increase in the density of the apical infolding network may aid the overall osmotic condition of the crab gill by increasing the surface area for the potential location of, not only the Na^+/H^+ antiporter, but also the Na^+/NH_4^+ exchange systems. The decrease in density of the apical membrane infolding network in the chief cells at 10‰ seawater is difficult to explain in this regard, but may suggest that the apical surface of the chief cells was more adversely affected by a decrease in salinity than the apical surface of other epithelial cell types.

In the epithelial cells, the basal membrane infolding network also increased in density on transfer to low salinity. This alteration may provide an increased surface area for the location of sites for active transport, in this case the Na^+/ K^+ exchange system. At low salinity, the activity of Na^+K^+ATPase in the posterior gills of crustaceans increases (Mantel & Olson, 1976; Pequeux & Gilles, 1977, 1978; Towle *et al.*, 1976; Mantel & Landesman, 1977; Spencer *et al.*, 1979; Neufeld *et al.*, 1980; Siebers *et al.*, 1982, 1983, 1985; Pequeux *et al.*, 1984, 1988; Holliday, 1985; Wanson *et al.*, 1984) presumably so Na^+ ions can be pumped across the gills into the haemolymph. This would maintain the haemolymph osmolality in spite of the increased loss of salts to the medium (Spencer *et al.*, 1979; Siebers *et al.*, 1985, 1990). Extensive development of the basal membrane infolding system may also provide an increased surface area for the location of carbonic anhydrase, an enzyme thought to participate in respiratory gas exchange and ionic regulation (for a review see Böttcher & Siebers, 1993) which has a basal membrane

associated fraction (Burnett *et al.*, 1981; Lucu, 1990). Carbonic anhydrase provides the counterion for the apical Na^+/H^+ exchange system (Böttcher & Siebers, 1993) and facilitates the diffusion of CO_2 from the haemolymph to the ambient medium (Burnett *et al.*, 1981; Burnett & McMahon, 1985). Increased surface area for the location of carbonic anhydrase could increase potentially the activity of this enzyme and aid the successful operation of respiratory gas exchange and ionic regulation under conditions of osmotic stress. Indeed, it has been shown previously that carbonic anhydrase activity increases in the posterior gills of crustaceans in dilute media (Böttcher *et al.*, 1990b; Böttcher & Siebers, 1990). It is not known whether this increase in activity arises due to a greater number of enzyme sites or whether the same number of original sites remain but have greater activity.

The significance of the greater development of basal, compared to apical, membrane infoldings seen particularly in chief cells cannot be explained conclusively. The structural difference implies that the integrity and extensive development of basal membrane infolding systems is of greater importance than the development of apical infolding systems at times of salinity stress. This is unexpected as the apical cell surface is the first line of contact with the external medium. On the other hand, the differential development may imply that the enzyme systems located on the basal membrane of the cell have a greater involvement in the active transport of ions than those located on the apical cell surface. Indeed, it has been stated already that, in some cases, the exchange systems on the basal membrane actually provide the counterions for the apical exchange systems. Thus, it may be possible that if basal membrane associated exchange systems do not function correctly neither do exchange systems sited on the apical membrane of the cell.

Since active transport in gill epithelial cells increases on transfer to a dilute medium it is not surprising that there is a simultaneous rise in the number of mitochondria in the

cell cytoplasm, presumably aiding the supply of energy for these processes. A faster rate of ATP turnover and energy consumption, and an increase in the activity of ATP synthetase found in mitochondria, have been observed previously in the gills of crustaceans acclimated to a low salinity (Engel *et al.*, 1975; Siebers *et al.*, 1990). A change in the conformation of the mitochondria was also observed between the two salinities used in this study, long and thin at 10‰ seawater compared to rounded at 35‰ seawater. This change in mitochondrial shape and size could result in an increase in the surface area for mitochondrial enzyme activity.

Comparable ultrastructural changes, to those described so far, in response to changes in external salinity have been documented previously in the gills of various crustaceans including *Callinectes sapidus* (Copeland & Fitzjarell, 1968), *Carcinus maenas* (Compere *et al.*, 1989), *Gammarus oceanicus* (Milne & Ellis, 1973), *Goniopsis cruentata* (Martelo & Zanders, 1986) and *Penaeus aztecus* (Foster & Howse, 1978). In contrast, no ultrastructural changes were observed in the gills of *Jaera nordmanni* (Bubel, 1976) nor *Uca mordax* (Finol & Croghan, 1983) following a lowering of the external salinity. Such differences in response to low salinities may be explained by differences in experimental seawater systems, salinity regimes and acclimation times and also interspecific variation in gill ultrastructure.

Vacuolation of the epithelial layer, as seen in the gills at 10‰ seawater, has also been documented previously in ultrastructural studies of crustaceans. In the present study, a few sub-cuticular vacuoles were observed, mainly in striated and pillar cells; however, this vacuolation was not extensive and the vacuoles were restricted to the apical cell surface. Sub-cuticular vacuoles may provide a longer pathway for water movement, decreasing its rate of entry into the cell and minimising the osmotic shock the cells have to withstand at reduced salinities. Alternatively, the presence of vacuoles may simply indicate a failure to regulate gill intracellular water content.

Another ultrastructural alteration observed in the hour-glass pillar cells at 10‰ compared to 35‰ seawater was a decrease in the density of the microtubule network in the cell cytoplasm. This ultrastructural change at low salinity has been demonstrated previously in *Goniopsis cruentata* (Martelo & Zanders, 1986); however, the effect of this structural change on the crab was not discussed. Since the pillar cells are thought to be involved in structural support and the flow of haemolymph through the lamellae (Table 1), it may be anticipated that an alteration to the microtubule density may affect either or both of these gill functions. It cannot be confirmed whether this change has a deleterious effect on the functioning of the gill or whether it is just a compensatory, adaptive ultrastructural change in response to decreased salinities. For example, the microtubule density may be reduced at low salinities to alter the rate of flow of haemolymph through the lamellae, or to change the structural orientation and available surface area of the lamellae, to maximise active ion uptake.

Following acclimation to 10‰ seawater, it was also evident that the ultrastructure of the intercellular junctional complexes of the epithelial layer was altered compared to the structure observed at 35‰ seawater. The junctions were longer, often branched and did not always connect apical and basal cell surfaces. In areas where subcuticular vacuoles formed, the *zonula adherens* was moved some distance from the apical cell surface. Since intercellular junctions are thought to be involved in cellular adhesion and the restriction of intercellular permeability (Loewenstein & Kano, 1964; Bubel, 1976), the change in ultrastructure seen at 10‰ seawater may imply that acclimation to low salinities had a qualitative effect on these roles. Whether a deleterious effect on intercellular permeability occurs in the crab gill or whether the ultrastructural change is just an alteration to enhance the stability of intercellular junctional complexes, under conditions of osmotic stress, cannot be stated.

Glycocytes did not change their ultrastructure, abundance or distribution when the gill

was acclimated to 10‰ compared to 35‰ seawater. Since glycocytes are thought to be involved in glycogen storage (Table 1), an absence of ultrastructural or distributional change following acclimation to a low salinity may imply that an increased availability of glycogen from these cells is not a necessary adaptation to low salinity. Glycogen stores from the existing cells may, however, be utilised as an alternative energy source at times of low salinity. Nephrocytes were observed more frequently in the distal gill region at 10‰ than at 35‰ seawater. Since nephrocytes are thought to play a role in the endocytosis of foreign material from the haemolymph (Table 1), it may be that endocytosis of foreign particles is a more important factor in the distal gill region at low than at high salinity. For example, a decrease in salinity may increase the rate of entry of foreign particles into the gill. In support of this hypothesis, the number of haemocytes (cells thought to be involved in defence mechanisms; Table 1) circulating in the gill haemolymph increased at 10‰ compared to 35‰ seawater. In addition, there was a greater proportion of large-granule, compared to agranular and small-granule, haemocytes in the gill at 10‰ compared to 35‰ seawater, the reason for this difference is not conclusively known. Apart from their defensive properties, haemocytes also have many other functions, including carbohydrate transport and metabolism, and amino-acid storage (Table 1). Thus, an increase in the number and size of granules within the haemocytes at the low, compared to the high, salinity may be necessary to supply the gill with more osmotic effectors (carbohydrates and amino-acids) at times of osmotic stress.

Changes in respiration rates in response to a low salinity have been documented previously in crustacean studies and it is interesting to compare these physiological changes with the ultrastructural observations documented in this study. Some crustaceans show an increase in their respiration rate (or oxygen consumption) at low salinities (Schlieper, 1929; Schwabe, 1933; Flemister & Flemister, 1951; Lofts, 1956;

Rao, 1958; King, 1965; Siebers *et al.*, 1972; Dimock & Groves, 1975; Engel *et al.*, 1975; Taylor, 1977; Shumway & Jones, 1981) and it has been proposed by many of these authors that this increase is a result of increased energy requirements for the active uptake of ions to maintain haemolymph osmolality. The ultrastructural observation from the present study of an increase in the number of mitochondria in the epithelial cells at 10‰ seawater could suggest that the amount of energy needed at the lower salinity is greater than at 35‰ seawater. Alternatively, the increased respiration rates may result from a deleterious effect of low salinity on the mechanisms of respiratory gas exchange. The present study showed that the apical membranes of cells implicated in respiratory gas exchange (chief cells) decreased in density at 10‰ compared with 35‰ seawater. This reduction may imply that chief cell ultrastructure and the physiological process of respiratory gas exchange were adversely affected by low salinity.

It is not universal that low salinity induces an increase in crustacean respiration rates and some crustaceans show no change in respiration rate at low salinity (Eltringham, 1965; McLusky, 1969; Jones, 1974). One interpretation of this finding is that the amount of energy needed for the active uptake of ions is not increased under conditions of osmotic stress. Variation in the length of acclimation to the holding salinity, the type of experimental seawater system and the salinity of the medium or interspecific variation may explain some of the documented inconsistencies.

Carcinus maenas has a preferred salinity range between 17-41‰ (Thomas *et al.*, 1981; Ameyaw-Akumfi & Naylor, 1987) although it can survive indefinitely in 5‰ seawater (Brockhuysen, 1936). Since 10‰ seawater is below the preferred salinity range, it may be anticipated that adverse effects on gill ultrastructure would occur at this salinity. Although differences in gill ultrastructure were observed between crabs acclimated to 10‰ and 35‰ seawater there was no evidence of cell degeneration at 10‰ seawater,

suggesting the gill can operate well at a low salinity. Of course, ultrastructural alteration of the gill is only one step in a complex sequence of expected changes following adaptation to a low salinity environment. Other physiological and biochemical changes, such as a change in membrane permeability or in the volume and/or ionic composition of urine, may occur, rendering a more successful acclimation to a low salinity medium.

Figure 5.1. Transmission electron micrographs of the epithelial cells of the untreated gill of *Carcinus maenas* kept in 10‰ seawater for three weeks. **(a)** Striated cell taken from the proximal gill region near the central raphe. **(b)** Chief cell taken from the mid gill region in the middle of the lamella. **(c)** Hour-glass pillar cell taken from the proximal gill region near the central raphe. **(d)** Thin pillar cell taken from the mid gill region near the marginal canal (A: apical infoldings; B: basal infoldings; C: cuticle; G: glycogen; I: intercellular junction; M: mitochondria; N: nucleus; R: electron-dense rods; S: intralamellar septum; T: microtubules; V: vacuole; Z: zig-zag border).

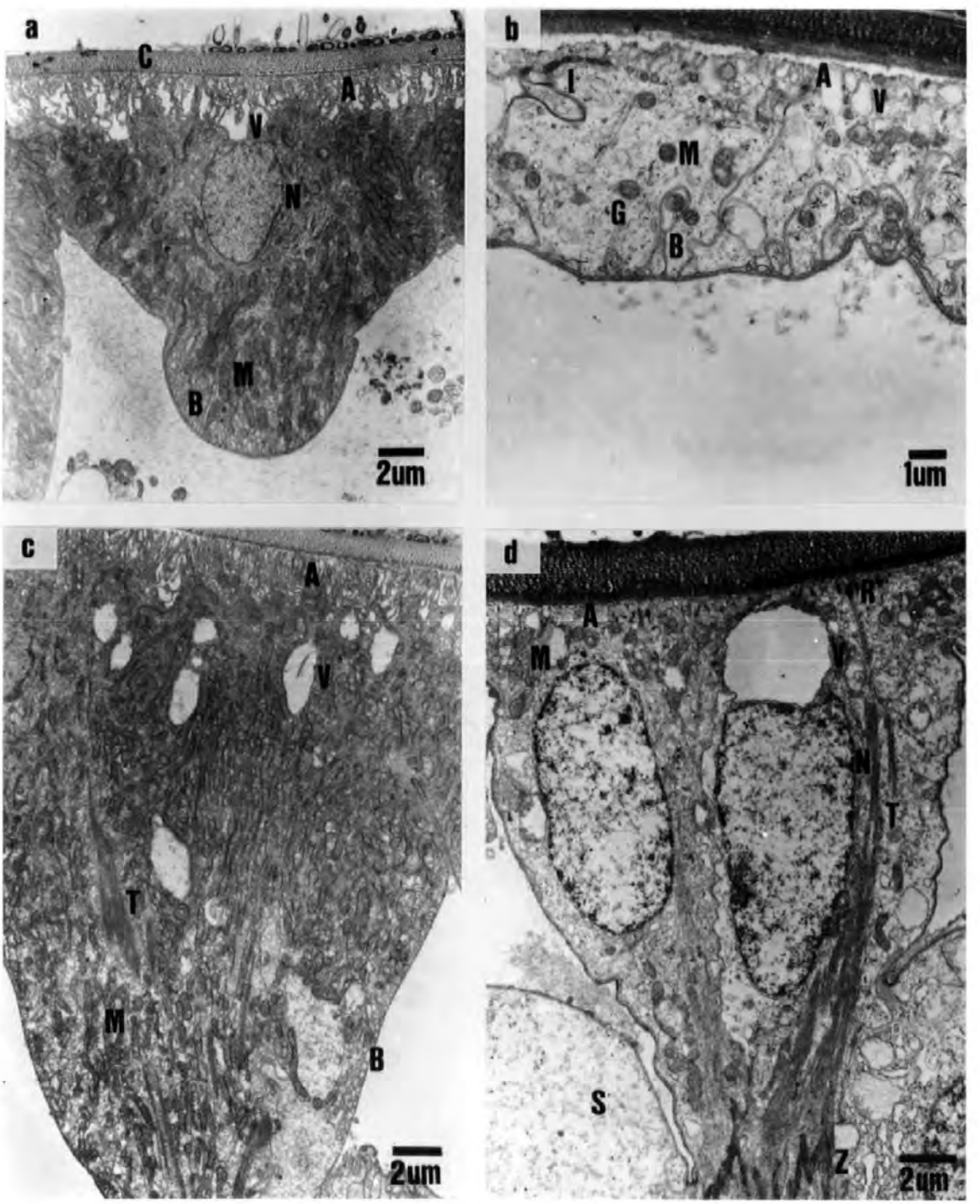

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Figure 5.2. Transmission electron micrographs of the intercellular junctional complexes seen in the untreated gill of *Carcinus maenas* kept in 10‰ seawater for three weeks. **(a)** Intercellular junction in a chief cell. **(b)** Part of an intercellular junction in a striated cell (A: apical infoldings; B: basal infoldings; G: glycogen; I: intercellular junction; M: mitochondria; SJ: septate junction).

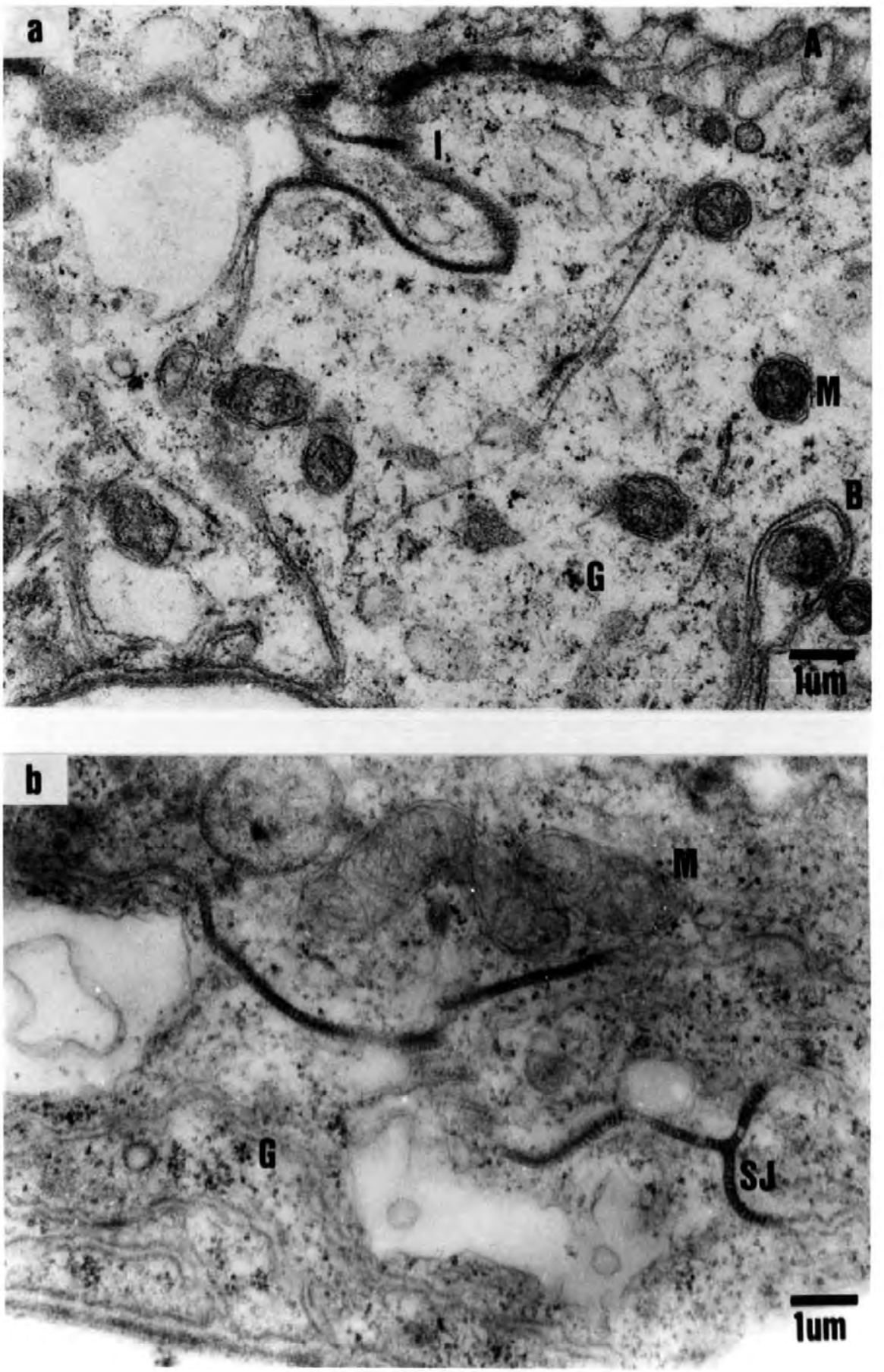
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Figure 5.3. Semi-thin methylene blue stained section of gill lamellae taken from the proximal region (near the central raphe) of the untreated gill of *Carcinus maenas* kept in 10‰ seawater for three weeks (C: cuticle; P: pillar cell; S: intralamellar septum).

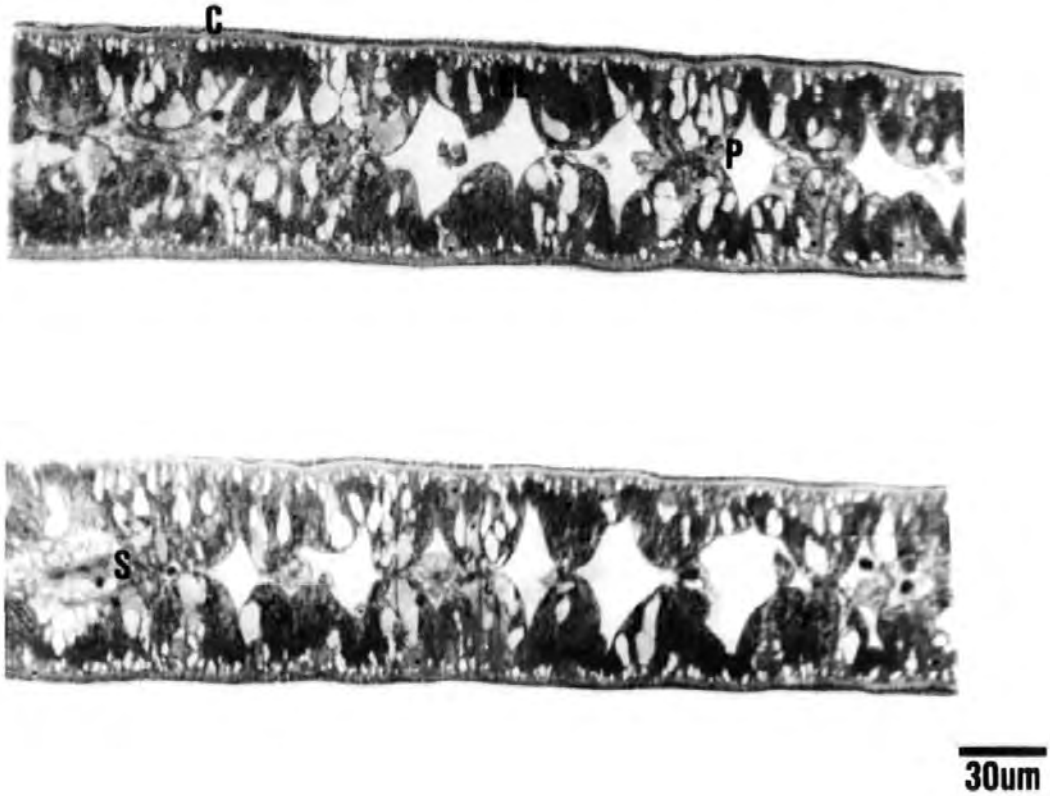
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Figure 5.4. Semi-thin methylene blue stained section of a gill lamella taken from the distal region (near the marginal canal) of the untreated gill of *Carcinus maenas* kept in 10‰ seawater for three weeks (C: cuticle; EL: epithelial layer; MC: marginal canal; NE: nephrocyte; P: pillar cell; S: intralamellar septum).


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Figure 5.5. Transmission electron micrographs of some of the other cells found in the haemolymph space of the untreated gill of *Carcinus maenas* kept in 10‰ seawater for three weeks. (a) Glycocyte in the intralamellar septum. (b) Two nephrocytes attached to the intralamellar septum (CP: capillary; CV: central vacuole; G: glycogen; H: haemocyte; M: mitochondria; N: nucleus; PE: pedicels; S: intralamellar septum; SV: satellite vacuoles).

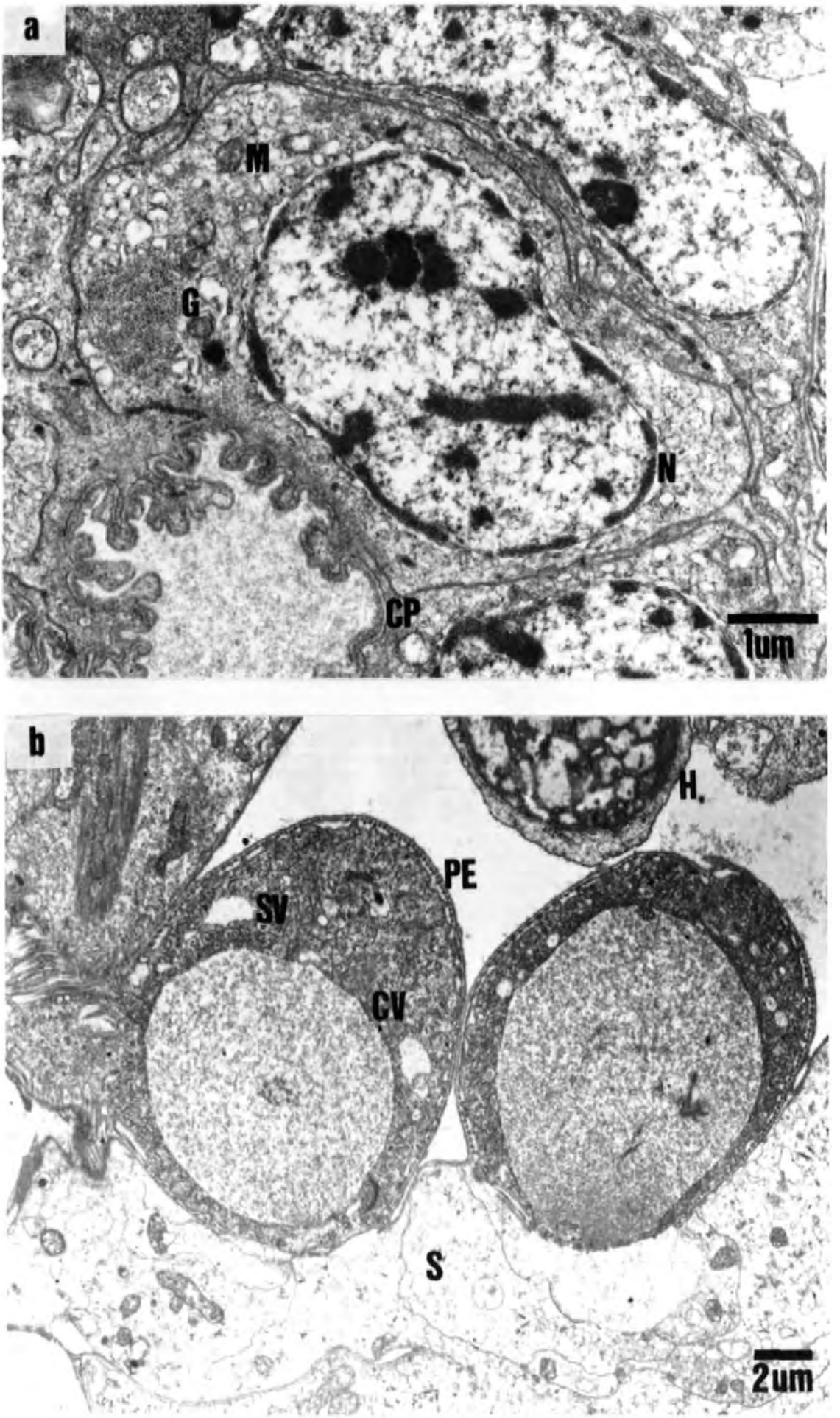
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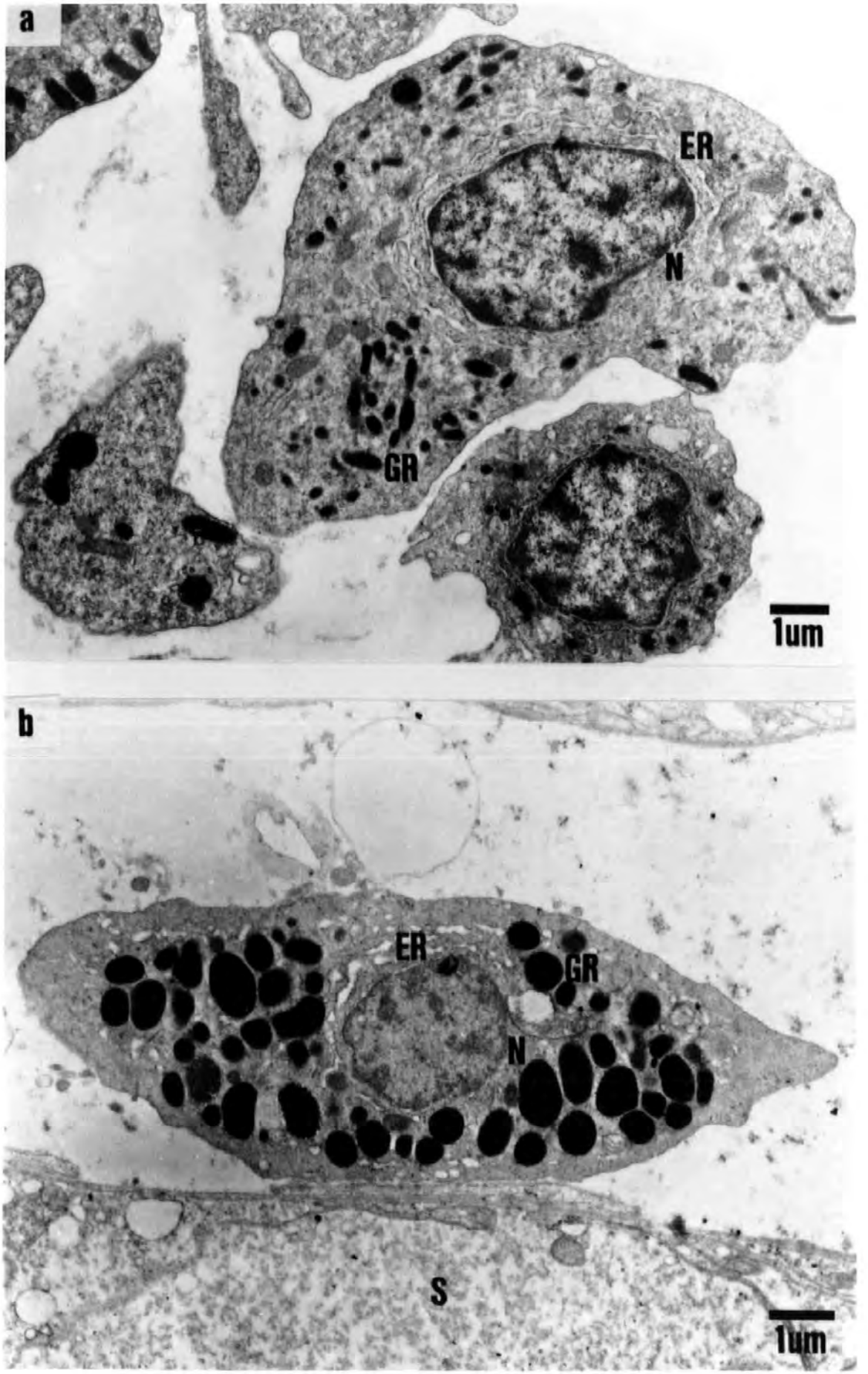
Figure 5.6. Transmission electron micrographs of the haemocytes observed in the untreated gill of *Carcinus maenas* kept in 10‰ seawater for three weeks. **(a)** Group of small-granule haemocytes in the haemolymph space of a lamella from the mid gill region. **(b)** Large-granule haemocyte attached to the intralamellar septum in the distal gill region (ER: endoplasmic reticulum; GR: granule; N: nucleus; S: intralamellar septum).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CHAPTER 6

STRUCTURE AND DISTRIBUTION OF THE GILL CELLS OF *CARCINUS MAENAS* EXPOSED TO COPPER IN 35‰ SEAWATER

[Aspects of this chapter were presented at the Society for Experimental Biology meeting, Canterbury, April, 1993 and at the 3rd International Conference on Trace Metals in the Aquatic Environment, Denmark, May, 1994. Some features of this chapter are also included in Lawson *et al.* "Effect of copper on the ultrastructure of the gill epithelium of *Carcinus maenas* (Decapoda: Brachyura). *Marine Pollution Bulletin*, in press.]

6.1 Introduction

Trace concentrations of heavy metals are normal constituents of marine organisms and aquatic environments (Bryan, 1976). However, elevated levels of heavy metals occur in heavily industrialised areas which are often sited in estuarine or coastal regions. As the shore crab *Carcinus maenas* (L.) is found frequently in such areas (Crothers, 1967) it is especially prone to heavy metal exposure. *Carcinus maenas* can regulate essential heavy metals (for example, copper) over a wide range of metal bioavailabilities (White & Rainbow, 1982; Rainbow, 1985), however, regulation breaks down, metals accumulate and toxic effects are observed above a threshold concentration. Crustacean gills are considered the first target of water-borne pollutants and it has been established that tissues, such as the gill, are involved in the concentration and/or redistribution of exogenous and naturally occurring heavy metals (Kerkut *et al.*, 1961; Djangmah & Grove, 1970; Bryan *et al.*, 1986; Aramugam & Ravindranath, 1987; Engel, 1987). It has been shown previously that elevated concentrations of heavy metals induce marked osmoregulatory and respiratory responses in crustaceans (Hunter, 1949; Kerkut & Munday, 1962; DeCoursey & Vernberg, 1972; Vernberg & Vernberg, 1972; Collier *et al.*, 1973; Thurberg *et al.*, 1973; Vernberg *et al.*, 1974; Jones, 1972, 1975a,b; Bouquegneau & Gilles, 1979; Depledge, 1984; Bjerregaard & Vislie, 1986; Spicer & Weber, 1991). As crustacean gills are known to play a prominent role in ionic regulation, osmoregulation and respiratory gas exchange (for a review see Taylor & Taylor, 1992), it may be anticipated that factors that influence these gill physiological processes will also affect gill ultrastructure. Early investigations of pollution effects in fish attributed the death of animals exposed to lethal concentrations of heavy metal to the coagulation and precipitation of mucus on the gills, resulting in an increased diffusion distance for oxygen and death by suffocation. In more recent years, cytological damage has been cited as the reason for the increased diffusion distance between the

water and blood (Mallat, 1985; Evans, 1987). There are many accounts of ultrastructural damage to crustacean gills as a result of exposure to heavy metals (Bubel, 1976; Couch, 1977; Ghate & Mulherkar, 1979; Papathanassiou & King, 1983; Papathanassiou, 1985; Anderson & Baartrup, 1988; Boitel *et al.*, 1990; Nonnotte *et al.*, 1993). Unfortunately, many of these studies employed levels of heavy metal not found in nature, making it difficult to extrapolate to field situations. A further problem in extrapolation to the field is that many studies have utilised static exposure systems, yet it has been shown that metal uptake, physiological response and mortality rate differ between animals maintained in a static compared to flow-through seawater systems (Vernberg *et al.*, 1977; Ritz, 1980).

Little work has been published on the effect of environmentally realistic concentrations of heavy metal on the ultrastructure of crustacean gills. Similarly, the potential effects of sublethal levels of heavy metal on gill physiological processes have not been discussed. Copper levels between 0.5-20μgl^{-1} have been measured in unpolluted marine waters (Baker, 1969; Bjerregaard & Vislie, 1986) but anthropogenic inputs can result in concentrations of around 0.6mgl^{-1} (Bryan, 1976). This chapter examines the effect of an environmentally realistic copper concentration (50μgl^{-1}) administered in a flow-through seawater (35‰) system on the ultrastructure and distribution of the six cell types found in the posterior gill of *Carcinus maenas*. Such a study enables a better understanding of the mechanisms by which crustacean ionic regulation, osmoregulation and respiratory gas exchange are affected by heavy metals.

6.2 Materials and methods

The general materials and methods used in this chapter follow those described in Chapter 3. Crabs were exposed to a copper concentration of 50μgl^{-1} in 35‰ seawater for 10 days. Individuals were removed at 4, 7 and 10 days.

6.3 Results

The gill cuticle had the same ultrastructure after exposure to copper as observed in untreated gills at 35‰ seawater (Chapter 4). The distribution of algal and/or bacterial cells on the epicuticle was also as observed previously in the untreated gill. The ultrastructure of the epithelial cells (striated, chief and pillar), however, was altered markedly following copper exposure. Irregularly-shaped striated cells of approximately the same size range (8-14μm apical to basal depth) and with the same distribution as observed in the untreated gill at 35‰ seawater were present. After 4 days copper exposure, the striated cell cytoplasm contained numerous vacuoles, some of which extended to the basal side of the cell, the basal membrane contained only a few infoldings and only a scattering of mitochondria were observed. Following 10 days exposure to copper, the number of basal folds in the striated cells was reduced dramatically compared to 4 days exposure and only very few, underdeveloped folds remained (Fig. 6.1a). In addition, the apical infolding network of the striated cells was highly disrupted, fewer mitochondria were present compared to observed in the untreated gill and the cell assumed a granular appearance with many free ribosomes and swollen endoplasmic reticulum in the cell cytoplasm (Fig. 6.1a).

Chief cells showed similar changes to those described for striated cells. Following 10 days copper exposure, the apical infolding network was highly disrupted, with very few folds present and most of the apical cell surface pulled away from the cuticle. The chief cells became highly vacuolated and mitochondria were less abundant and more swollen than observed in the untreated gill (Fig. 6.1b).

Pillar cells exhibited the same variation in shape and distribution following copper exposure as observed in the untreated gill. Hour-glass pillar cells were found towards the central raphe and thin pillar cells towards the marginal canal of a lamella (both areas are shown in Fig. 1.3). Both types of pillar cell were disrupted by the addition of

copper. After 10 days exposure to copper, the pillar cells contained numerous vacuoles, the number of apical folds and mitochondria in the cell cytoplasm decreased compared to the untreated equivalent and the cell assumed a granular appearance with many areas of swollen endoplasmic reticulum in the cytoplasm (Fig. 6.1c,d). In addition following exposure to copper, the microtubular network was less dense than observed in the untreated gill and the microtubules were only observed in discrete patches. Areas of the apical cell surface of the pillar cells, where electron-dense rods formed, pulled away from the cuticle. Small and large-granule haemocytes were observed attached to the basal side of the pillar cell (Fig. 6.1c) and also occurred within the pillar cell cytoplasm in the 7 and 10 day gills (Fig. 6.1d). In some areas of the gill, particularly the distal region, the pillar cell junctions were also disrupted following exposure to copper and the cells did not traverse the haemolymph space in a straight course but were displaced to one side (Fig. 6.2). Intercellular junctions in the epithelial layer remained relatively intact after copper exposure and only appeared to be affected when vacuoles were formed near the apical side of the epithelial cell, effectively pushing the adherence zone further towards the basal side of the cell.

In general, all epithelial cells, independent of gill position, were affected uniformly by exposure to copper at 35‰ seawater and a summary of these changes is provided in Figures 6.3-6.5. Figure 6.3 shows that over the 10 day exposure period striated cells became increasingly vacuolated and contained fewer apical and basal plasma membrane infoldings and mitochondria. The striated cell cytoplasm contained increasing amounts of swollen endoplasmic reticulum and free ribosomes as the exposure time proceeded. Similarly, chief cells became increasingly vacuolated and the density of the apical infolding network became progressively less over 4, 7 and 10 days of exposure (Fig. 6.4). In addition, the number of mitochondria in the chief cell cytoplasm decreased and those remaining became swollen. Figure 6.5 shows that the microtubular network of the

pillar cells became increasingly disrupted as exposure progressed. The pillar cell cytoplasm became increasingly vacuolated and contained more swollen endoplasmic reticulum and free ribosomes in the 10 day compared with the 4 day copper-exposed gill. After 10 days exposure, haemocytes were frequently observed attached to the basal edge of the pillar cells or infiltrated into the cell cytoplasm (Fig 6.5d).

The ultrastructure and distribution of the remaining gill cells (nephrocytes, glycocytes and haemocytes) were not altered uniformly following exposure to copper at 35‰ seawater. Glycocytes showed no ultrastructural or distributional change following copper exposure from that observed in the untreated gill. Nephrocytes were observed frequently in the copper-exposed gill, were of variable size (10-24μm diameter) and had the same ultrastructure as described earlier for the untreated gill (Chapter 4). Following copper exposure, nephrocytes were sited more frequently in the distal gill region of the copper--exposed compared to the untreated gill.

One of the most striking ultrastructural alterations in the gill lamellae following exposure to copper at 35‰ seawater was the massive increase in the number of haemocytes in the haemolymph space compared to the untreated gill at the same salinity. This increase in abundance was evident after 4 days copper exposure when the haemolymph space was packed with haemocytes (Fig. 6.6); the distal gill region contained the greatest number of haemocytes. The same three forms (agranular, small-granule and large-granule) of haemocyte were observed in the copper-exposed as in the untreated gill, however, the relative abundance of each form was markedly different between copper-exposed and untreated gills. Large-granule haemocytes were the most frequently observed haemocyte in the gill lamellae following copper exposure. Small-granule haemocytes were also seen, however, agranular haemocytes were very scarce. After 10 days exposure to copper, haemocytes were observed within epithelial cells (Fig. 6.1d), within gill capillaries (Fig. 6.7a) and also in clumps in the haemolymph

space of a lamella (Fig. 6.7b), many cells within the clumps appeared to have lysed.

6.4 Discussion

At 35‰ seawater, the gill ultrastructure changed markedly following exposure to copper. As the exposure period proceeded, the gill epithelial layer became highly vacuolated, the number of plasma membrane infoldings and mitochondria became reduced, the microtubule network became distorted, the endoplasmic reticulum became swollen and many free ribosomes appeared in the cell cytoplasm. The number of haemocytes increased and some became attached to the basal epithelial cell surface or actually infiltrated into the epithelial cell cytoplasm. These changes correlate well with previous accounts of ultrastructural damage to crustacean gills in the presence of heavy metals (Bubel, 1976; Couch, 1977; Ghate & Mulherkar, 1979; Doughtie & Rao, 1981; Papathanassiou, 1985; Anderson & Baartrup, 1988; Boitel *et al.*, 1988; Nonnotte *et al.*, 1993). Since the copper concentration used in this study was at least one order of magnitude lower than those used in previous studies, it is somewhat surprising that this extent of ultrastructural change occurred. It may be that exposure to copper in a flow-through seawater system increases the degree of metal toxicity compared to a static system. Vernberg *et al.* (1977) and Ritz (1980) showed that metal uptake, physiological response and the rate of mortality was greater in animals maintained in a flow-through compared to a static seawater system. Alternatively, the documented levels of metal used in previous studies may have been nominal and may have reduced during the experimental period due to loss of toxicant via aeration, absorption to the container or uptake by the test organism. On exposure to relatively high sublethal concentrations of metal, some crustaceans show more severe changes in the gill structure than documented here. Examples include thickening of the epithelial layer and reduction of the haemolymph space (Nonnotte *et al.*, 1993), and extensive cell necrosis (Ghate &

Mulherkar, 1979). Such extensive change was not observed in this study, suggesting either the ultrastructural observations from this work were very much sublethal effects or that there is a great deal of variability in the effect of heavy metals on the gill, dependant on experimental seawater systems, heavy metal types and test organism species.

All epithelial cells were affected uniformly by copper in 35‰ seawater. This was surprising as there was variation in the thickness of the respective epithelial cell types. On the criterion of cell shape, chief cells (the thinnest epithelial cell type) would be expected to show the greatest degree of ultrastructural alteration as this cell had the greatest surface area to volume ratio and thus was potentially more permeable to heavy metals. As this was not the case, it could be implied that copper does not preferentially enter into the gill via a single cell type and exerts its effect independent of cell thickness.

The extensive literature on the physiological effects of heavy metal exposure reveals that heavy metals generally decrease the oxygen consumption of crustaceans (Jones, 1942; Corner & Sparrow, 1956; Kerkut & Munday, 1962; DeCoursey & Vernberg, 1972; Vernberg & Vernberg, 1972, Collier *et al.*, 1973; Thurberg *et al.*, 1973; Depledge, 1984). This response is not universal, however, and some crustaceans demonstrate no change in oxygen consumption following heavy metal exposure, particularly when sublethal concentrations are used (Thurberg *et al.*, 1973; Depledge & Phillips, 1987; Spicer & Weber, 1991). The changes in osmoregulatory ability of crustaceans following heavy metal exposure is conclusive, and demonstrates that decreased haemolymph osmolalities and disrupted osmoregulatory ability are characteristic changes following heavy metal exposure (Thurberg *et al.*, 1973; Vernberg *et al.*, 1974; Jones, 1975a,b; Bouquegneau & Gilles, 1979; Bjerregaard & Vislie, 1986). Unfortunately, the majority of these studies have used levels of heavy metal much greater than used in this study,

making direct comparison of physiological response and ultrastructural change difficult. It is interesting, however, to discuss the potential physiological and biochemical effects that could result from the ultrastructural changes observed in this study.

It has been suggested previously that the disruption of gill physiological processes is a direct result of anatomical gill damage (Boitel & Truchot, 1989). It is true that the structural integrity of the apical and basal plasma membrane infoldings is essential for the correct functioning of ionic regulation and respiratory gas exchange and, as exposure to copper at 35‰ seawater results in severe disruption of the membrane infolding networks, an effect on these physiological processes may be anticipated. Exposure to copper may affect the structure of the apical and basal plasma membrane infolding networks since copper has a high affinity for the sulphydryl groups of proteins (Viarengo & Nott, 1993) and may bind to membrane proteins and phospholipids and, in so doing, alter their structure and function (Webb, 1979; Evtushenko *et al.*, 1986). In addition, copper may alter the structure of the plasma membranes by lipid peroxidation processes (Viarengo, 1985; Viarengo *et al.*, 1988). Many *in vitro* studies indicate that transition metals, such as copper, are involved, probably through the formation of oxyradicals, in several oxidative processes resulting in the degradation of enzymes, glycoproteins and DNA damage, for example. Indeed, recent studies on mussels have demonstrated that sublethal concentrations ($40\mu gl^{-1}$) of Cu^{2+} stimulated lipid peroxidation in the gills and digestive gland (Viarengo *et al.*, 1988).

Disruption of apical and basal plasma membrane infoldings in the gill epithelial layer decreases the surface area available for the location of exchange pumps, such as Na^+/H^+ and Na^+/K^+ exchange systems, and could decrease the ionic regulatory capabilities of the crab. Active Ca^{2+} transport also takes place through a membrane located Na^+/Ca^{2+} exchange system (Roer, 1980; Bjerregaard & Vislie, 1986). If active Ca^{2+} transport is decreased, the haemolymph calcium levels may increase due to the passive influx of

Ca^{2+}. It is known already that a sustained increase in the cytosolic Ca^{2+} concentration is toxic to the cell and, in extreme cases, could lead to cell death (Nicotera *et al.*, 1989). Carbonic anhydrase, an enzyme thought to participate in respiration and ionic regulation (for a review see Böttcher & Siebers, 1993), has soluble and basal membrane associated fractions (Burnett *et al.*, 1981, 1985; Henry, 1988; Böttcher *et al.*, 1990a,b; Lucu, 1990). Disruption of the basal membrane could also indirectly disrupt ionic regulation, since carbonic anhydrase provides the counterion for the apical ion exchange system (Böttcher & Siebers, 1993). Basal membrane disruption may also disturb respiratory gas exchange, as carbonic anhydrase facilitates the diffusion of CO_2 from the blood to the ambient medium (Burnett *et al.*, 1981; Burnett & McMahon, 1985).

Thus, the previously documented physiological effects of heavy metals may result from a decrease in the sites of active ion or respiratory gas exchange. Alternatively, the physiological changes may be caused by a direct inhibitory effect of copper on the enzymes associated with these exchange systems. It has been stated already that copper has a high affinity for sulphydryl groups of enzymes (Viarengo & Nott, 1993) and the activity of enzymes such as Na^+K^+ATPase (Haya & Waiwood, 1983; Dhavale & Masurekar, 1988; Hansen *et al.*, 1992a), Ca^{2+}ATPase (Bjerregaard & Vislie, 1986; Viarengo *et al.*, 1993) and respiratory enzymes (Corner & Sparrow, 1956; Kerkut & Munday, 1962; Hubschman, 1966, 1967) has been shown to be inhibited in crustaceans exposed to heavy metals. The initial inhibition of the enzymes associated with the exchange systems may trigger the decrease in the number of exchange sites and limit the success of ionic regulation, thus leading to an increase in the influx of copper and subsequently resulting in membrane disruption. Another explanation for the decrease in plasma membrane infoldings (particularly the apical infoldings) could be that the gill is structurally changed to decrease the possible sites of entry of copper into the cell. This, of course, assumes that copper is taken actively into the cell through these

exchange sites. Although this is probably true, it is also likely that copper enters the cell by passive diffusion, questioning the theory that the number of active absorption sites in the gill is reduced to decrease the rate of entry of copper into the gill.

Plasma membranes may be rendered leaky after structural disorientation and this may lead to cell vacuolation. Extensive vacuolation was observed frequently in the gill cells of *Carcinus maenas* following copper exposure at 35‰ seawater. Such vacuolation may indicate a failure to regulate water content. Vacuolation of epithelial cells could potentially affect the physiological processes in the gill by increasing the diffusion distance for respiratory gases and ions. In addition, if cell volume is maintained by the regulation of the Na^+ ion content, any decrease in the activity of the ionic exchange pumps would lead to cell swelling. Evidence of swollen mitochondria in copper-exposed epithelial cells is consistent with this suggestion. It is well known that heavy metals affect mitochondrial physiology and ATP production (Webb, 1979; Viarengo *et al.*, 1985), and *in vitro* mitochondrial Ca^{2+} uptake (Zaba & Harris, 1976; Jarvisalo *et al.*, 1980; Chàvez *et al.*, 1985), resulting in increased permeability to water. A decrease in the number of mitochondria, or an increase in disruption of the mitochondrial membranes, equates to a decrease in the available energy for ionic regulation and respiratory gas exchange. It has been shown previously that the addition of copper to fish mitochondria alters the influx of cations through the inner membrane, finally inhibiting respiration (Zaba & Harris, 1976).

A change in ribosomal distribution was also observed following copper exposure at 35‰ seawater. Metals have been shown previously to alter ribosomal distribution between cytosol and endoplasmic reticulum (Gamulin *et al.*, 1978; Ord & Al-atia, 1979). Alteration of the ribosomal distribution, as observed in this study, may suggest a switch in protein synthesis from the production of structural and enzyme proteins (such as Na^+K^+ATPase, Ca^{2+}ATPase and carbonic anhydrase) to the production of soluble

proteins (such as metallothioneins) which play an important role in heavy metal regulation (Viarengo, 1985). This, in turn, would affect indirectly ionic regulation and respiration by decreasing the production of the critical enzymes involved in these processes. As stated already, however, heavy metals also act directly on enzymes by combining with sulphydryl groups. Theoretically, the animal may lose little from switching protein synthesis, as structural and enzymatic proteins may be inevitably destroyed directly by heavy metals in the long term.

Since pillar cells are thought to be involved in structural support and haemolymph flow through the gill (Copeland & Fitzjarrell, 1968; Fisher, 1972; Johnson, 1980; Farrelly & Greenaway, 1992), disruption of their microtubular network could affect these roles. A collapse of the lamellae would decrease the surface area for exchange of ions and gases. A disruption of haemolymph flow would affect the transport of ions and gases in and out of the gill and may result in vascular stasis. The latter may be a compensatory mechanism to inhibit the passage of copper to other parts of the animal; however, it would also inevitably lead to a build up of copper compounds in the gill.

The remaining gill cells (glycocytes, nephrocytes and haemocytes) were not affected uniformly by copper at 35‰ seawater. For instance, glycocytes and nephrocytes did not undergo any ultrastructural change in the presence of copper. However, many more nephrocytes were present in the distal gill region than observed in the same region of the untreated gill at 35‰ seawater. Since nephrocytes are thought to play a role in the endocytosis of foreign material from the haemolymph (Table 1), this distributional change resulting from copper exposure may indicate that the gill defence mechanisms are under increased stress after exposure to copper and a greater number of nephrocytes are required in the gill to keep pace with this change. The entry of copper into the gill, or the effect of copper on the membrane structures, may allow an increased entry of foreign particles into the gill. Alternatively, nephrocytes may be involved directly in the

removal of copper from the gill and may increase in number due to a greater influx of copper ions into the gill. The relevance of an increase in nephrocyte number solely in the distal gill region is not fully understood. However, since the epithelial layer of the distal gill region consisted predominantly of chief cells, with a greater surface area to volume ratio than striated cells, concentration of nephrocytes in the distal gill region may imply that copper (or foreign material when the gill is exposed to copper) enters the gill through chief cells at a quicker rate than through the other epithelial cell types. The requirement for a successful defence strategy may, therefore, be greater in areas where chief cells are the most prominent epithelial cell type. Haemocytes, also thought to have an extensive involvement in cellular defence mechanisms (Table 1), increased in number following exposure to copper. Again, this increase was most pronounced in the distal gill region. Haemocytes not only increased in number but also altered in conformation following exposure to copper. Many more large-granule haemocytes were observed following copper exposure than in the untreated gill at 35‰ seawater and the agranular haemocytes were very scarce. This change in numbers may imply that the large-granule haemocytes were involved predominantly in the removal of copper from the gill (discussed further in Chapter 8). High numbers of haemocytes were observed in the gill lamellae following 4 days of copper exposure. This very rapid influx of haemocytes has been documented previously. For example, Bubel (1976) reported an increase in haemocyte number in the gill of *Jaera nordmanni* within 24h of heavy metal exposure. The origin of such large numbers of cells is unknown. Haemocytes may infiltrate the gill lamellae from other organs of the crab or, alternatively, a disruption of haemolymph flow may trap the cells within the gill and give the appearance of an apparent influx of cells into this region. Small and large-granule haemocytes were observed frequently attached to epithelial cells and, in many cases, infiltrated the cell cytoplasm. Infiltration of the epithelial cell cytoplasm by haemocytes may imply that

cell debris, caused by exposure to copper, was produced in the epithelial cells, supporting Bubel (1976) who stated that movement of haemocytes into the gill epithelial cells generally indicated the cell was undergoing some degree of degenerative change. Alternatively, haemocytes may be involved in the removal of copper from the gill and are placed in close proximity to the apical cell surface of the epithelial layer to remove the copper from the cell as soon as it passes through the apical cell surface. Another possibility, as mentioned previously, is that haemocytes may serve as provisors of energy from sugars held in glycogen stores and granules and aid in the fuelling of the gill physiological processes.

In conclusion, exposure to copper at 35‰ seawater caused extensive ultrastructural alteration to the cells of the posterior gill of *Carcinus maenas*. The epithelial cells were affected more than the cells in the haemolymph space (glycocytes, nephrocytes and haemocytes). If the observed ultrastructural alterations were deleterious to the crab then copper exposure could potentially affect gill physiological processes by decreasing the sites of exchange of ions and respiratory gases and the energy supply supporting these systems. In addition, copper may exert adverse effects by inhibiting the enzymes involved in these exchange processes or by disrupting haemolymph flow and impairing the transport of ions and gases to and from the gill surfaces. The definite sequence of change is not known, and it cannot be stated from this study which aspects of the gill exchange system were disrupted initially by copper and triggered the change in gill ultrastructure.

Figure 6.1. Transmission electron micrographs of the epithelial cells in the gill of *Carcinus maenas* following 10 days copper exposure at 35‰ seawater. **(a)** Striated cell taken from the proximal gill region near the central raphe. **(b)** Chief cell taken from the mid gill region towards the marginal canal end of the lamella. **(c)** Hour-glass pillar cell taken from the mid gill region near the central raphe. **(d)** Thin pillar cell taken from the distal gill region near the marginal canal, containing a haemocyte in the cell cytoplasm (A: apical infoldings; B: basal infoldings; ER: endoplasmic reticulum; G: glycogen; H: haemocyte; I: intercellular junction; M: mitochondria; N: nucleus; R: electron-dense rods; RB: ribosomes; S: intralamellar septum; T: microtubules; V: vacuole).

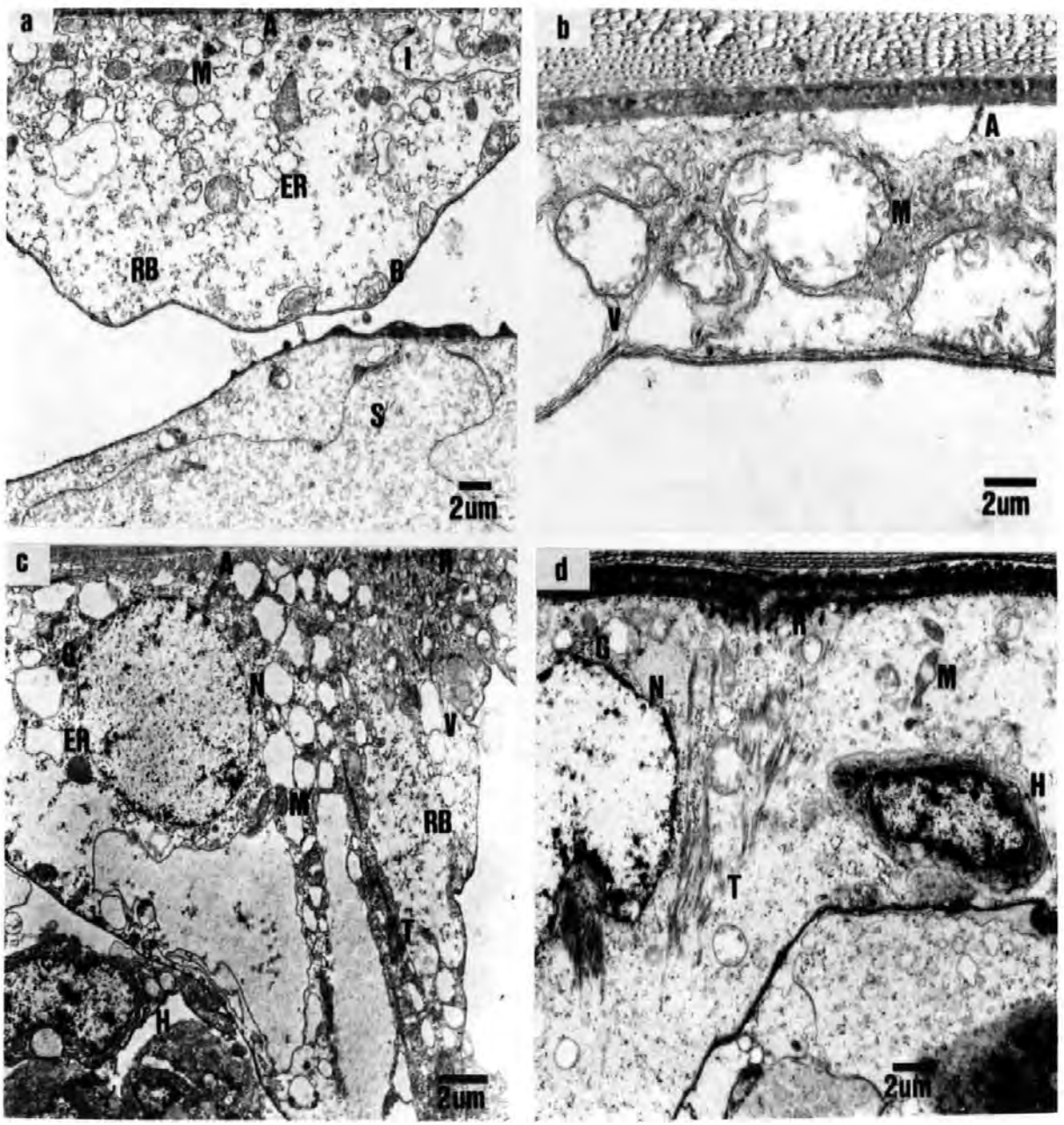
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Figure 6.2. Semi-thin methylene blue stained section of a gill lamella taken from the distal region (towards the marginal canal) of the gill of *Carcinus maenas* following 10 days copper exposure at 35‰ seawater (C: cuticle; EL: epithelial layer; H: haemocyte; P: pillar cell; S: intralamellar septum).

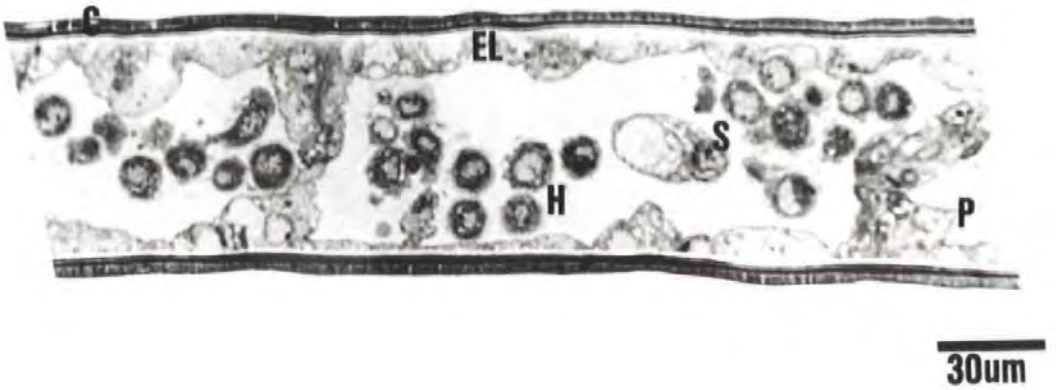
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Figure 6.3. Diagrammatic representation of the major stages undergone in the striated cell following exposure to copper at 35‰ seawater. (a) Untreated gill. (b) Following 4 days copper exposure. (c) Following 7 days copper exposure. (d) Following 10 days copper exposure (A: apical infoldings; B: basal infoldings; C: cuticle; ER: endoplasmic reticulum; G: glycogen; M: mitochondria; RB: ribosomes; V: vacuole).

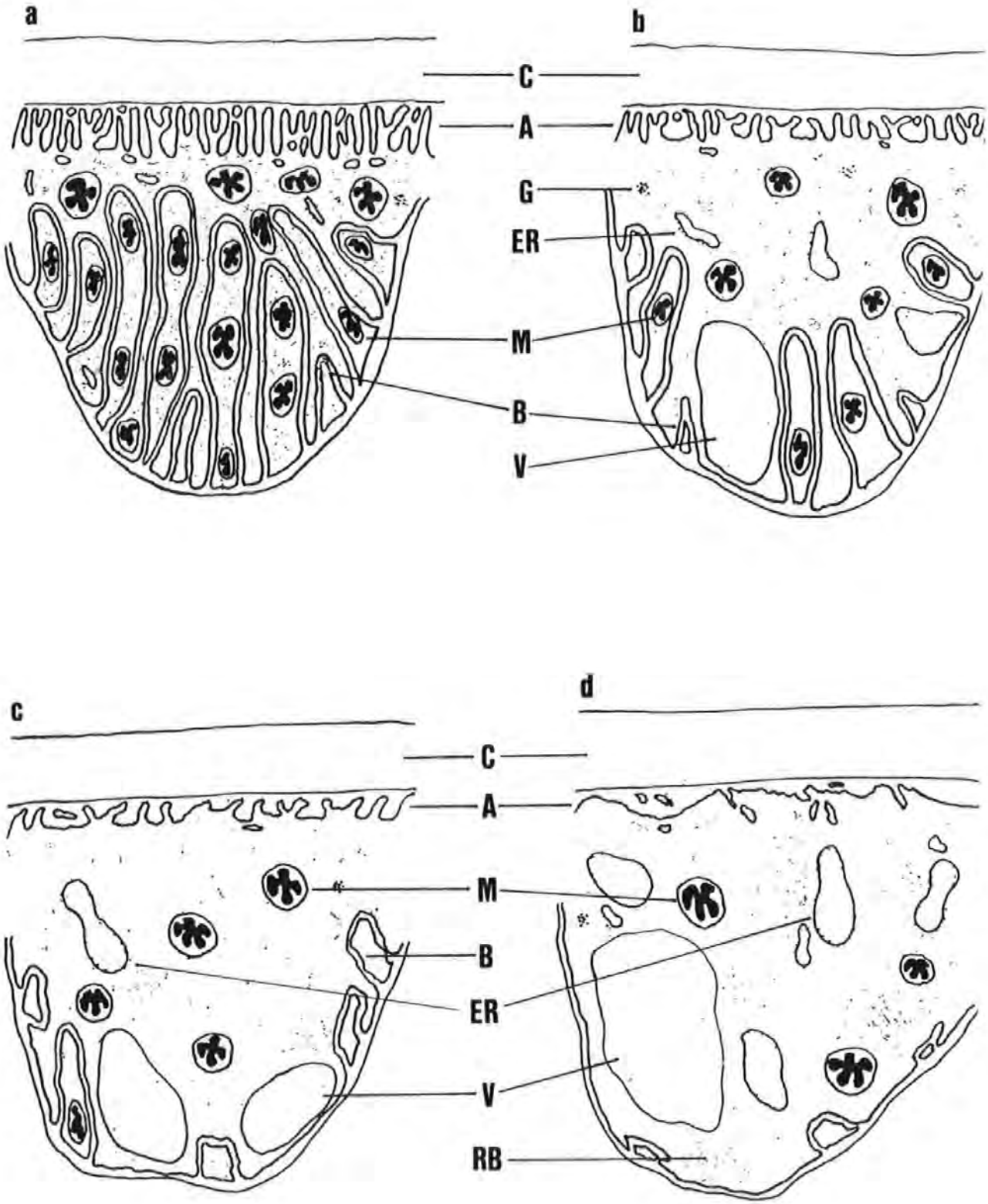
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Figure 6.4. Diagrammatic representation of the major stages undergone in the chief cell following exposure to copper at 35‰ seawater. (a) Untreated gill. (b) Following 4 days copper exposure. (c) Following 7 days copper exposure. (d) Following 10 days copper exposure (A: apical infoldings; B: basal infoldings; C: cuticle; ER: endoplasmic reticulum; G: glycogen; M: mitochondria; RB: ribosomes; V: vacuole).

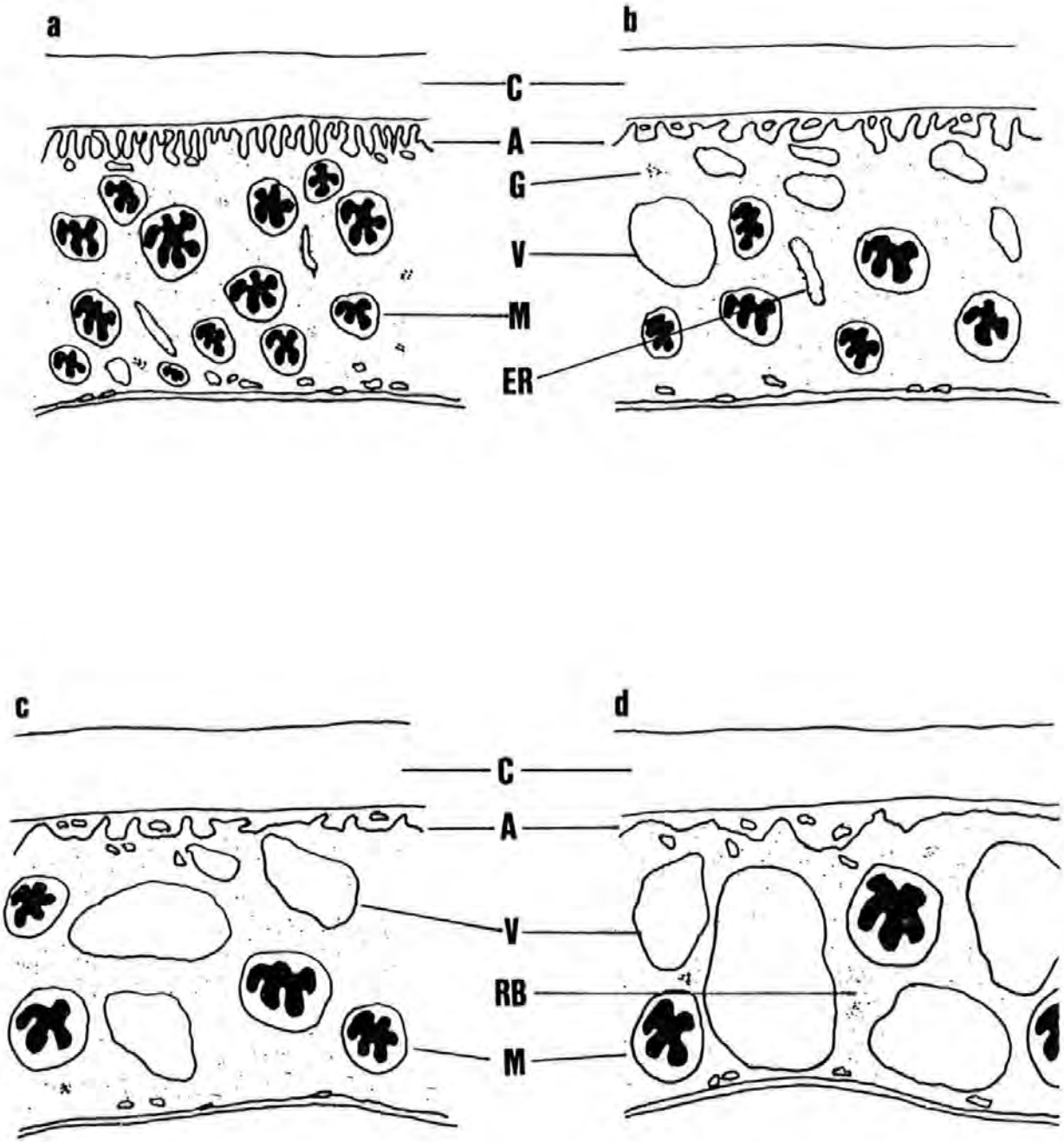
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Figure 6.5. Diagrammatic representation of the major stages undergone in the pillar cell following exposure to copper at 35‰ seawater. **(a)** Untreated gill. **(b)** Following 4 days copper exposure. **(c)** Following 7 days copper exposure. **(d)** Following 10 days copper exposure (A: apical infoldings; C: cuticle; ER: endoplasmic reticulum; G: glycogen; H: haemocyte; M: mitochondria; R: electron-dense rods; RB: ribosomes; T: microtubules; V: vacuole; Z: zig-zag border).

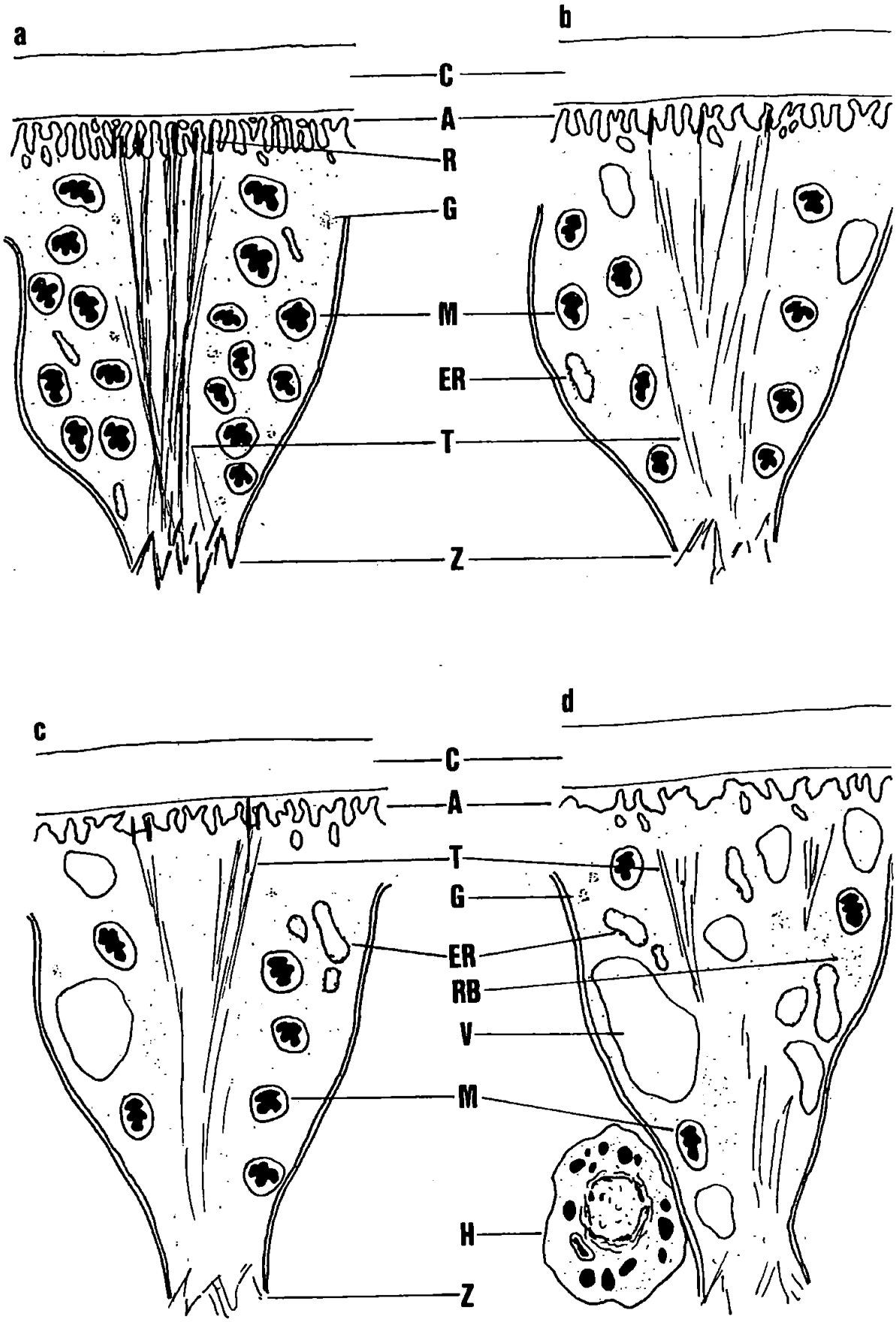
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Figure 6.6. Semi-thin methylene blue stained section of gill lamellae taken from the mid region (in the middle of the lamellae) of the gill of *Carcinus maenas* following 4 days copper exposure at 35‰ seawater (C: cuticle; EL: epithelial layer; H: haemocyte; P: pillar cell).

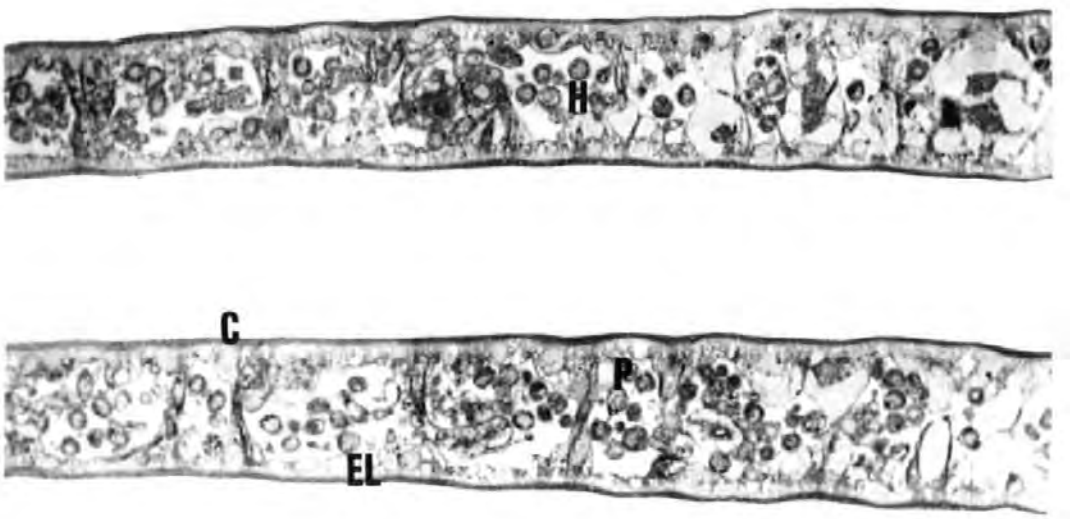
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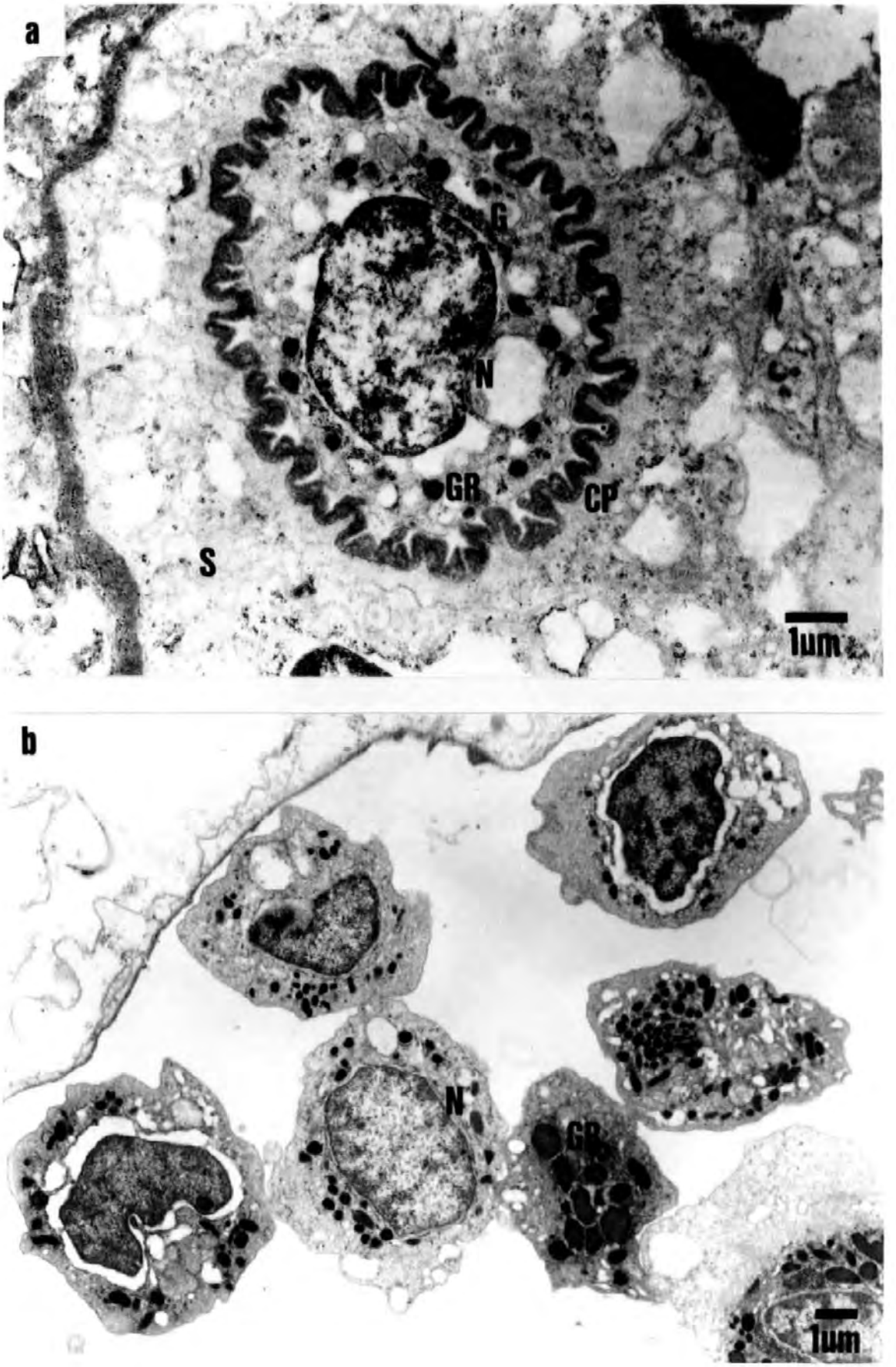
Figure 6.7. Transmission electron micrographs of the haemocytes observed in the gill of *Carcinus maenas* following 10 days copper exposure at 35‰ seawater. **(a)** Small-granule haemocyte within a gill capillary. **(b)** Small and large-granule haemocytes in the haemolymph space of the distal gill region (CP: capillary; GR: granule; N: nucleus; S: intralamellar septum).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CHAPTER 7

STRUCTURE AND DISTRIBUTION OF THE GILL CELLS OF *CARCINUS MAENAS* EXPOSED TO COPPER IN 10‰ SEAWATER

[Aspects of this chapter were presented at the 3rd International Conference on Trace Metals in the Aquatic Environment, Denmark, May, 1994 and are included in Lawson *et al.* "Effect of copper on the ultrastructure of the gill epithelium of *Carcinus maenas* (Decapoda: Brachyura)". *Marine Pollution Bulletin*, in press.]

7.1 Introduction

Carcinus maenas is found in estuarine and coastal regions where it is likely to be exposed to a combination of heavy metal pollution and large fluctuations in salinity. While there are many accounts of the ultrastructural damage to crustacean gills resulting from heavy metal exposure (Bubel, 1976; Couch, 1977; Ghate & Mulherkar, 1979; Papathanassiou & King, 1983; Papathanassiou, 1985; Anderson & Baartrup, 1988; Boitel *et al.*, 1990; Nonnotte *et al.*, 1993), information on the combined effects of sublethal metal concentrations and environmental variables, such as salinity, on gill ultrastructure is relatively scarce (but see Bubel, 1976). Most metal toxicity studies are conducted at one salinity and, for marine species, this is usually seawater (but often the salinity is not stated) (McLusky *et al.*, 1986). In general for marine crustaceans, a decrease in salinity has been associated with increased metal uptake rates (O'Hara, 1973a,b; Hutcheson, 1974; Jones, 1975a,b; Wright, 1977; Nugegoda & Rainbow, 1989a,b; Rainbow *et al.*, 1993) and enhanced metal toxicity (Bryan, 1976; Chen & Slinn, 1980; McLusky *et al.*, 1986; McLusky & Hagerman, 1987). If more metal enters the gill at low salinity, it may be anticipated that the degree of ultrastructural change in the gill cells following heavy metal exposure may differ depending on the external salinity.

As stated previously, the gill of *Carcinus maenas* consists of cells involved in ionic regulation, osmoregulation and respiratory gas exchange (Chapter 4). It has been shown that the ultrastructure and distribution of the gill cells, particularly those thought to be involved directly in these physiological processes, changed markedly between crabs acclimated to 10‰ and 35‰ seawater (Chapter 5). In addition, it has also been demonstrated that considerable gill ultrastructural change results from copper exposure at 35‰ seawater (Chapter 6).

This chapter describes the change in ultrastructure and distribution of the six major gill

cell types following exposure to copper ($50\mu gl^{-1}$) at 10‰ seawater. This study enables an assessment to be made of whether gill ultrastructure was additionally affected by copper exposure under conditions of osmotic stress.

7.2 Materials and methods

The materials and methods used in this chapter are as detailed in Chapter 3. Crabs were exposed to copper at a concentration of $50\mu gl^{-1}$ in 10‰ seawater for 10 days. Individuals were removed at 4, 7 and 10 days.

7.3 Results

The gill cuticle ultrastructure and the distribution of algal and bacterial cells on the epicuticle, of *Carcinus maenas* exposed to copper at 10‰ seawater did not differ from those described previously (Chapters 4-6). At 10‰ seawater, copper exposure altered the ultrastructure of striated, chief and pillar cells, and the changes observed were dependent on cell type, unlike at 35‰ seawater. The extent of gill cell ultrastructural change following copper exposure at 10‰ seawater was not as great as observed previously at 35‰ seawater. After 4 days copper exposure, striated cells became vacuolated, particularly in the area around the apical infolding network, and the apical and basal plasma membrane infoldings decreased in density (apical more so than basal) compared to that observed in the untreated gill. In addition, the mitochondria within the striated cells became more rounded in profile and swollen in appearance. After 10 days copper exposure, the structural integrity of the apical infolding network of the striated cells was highly disrupted and, in some cases, the apical infoldings became detached from the cuticle and large vacuoles formed (Fig. 7.1a). Some sub-cuticular vacuoles containing loosely arranged apical plasma membrane material were also observed. The density of the basal plasma membrane infolding network of the striated cells did not

decrease with increasing exposure time as great at 10‰ as seen at 35‰ seawater. The majority of mitochondria were swollen in the 10 day copper-exposed gill. The distribution of striated cells was not altered following copper exposure from that described in Chapter 5; the proximal gill region housed the greatest, and the distal gill region the least, proportion of striated cells.

After 10 days copper exposure, the chief cells also became extensively vacuolated, the apical infolding network was almost completely disrupted, and large, swollen mitochondria were frequently observed in the cell cytoplasm (Fig. 7.1b). The basal surface of the chief cells was less affected by copper exposure than the apical cell surface; many basal infoldings remained even after 10 days exposure to copper. Chief cells exhibited the same distributional pattern, dependant on gill position, as described for the untreated gill at 10‰ seawater (Chapter 5). Chief cells were most abundant in the distal gill region and very scarce in the proximal gill region.

The ultrastructural alterations seen in the pillar cells following copper exposure at 10‰ seawater were dependant on pillar cell shape. After 10 days exposure, very few basal folds were observed in hour-glass cells (Fig. 7.1c); only those in the proximal gill region, near the central raphe, retained these structures. As the number of basal folds diminished the number of mitochondria within the cell cytoplasm simultaneously decreased, and the mitochondria became swollen in appearance. The number of apical infoldings in hour-glass pillar cells also decreased following exposure to copper, some areas of the apical cell surface pulled away from the cuticle. Some hour-glass pillar cells (in the mid and distal gill region) contained more vacuoles than observed in the untreated gill at 10‰ seawater. Exposure to copper at 10‰ seawater altered the ultrastructure of thin pillar cells to a greater extent than hour-glass pillar cells. After 10 days exposure, thin pillar cells became more vacuolated and the cell cytoplasm contained many more free ribosomes than in the untreated gill, giving the cell a diffuse

and granular appearance (Fig. 7.1d). A reduction in the density of the microtubular network was observed only in thin pillar cells following exposure to copper at 10‰ seawater. The distribution of the different pillar cell forms was not altered after copper exposure, hour-glass pillar cells were found towards the central raphe and thin pillar cells towards the marginal canal end of a lamella.

The degree of ultrastructural change in the epithelial layer increased as the length of exposure increased (Figs 7.2-7.5). Figure 7.2 shows that the striated cells became increasingly vacuolated over 10 days of exposure and the number of basal folds gradually reduced. Fewer mitochondria were contained in the cell cytoplasm after 10 days compared to 4 and 7 days of exposure. Similarly, Figure 7.3 shows that chief cells became increasingly vacuolated as the exposure time increased, vacuoles formed initially at the apical cell surface (4 days) and eventually occupied all areas of the cell cytoplasm (10 days). Mitochondria became less prevalent in the chief cell cytoplasm and appeared swollen after 7 and 10 days of exposure. Also, the density of the basal membrane infolding network was reduced as exposure time proceeded. Hour-glass pillar cells became more vacuolated and contained reduced numbers of mitochondria as the exposure time increased (Fig. 7.4). In addition, the hour-glass pillar cell basal membrane progressively lost its infoldings over the 10 day period. Figure 7.5 shows that the microtubular network of thin pillar cells decreased in density after 10 days copper exposure and the cell became highly vacuolated. Thin pillar cells contained increasing amounts of swollen endoplasmic reticulum and free ribosomes as exposure time increased and fewer mitochondria were observed in the cell cytoplasm.

Following exposure to copper at 10‰ seawater, chief cells were the most and striated cells the least disrupted cell type. Thus, the proximal gill region (where the epithelial layer consisted mainly of striated cells) was ultrastructurally altered to the least extent, and the distal gill region (where chief cells were the predominant cell type) to the

greatest extent. The apical side of all epithelial cells became more vacuolated than the basal surface (Fig. 7.6) and the epithelial cell apical infolding network was less dense than the basal infolding network after 10 days of copper exposure.

In general, the intercellular junctions were of the same ultrastructure following copper exposure as observed in the untreated gill at 10‰ seawater (Chapter 5). Although, in some cases, the adherence zone of the junction pulled further away from the apical side of the cell than in the untreated gill when the epithelial cell became vacuolated due to the effect of copper.

The gill cells outside the epithelial layer were also affected by exposure to copper at 10‰ seawater but in a non uniform manner. Glycocytes remained abundant within the intralamellar septum and did not show any ultrastructural or distributional change from that described in Chapter 5. The distribution and abundance of nephrocytes was also similar to that observed in the untreated gill, however, there appeared to be more small nephrocytes, particularly in the distal gill region, in the copper-exposed than the untreated gill. Nephrocytes prevailed in all gill regions even after 10 days exposure to copper. Haemocytes were seen more frequently in the copper-exposed than the untreated gill at 10‰ seawater. The same three haemocyte forms, as documented previously (Chapters 4-6), were seen. Small and large-granule haemocytes were observed in approximately the same number following copper exposure as seen in the untreated gill. Agranular haemocytes were seldomly sited in the copper-exposed gill. After copper exposure, haemocytes were seen within the capillaries of the intralamellar septum (Fig. 7.7a) and occasionally infiltrated into the gill epithelial cells (Fig. 7.7b). The latter was an infrequent occurrence in 10‰ seawater compared to observed in the copper-exposed gill at 35‰ seawater. After 10 days exposure, haemocytes occurred in agglutinating clumps of both small and large-granule haemocytes, rather than singularly. Within these clumps, a wide range of developmental stages was observed. For example, some of the

haemocytes undergo cell lysis. Haemocytes were seen in the greatest numbers in the distal, rather than the proximal and mid regions of the gill.

7.4 Discussion

Although the different types of ultrastructural alteration in response to copper were similar at 10‰ and 35‰ seawater, the extent of ultrastructural change resulting from copper exposure at 10‰ seawater was not as great as observed at 35‰ seawater. At 10‰ seawater, as the copper exposure period progressed, the epithelial cells became increasingly vacuolated, the plasma membrane infoldings decreased in number, and the mitochondria assumed a more rounded profile with some degree of cell swelling. It was difficult to compare these results at low salinity with previous ultrastructural studies as most authors have only looked at the effect of heavy metal exposure on the crustacean gill structure at high salinity (Couch, 1977; Ghate & Mulherkar, 1979; Papathanassiou & King, 1983; Papathanassiou, 1985; Anderson & Baartrup, 1988; Boitel *et al.*, 1990; Nonnotte *et al.*, 1993). The exception is Bubel (1976) who observed the change in ultrastructure of the gill of *Jaera nordmanni* following exposure to mercury and cadmium at two salinity regimes. Direct comparison to this work (Bubel, 1976) is difficult because of the different metals, different salinity regimes and different test organisms used. Similar ultrastructural changes in response to heavy metals were, however, documented and Bubel (1976) noted dissociated ribosomes, decreased numbers of plasma membrane infoldings and the presence of swollen mitochondria in the epithelium of the gill.

One particularly noticeable, and previously undocumented, observation was the difference in densities of the apical and basal plasma membrane infoldings in the epithelial cells following copper exposure at 10‰ seawater; this trend was not observed in copper-exposed gills at 35‰ seawater. Following exposure at 10‰ seawater, the

density of the basal infoldings in striated and chief cells remained higher than the apical infoldings. This difference may be explained in a number of ways. Firstly, copper enters the epithelial layer via the apical cell surface, thus, it may be expected that this region (as it is the first line of contact with the heavy metal) may be structurally altered to a greater extent than the basal cell surface. Secondly, as stated already, many of the important exchange systems and respiratory enzymes are located on the basal membrane infoldings; for example, the Na^+/K^+ exchange and one fraction of carbonic anhydrase, the enzyme providing a counterion for the apical Na^+/K^+ exchange system. Hence, retention of the structural integrity of the basal membrane, rather than the apical membrane, may be more important for the correct functioning of ionic and osmotic regulation in the gill.

Pillar cells undergo ultrastructural alteration following copper exposure and this change was dependent on cell shape at 10‰ seawater, unlike at 35‰ seawater when both pillar cell types were affected uniformly by exposure to copper. Ultrastructural change was greater in thin than in hour-glass pillar cells. This difference may imply that hour-glass pillar cells are a more stable cell type, able to withstand exposure to copper more successfully than the thin cell type. Denser plasma membrane infoldings were present in hour-glass than in the thin pillar cells, creating potentially a greater surface area for the copper ions to structurally alter. The high density of membrane infoldings could result in the ultrastructural alteration taking longer in hour-glass than in thin pillar cells. In addition to the pillar cell ultrastructural variation seen at 10‰ seawater, it was noticeable that chief cells showed more ultrastructural alteration than striated cells following copper exposure at dilute seawater. This latter observation corresponds with the pillar cell ultrastructural changes described above, as chief cells are located in areas where thin pillar cells prevail, striated cells in regions where hour-glass pillar cells dominate the epithelial layer. The distal and mid gill regions (containing more chief and

thin pillar cells than the proximal gill region) were more structurally altered than the proximal gill region. This difference may be due to the differences in the relative amounts of mitochondria and plasma membrane infoldings in the cells in the respective gill regions, as discussed previously. Additionally, since chief cells are thought to be involved predominantly in respiratory gas exchange (Table 1) and are the most ultrastructurally altered epithelial cell type, it may be suggested that the physiological process of respiratory gas exchange was more affected than ionic regulation and osmoregulation following copper exposure at 10‰ seawater.

The ultrastructure of the epithelial layer was more extensively altered in the untreated gill at 10‰ compared to 35‰ seawater (c.f. Chapters 4 & 5), thus, gill epithelial cells are potentially capable of much structural change in a short period of time (the acclimation period was three weeks). The absence of epithelial cell distributional change following copper exposure at each salinity implies that it is not ultrastructurally advantageous to have one particular cell type in the gill epithelium, to limit the uptake of copper into the gill or the degree of effect of copper on gill cell ultrastructure. Alternatively, copper exposure may inhibit the ability of the gill epithelium to undergo rapid ultrastructural alteration, for example, from an epithelium consisting predominantly of striated cells to one of chief cells. Unfortunately, there is no information available on the mechanisms and rate of epithelial cell turnover in crustaceans to allow further consideration of these findings.

Glycocytes did not undergo any ultrastructural or distributional alteration following copper exposure at 10‰ seawater implying that sufficient glycogen sources were available in the gill and exposure to copper did not increase glycogen demand. Nephrocyte numbers in copper-exposed gills at 10‰ seawater were similar to those found in the untreated gill at 10‰ and the copper-exposed gill at 35‰ seawater; however, nephrocyte numbers were higher than in the untreated gill at 35‰ seawater.

This finding implies that a decrease in salinity induced similar nephrocyte distributional changes in the gill as exposure to copper. There was an abundance of small nephrocytes in the gill exposed to copper at 10‰ seawater. It cannot be stated whether this was a specific response to copper exposure at low salinity or whether differences in the plane of sectioning or stages in the moult cycle (Drach, 1930; Johnson, 1980) caused this effect.

Increase in haemocyte numbers in the gill following heavy metal pollution has been documented previously in this study (Chapter 6). Increased numbers of haemocytes circulating in the gill haemolymph space could imply that these cells are involved in the phagocytosis of copper ions, or, as stated in Chapter 6, haemocytes may also be involved in the phagocytosis of foreign particles or cell debris. The lysing of haemocytes after 10 days of copper exposure at 10‰ seawater implies that the cell defensive systems cannot keep up with the cellular effects of copper and that after 10 days exposure the haemocytes themselves are affected directly by copper ions. Alternatively, haemocyte lysis may be a natural phase in the developmental cycle of haemocytes and may be independent of copper exposure. Cell contents released from the lysed haemocytes may be engulfed by neighbouring cells. It is pertinent to note that the greatest numbers of nephrocytes and haemocytes were observed in the distal gill region following copper exposure at 10‰ seawater (as observed at 35‰ seawater). Since these cells are thought to be involved in the gill defence systems, these observations may imply that the distal gill region, where chief cells dominate, is under increased stress compared with other gill regions following exposure to copper.

In general, previous studies have shown that the toxicity of heavy metals increases as salinity decreases (Vernberg & Vernberg, 1972; Jones, 1973, 1974; O'Hara, 1973a,b; McLeese, 1974; Vernberg *et al.*, 1974; Bryan, 1976; McLusky *et al.*, 1986; McLusky & Hagerman, 1987). In the case of copper, this increase in toxicity is thought to be due

to an increased bioavailability and uptake of copper at low salinities due to several reasons. Firstly, because of changes in chemical speciation (Batley & Florence, 1976; Florence & Batley, 1977; Batley & Gardner, 1978; Mantoura *et al.*, 1978), increasing the proportion of the free uncomplexed metal ion in solution at low salinity (Fig. 1.1). The free uncomplexed ion being the most bioavailable species of metal for uptake by aquatic biota (Sunda *et al.*, 1978; Engel & Fowler, 1979; Zamuda & Sunda, 1982; Borgman, 1983; Florence, 1983; Campbell & Stokes, 1985; Wangersky, 1986; Nugegoda & Rainbow, 1988; Luoma,1989). Secondly, decreased competitive interactions with major cations (Mg^{2+}, Ca^{2+}) for the sensitive sites of ion transport occurs at low salinities (Bjerregaard & Vislie, 1986; McLusky *et al.*, 1986). Thirdly, because of the disruptive effect of copper on the mechanisms responsible for maintaining the correct osmotic and ionic concentrations in the organism at decreased salinities (Bjerregaard & Vislie, 1986). Thus, it is surprising that following exposure to copper at 10‰ seawater, the cells remained visually more intact than at 35‰ seawater. This unexpected result may be explained in a number of ways. One explanation is the difference in epithelial cell size and the relative proportions of plasma membrane infoldings and mitochondria contained in the cell cytoplasm. At 10‰ seawater, the gill epithelium contained denser apical and basal infolding networks, with many more associated mitochondria, than observed at 35‰ seawater. Hence, the copper ions had a much greater surface area to bind to and, as stated already for the pillar cells, it may take longer to alter the structure of such an expansive plasma membrane infolding network; the resulting visual change after 10 days copper exposure at 10‰ seawater not being as great as observed at 35‰ seawater. Unfortunately, the experimental period was not extended beyond 10 days. It would have been interesting to observe whether a similar degree of change in the gill epithelial layer resulted following copper exposure at 10‰ compared to 35‰ seawater, but after a longer period of exposure. In addition, at lower salinities it has been shown that there

is an increase in the activity of enzymes such as Na^+K^+ATPase, carbonic anhydrase and ATP synthetase (Chapter 5). This increase would result in a greater number of potential protein sulphydryl sites for the copper ions to bind to. Again, it may take longer for the copper ions to disrupt a more expansive amount of enzyme material and subsequently alter cell ultrastructure at 10‰ than at 35‰ seawater.

The data on metal uptake rates at decreased salinities are very confusing and erratic and it has been difficult to interpret the ultrastructural differences in effect with salinity, using data on heavy metal uptake rates at varied salinities. Many investigators have documented an increase in metal uptake rate at low salinities (Wright, 1977; Fales, 1979; O'Hara, 1973a; Johnson & Jones, 1989; Nugegoda & Rainbow, 1989a) and have postulated that this is caused by an increase in the active uptake of salts necessary to maintain a hyperosmotic internal medium, a concept discussed previously (Chapter 5). More recently, investigators have found no change, or an increase, in the metal uptake rate with decreasing salinity; examples include amphipods (Weeks & Rainbow, 1992; Rainbow *et al.*, 1993) and, more pertinently, *Carcinus maenas* (Chan *et al.*, 1992; Weeks *et al.*, 1993; Black, 1994; Rasmussen *et al.*, 1994). These inconsistencies may be explained by variations in experimental seawater system, salinity regimes, concentration of heavy metal, species of metal or simply by interspecific variation, as *C. maenas* In particular is a very resilient, euryhaline crustacean (Depledge, 1990). The decrease in metal uptake rates at low salinity in some crustaceans has been explained partly by decreased cuticle permeability to water. In some crustaceans, a salinity decrease results in a reduction of apparent water permeability in the gills (Smith, 1967, 1970; Lockwood *et al.*, 1973; Bolt, 1983; Campbell & Jones, 1990; Black, 1994; Rasmussen *et al.*, 1994). It has been suggested that this reduction reflects real changes in integumental permeability (Mantel & Farmer, 1983; Campbell & Jones, 1990). As a result of changes in water permeability in response to changes in salinity, it is possible that there could

be resulting changes in the metal uptake rates into the gill. A reduced uptake of copper at 10‰ compared to 35‰ seawater would explain the decrease in the ultrastructural changes observed in the gill epithelial cells at the higher salinity in the present study. Other explanations for the decrease in ultrastructural change following copper exposure at 10‰ compared to 35‰ seawater could include a change in demand for copper in the gill and an alteration in the rate of removal of copper ions away from the gill at low salinity. Copper is necessary for the manufacture of ferroproteins (Roeser *et al.*, 1970) and the formation of haemocyanin molecules. Thus, copper is involved indirectly in the processes of oxygen transport and electron transfer coupled to energy release (Spaargaren, 1983). A decrease in salinity would increase the energy requirement for active transport and possibly increase the demand for copper ions in the gill. However, data on the change in haemocyanin concentrations in the gill following a decrease in salinity are limited. Some authors report an increase in the haemocyanin concentration at low salinities (Taylor, 1977; Boone & Schoffeniels, 1979; Spicer & Taylor, 1987) whereas others have found no apparent change with salinity (Weeks *et al.*, 1993). Transport processes out of the gill may be more effective at 10‰ than at 35‰ seawater. It has been stated already that the number of nephrocytes and haemocytes (cells thought to be involved in the endocytosis or phagocytosis of foreign material) increase in the untreated gill at 10‰ compared to 35‰ seawater. Thus, the defence system may have already been primed during the acclimation period to 10‰ seawater, aiding a more rapid removal of copper out of the gill than at 35‰ seawater.

The physiological effects of heavy metal exposure at low salinities have also been studied in crustaceans. In general, there is greater impairment of respiratory gas exchange and ionic regulation when heavy metals are administered at decreased, rather than natural, salinities (Thurberg *et al.*, 1973, Jones, 1975a,b; Taylor, 1977; Bjerregaard & Vislie, 1986; Depledge, 1987). This finding does not correlate well with the ultrastructural

observations from this study, which would anticipate a greater impairment of the gill physiological processes at 35‰ than at 10‰ seawater, as the degree of ultrastructural alteration was greater at the higher salinity. Based on data from previous physiological studies it could, therefore, be suggested that the greater impairment of respiratory gas exchange and ionic regulation at decreased salinities may be due to a factor other than gill ultrastructural damage. Previous physiological studies have, however, employed higher levels of heavy metal than used in this study, thus a direct comparison is probably inappropriate. The potential physiological and biochemical effects of these ultrastructural changes in the gill following copper exposure have been discussed (Chapter 6).

In conclusion, comparison of the present results with previous ultrastructural observations is limited as only Bubel (1976) has presented results on the combined effects of low salinities and heavy metal exposure on crustacean gill ultrastructure. As Bubel (1976) used a different test organism, different heavy metals and different salinity regimes comparison is even more tenuous. Information on the effects of copper administered at low salinity on the functioning of the crustacean gill is contradictory. It was evident from the present study that the ultrastructure of the gill epithelium was altered to a lesser extent following copper exposure at 10‰ than at 35‰ seawater, and there are a number of explanations for this rather unexpected observation. Most authors regard animals living near their tolerance range as being more susceptible to any stress (McLusky *et al.*, 1986). However, contrary views are more recently being formulated, particularly in the case of *Carcinus maenas*. As Depledge (1990) stated, it is possible that physiological adaptation to dilute media may not only help the crab to minimise ionic and osmotic fluxes, but may also impair the uptake of dissolved trace metals. Indeed, Howell (1984) demonstrated that stenohaline nematode species were more susceptible to heavy metals than euryhaline nematodes. Metal uptake rates may also

fluctuate with habitat variability, experimental acclimation period and salinity regime. For instance, Chan *et al.* (1992) showed that the metal uptake rate in Scottish *C. maenas*, adapted to a high salinity environment, was markedly different at low salinities to the metal uptake rate in Danish crabs, exposed to a much wider salinity range in their natural environment.

The trend of explaining metal toxicity solely by chemical speciation changes with decreasing salinity is rapidly becoming inadequate for crustacean species. It is now being implied that in times of osmotic stress, physiological adaptive responses, particularly in the case of *C. maenas*, may play a more important role in determining heavy metal uptake rates than changes in chemical speciation.

Figure 7.1. Transmission electron micrographs of the epithelial cells in the gill of *Carcinus maenas* following 10 days copper exposure at 10‰ seawater. **(a)** Striated cell taken from the proximal gill region near the central raphe. **(b)** Chief cell taken from the mid gill region towards the marginal canal end of the lamella. **(c)** Hour-glass pillar cell taken from the proximal gill region near the central raphe. **(d)** Thin pillar cell taken from the mid gill region near the marginal canal (A: apical infoldings; B: basal infoldings; ER: endoplasmic reticulum; G: glycogen; M: mitochondria; N: nucleus; R: electron-dense rods; RB: ribosomes; T: microtubules; V: vacuole).

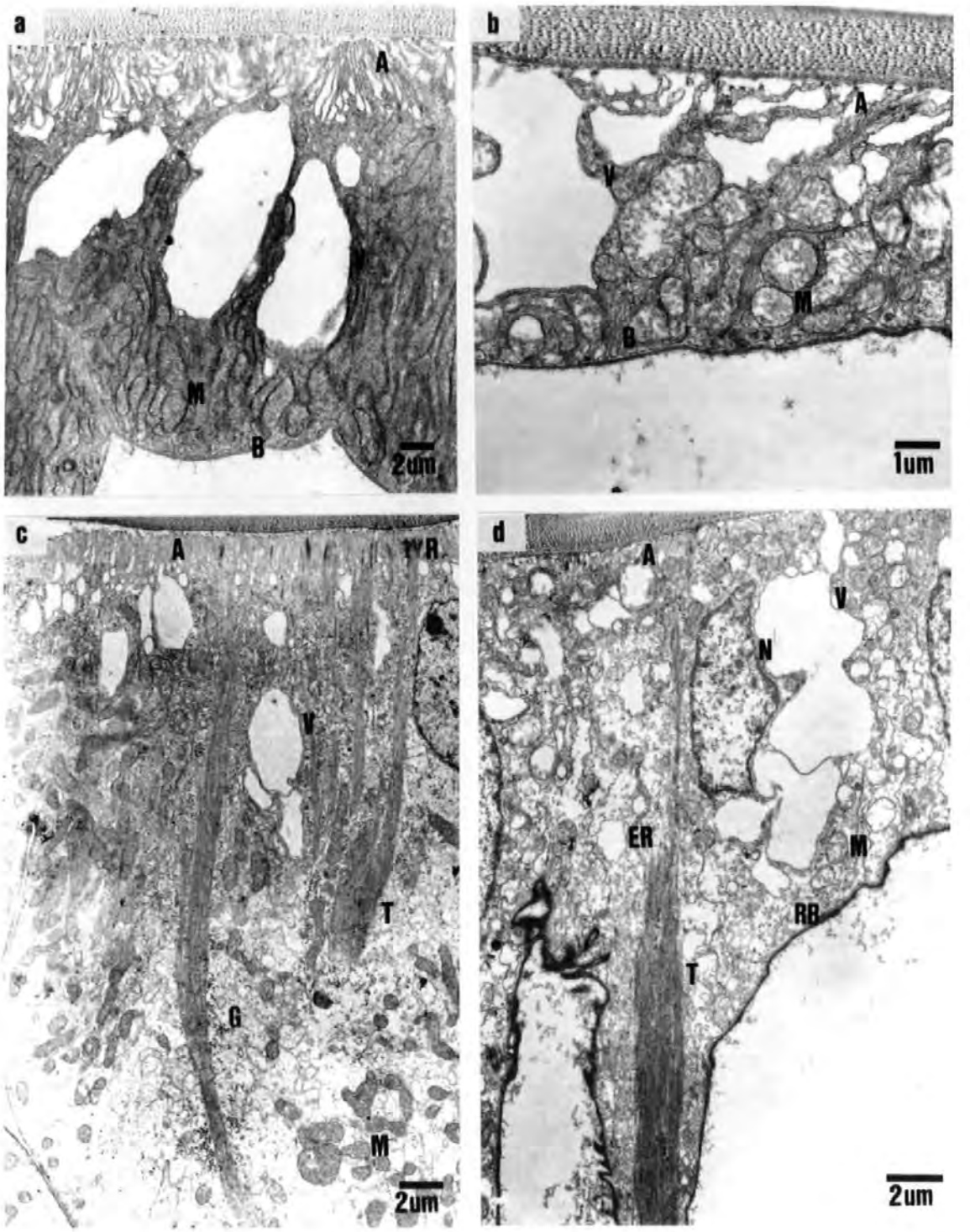
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Figure 7.2 Diagrammatic representation of the major stages undergone in the striated cell following exposure to copper at 10‰ seawater. **(a)** Untreated gill. **(b)** Following 4 days copper exposure. **(c)** Following 7 days copper exposure. **(d)** Following 10 days copper exposure (A: apical infoldings; B: basal infoldings; C: cuticle; ER: endoplasmic reticulum; G: glycogen; M: mitochondria; V: vacuole).

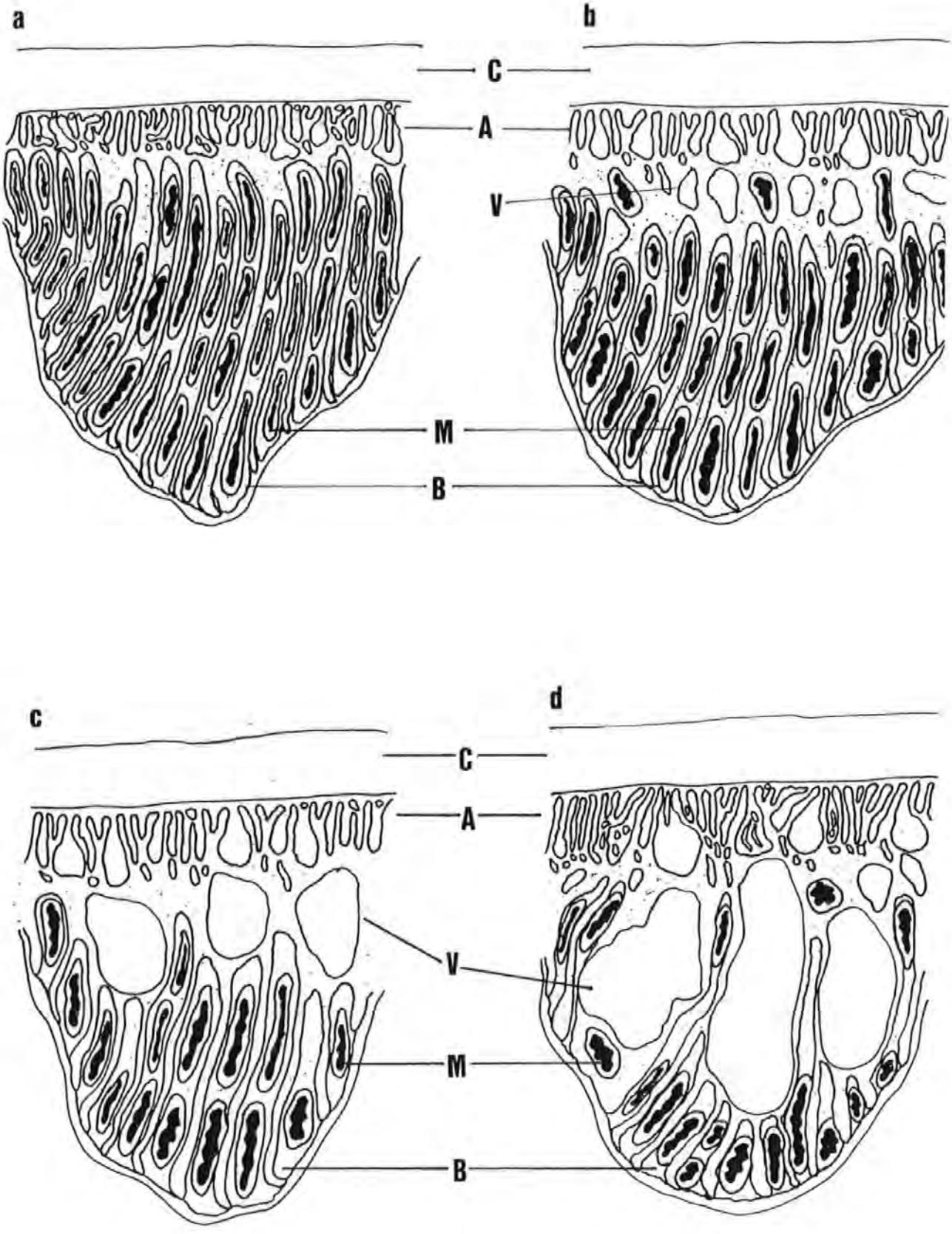
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Figure 7.3. Diagrammatic representation of the major stages undergone in the chief cell following exposure to copper at 10‰ seawater. **(a)** Untreated gill. **(b)** Following 4 days copper exposure. **(c)** Following 7 days copper exposure. **(d)** Following 10 days copper exposure (A: apical infoldings; B: basal infoldings; C: cuticle; G: glycogen; M: mitochondria; V: vacuole).

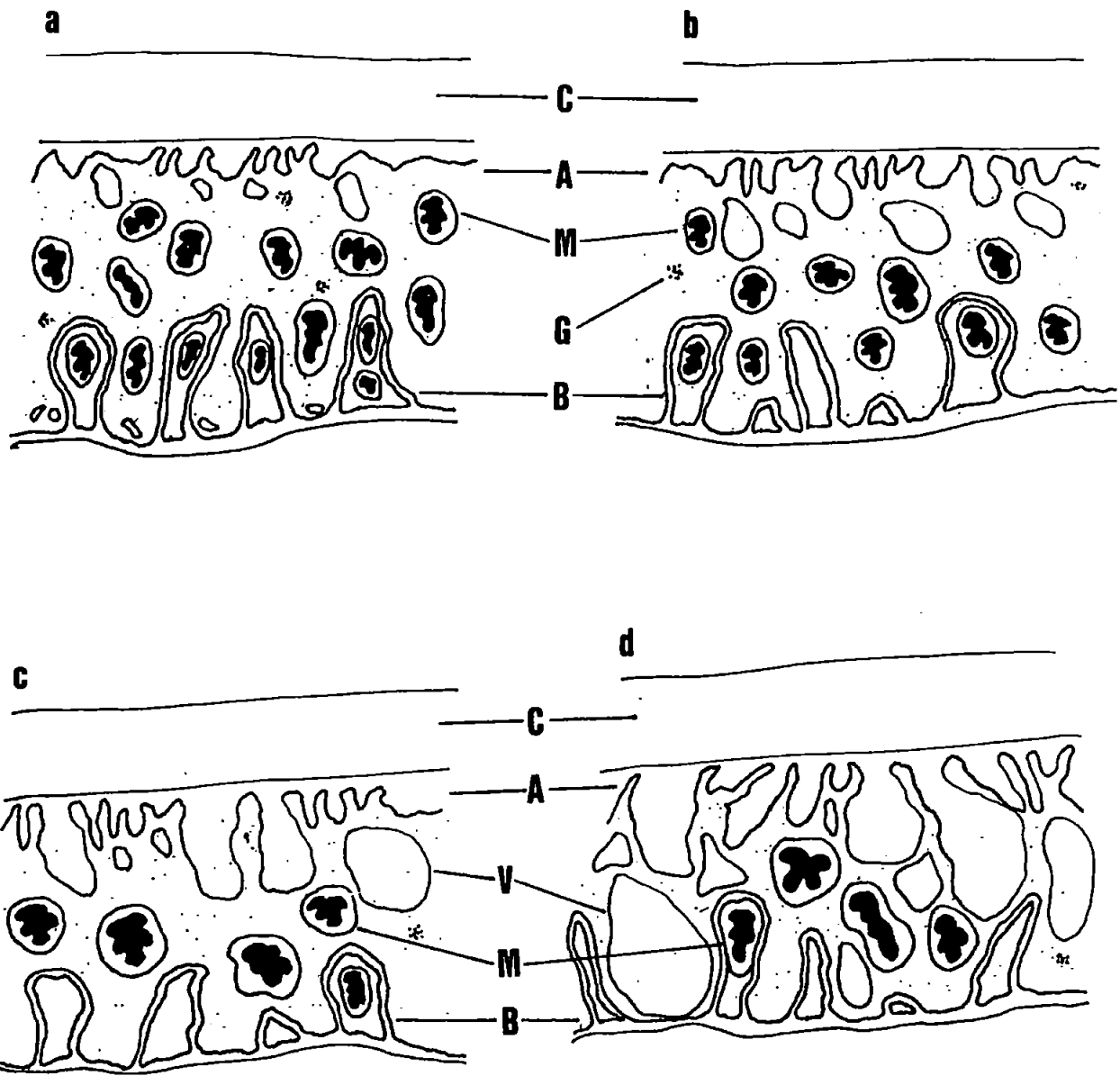
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Figure 7.4. Diagrammatic representation of the major stages undergone in the hour-glass pillar cell following exposure to copper at 10‰ seawater. (a) Untreated gill. (b) Following 4 days copper exposure. (c) Following 7 days copper exposure. (d) Following 10 days copper exposure (A: apical infoldings; B: basal infoldings; C: cuticle; G: glycogen; M: mitochondria; R: electron-dense rods; T: microtubules; V: vacuole; Z: zig-zag border).

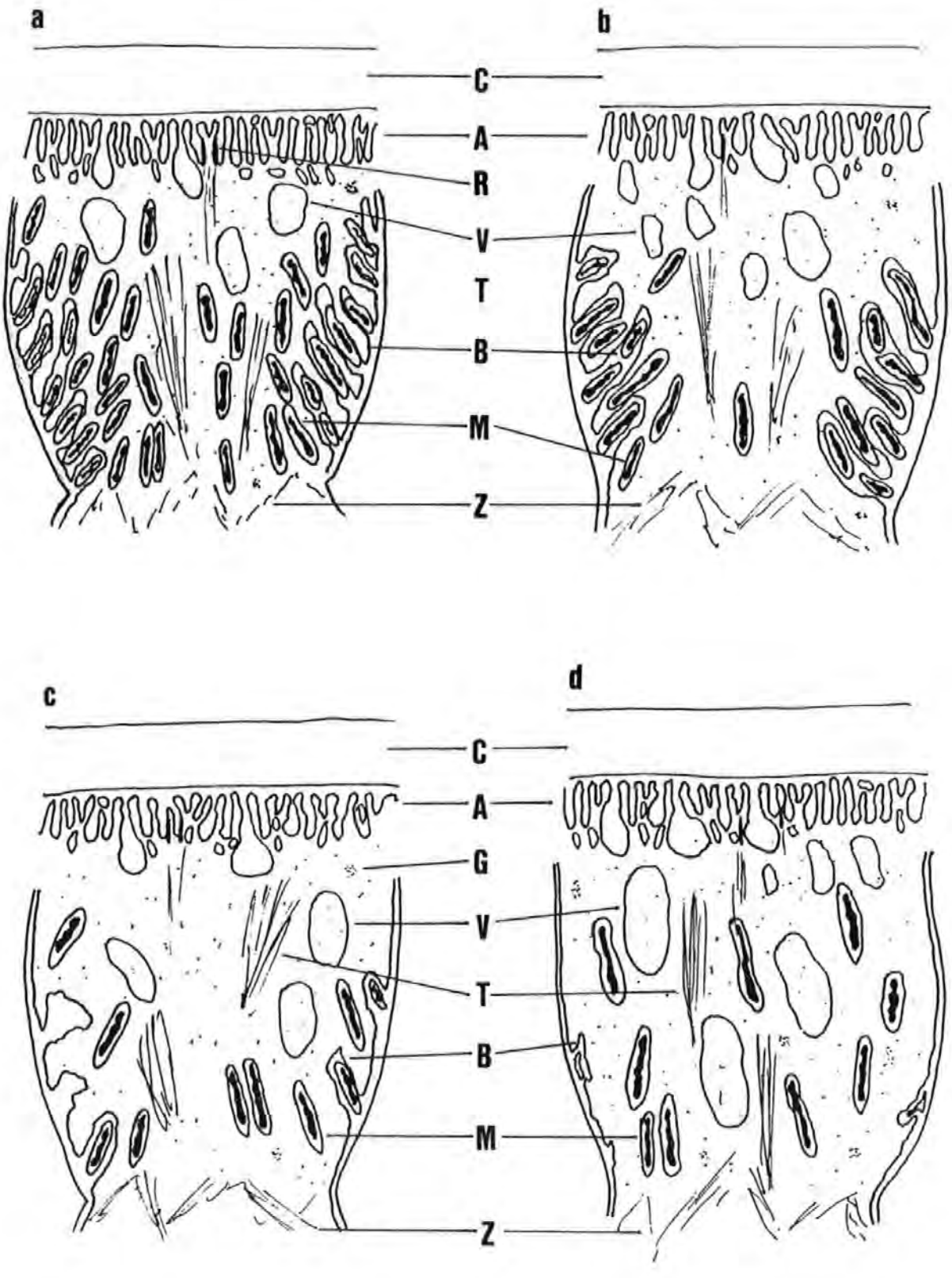
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Figure 7.5. Diagrammatic representation of the major stages undergone in the thin pillar cell following exposure to copper at 10‰ seawater. (a) Untreated gill. (b) Following 4 days copper exposure. (c) Following 7 days copper exposure. (d) Following 10 days copper exposure (A: apical infoldings; B: basal infoldings; C: cuticle; ER: endoplasmic reticulum; G: glycogen; M: mitochondria; R: electron-dense rods; RB: ribosomes; T: microtubules; V: vacuole; Z: zig-zag border).

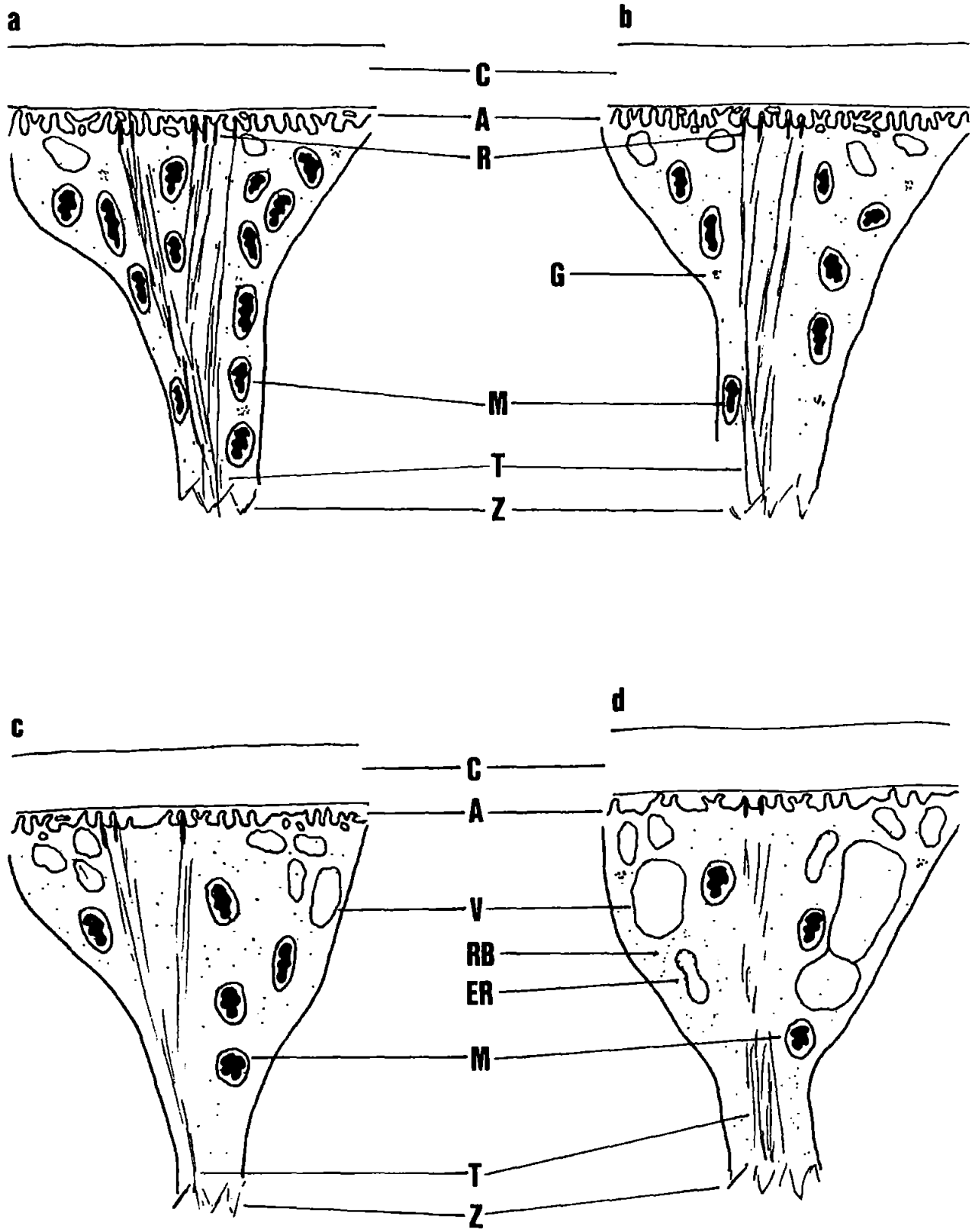
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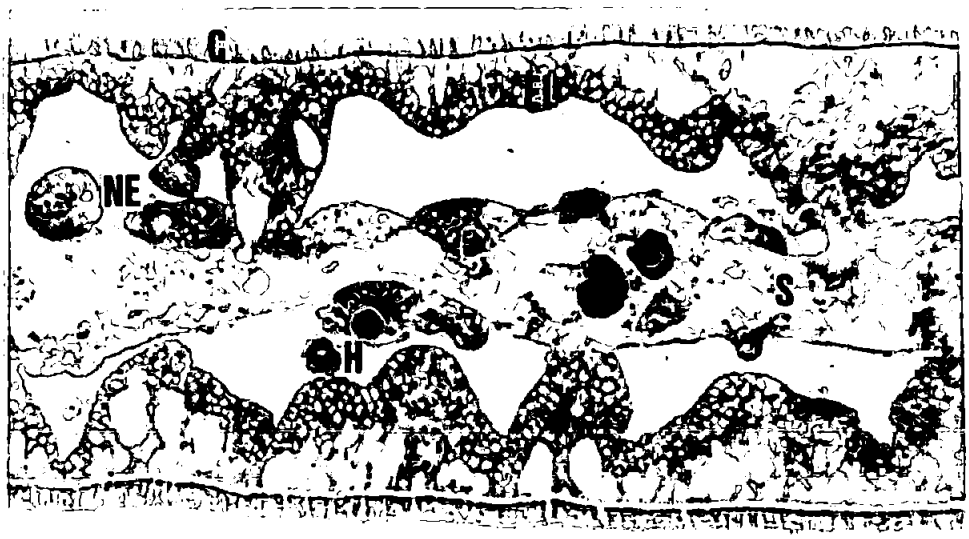
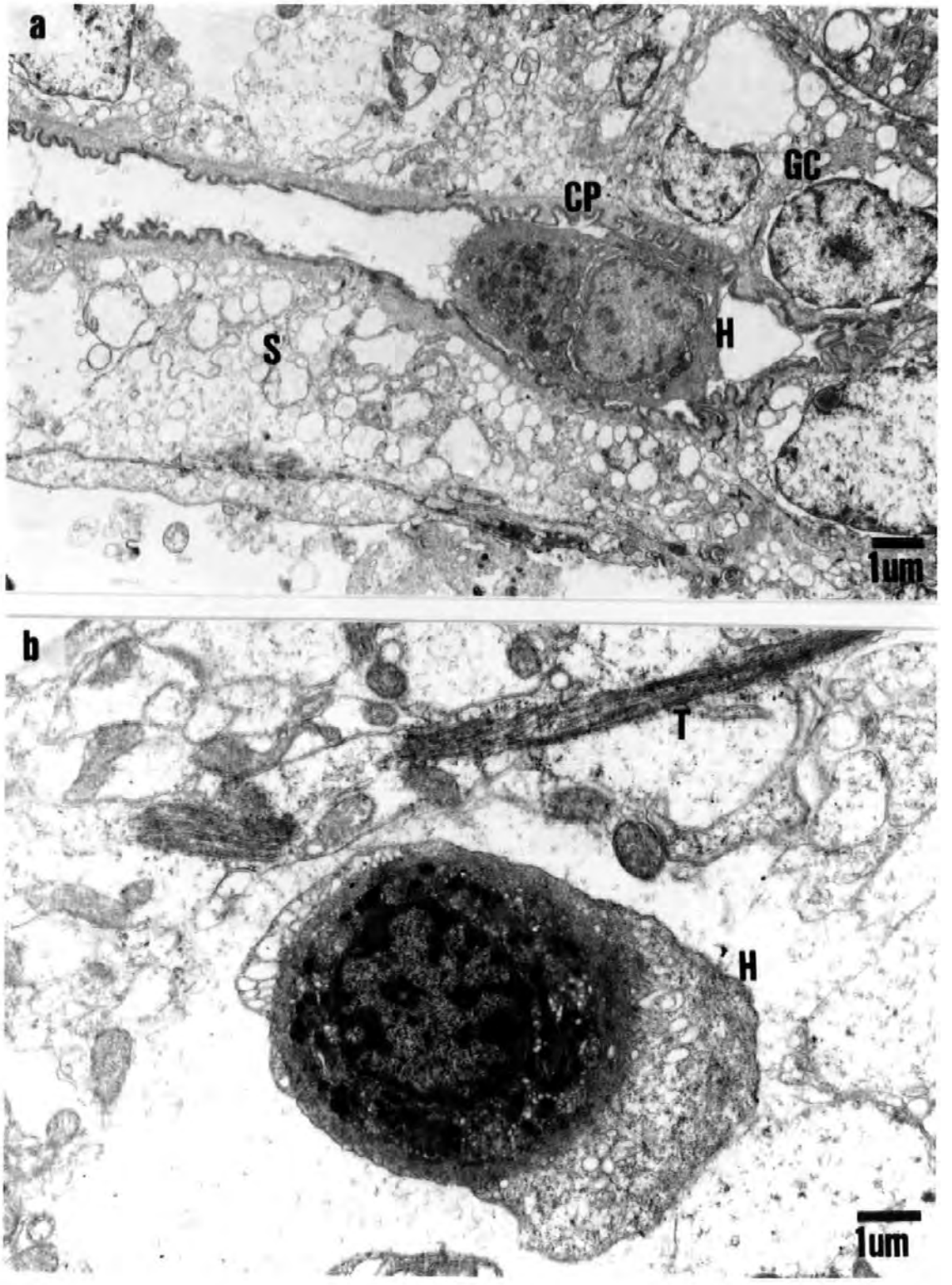
Figure 7.6. Semi-thin methylene blue stained section of a gill lamella taken from the proximal region (in the middle of the lamella) of the gill of *Carcinus maenas* following 10 days copper exposure at 10‰ seawater (C: cuticle; EL: epithelial layer; H: haemocyte; NE: nephrocyte; S: intralamellar septum).



10um

Figure 7.7. Transmission electron micrographs of the haemocytes observed in the gill of *Carcinus maenas* following 10 days copper exposure at 10‰ seawater. **(a)** Small-granule haemocyte in a gill capillary. **(b)** Small-granule haemocyte within a pillar cell (CP: capillary; GC: glycocyte; H: haemocyte; S: intralamellar septum; T: microtubules).


a
GC
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CHAPTER 8

X-RAY MICROANALYSIS OF THE HAEMOCYTES CIRCULATING IN THE GILL OF *CARCINUS MAENAS* FOLLOWING EXPOSURE TO COPPER

8.1 Introduction

The electron microscopical observations of Chapters 6 & 7 showed that the number of haemocytes circulating in the gill increased following exposure to copper. In addition, the proportion of cells containing granules in their cell cytoplasm increased. Since haemocytes are thought to be involved in the phagocytosis of foreign material (Table 1), the observed changes in haemocyte morphology and distribution following copper exposure implies that these cells play an important role in the uptake and immobilization of copper in the gill of *Carcinus maenas*.

Metal accumulation in granular deposits or membrane-limited vesicles is well documented for many invertebrate phyla (for reviews see Simkiss, 1976; Coombs & George, 1978; Coombs, 1980; Brown, 1982). The accumulation of heavy metals in granules has led to the suggestion that granules may function as part of a detoxification mechanism for surplus metal ions in invertebrate tissues (Simkiss, 1977). Although much work on the analysis of granules in invertebrate tissues has been completed, it has focused mainly on regions thought to be involved predominantly in heavy metal storage, such as the hepatopancreas and digestive gland (Zuckerkandl, 1960; Bryan, 1964, 1968; Cuadras *et al.*, 1981). Some authors have analyzed granules within invertebrate amoebocytes (George *et al.*, 1978; Marshall & Talbot, 1979; George & Pirie, 1980; Chassard-Bouchaud *et al.*, 1989, 1992), however, analysis of granules within gill haemocytes has not been completed previously.

This chapter describes the elemental composition of the granules of the haemocytes, found in great numbers in the gill following exposure to copper. Such a study allows an assessment of whether the haemocytes are directly involved in the immobilisation of copper, and its removal from the gill to other organs involved in detoxification processes.

8.2 Materials and methods

8.2.1 Collection of animals

In June 1993 intermoult, male *Carcinus maenas* (15-20g wet weight; carapace width 40-42mm) were collected from Portwrinkle, Cornwall, U.K. (4°18'W, 50°21'N). Crabs were transported to the University of Plymouth in aerated seawater (35‰) obtained from the collection site.

8.2.2 Experimental system

Crabs were maintained individually in four covered, plastic tanks (each of 25l volume) supplied with recirculating seawater (35‰, 12 ± 1°C); eight crabs were held in each tank. Crabs were acclimated to these experimental conditions for three weeks, during which they were fed every three days with chopped coley [*Pollachius virens* (L.)] until three days prior to copper exposure. Following the acclimation period, two tanks were exposed to a nominal copper ($Cu(NO_3)_2.3H_2O$) concentration of $0.5mgl^{-1}$. The actual initial concentration in the tanks was calculated using atomic absorption spectrophotometry (Varian-975) and was found to be $0.2 \pm 0.01mgl^{-1}$. Atomic absorption spectrophotometry also confirmed that the actual concentration of copper in the tanks was not depleted over the experimental period. Two tanks were not exposed to copper and served as controls. All crabs, from each of the four tanks, were dissected after 7 days.

Preliminary atomic absorption investigations showed that over a 20 day period, the concentration of copper in the gills was at its highest following 7 days of exposure (Table 2). For the preliminary atomic absorption investigations, crabs were maintained as described above and each tank was exposed to an initial actual copper concentration of $0.2mgl^{-1}$. Gill number 8 from the left and right branchial chambers was dissected from each crab, freeze-dried at 80°C for 18h and weighed (dry weights were used in

calculations). Five crabs were dissected at 2, 4, 7, 14 and 20 days from the start of the exposure. Freeze-dried gills were digested in concentrated nitric acid, made up to a set volume with distilled water and analyzed for copper using atomic absorption spectrophotometry techniques (Varian-975). The results of these preliminary studies (Table 2) indicated that the highest copper levels were detected after 7 days of exposure, thus, to optimise the detection of copper in X-ray microanalytical work, crabs were exposed to copper for a 7 day period.

Table 2. The accumulation of copper in the gills of *Carcinus maenas* over a 20 day exposure period .

Exposure Time (days)	Mean copper content (ugCu/g dry weight)
2	109.8
4	201.7
7	284.0
14	75.0
20	80.2

8.2.3 Preparation of specimens

Chemical fixation techniques, routinely used for transmission electron microscopy studies, are generally unsuitable for most microanalytical purposes. Chemical fixation removes labile, physiological elements and soluble pollutant metals; only the most insoluble intracellular metal species are retained (Nott, 1991). At the present time, chemical fixation techniques have been replaced by cryofixation methods. Cryofixation can give almost instantaneous preservation of the specimen in a near life-like state

(Ryan, 1992), avoiding structural shrinkage (Van Harreveld *et al.*, 1974; Boyde *et al.*, 1977), loss of diffusible ions (Morgan, 1980), and loss of sugars, amino-acids and proteins associated with chemical processing (Coetzee & Van der Merwe, 1984). The primary goal of cryofixation is to vitrify the specimen, which means cooling it so rapidly that its water molecules are captured in their normal random distribution and do not have time to organise into crystals (Ryan, 1992). Specimens can be cryofixed by plunging into liquid coolants, by jetting liquid coolants onto them or by contact with cold metal blocks ("slamming") (for a review see Ryan, 1992). In addition, a cryoprotectant can be used on the tissue, prior to cryofixation; ice crystal damage is thought to be reduced if the tissue is infiltrated with a cryoprotectant prior to freezing. For each crab, the carapace was removed and gill number 8 was extracted by cutting the basal attachment with dissection scissors. Each excised gill was then cut into small pieces (approx. 1mm diameter) and processed using one of the three methods outlined below (summarised in Fig. 8.1).

Method A: Metal mirror cryofixation followed by molecular distillation drying (MDD)

This technique was carried out in the Biological Sciences Department, Oxford Brookes University. Molecular distillation drying is an ultra-high vacuum, low temperature, freeze drying technique which reduces the chance of ice damage during processing. Crabs were transported to Oxford after 7 days copper exposure in the experimental seawater tanks and dissected just prior to cryofixation. Small pieces (1mm diameter) of tissue were rapidly frozen by "slamming" onto a metal mirror freezing device, a CF100 (a polished copper block cooled with liquid nitrogen) contained within a vacuum chamber to prevent ice formation. The gill pieces were removed subsequently into liquid nitrogen and transferred to the molecular distillation dryer (MDD). An ultra-high vacuum system and a computer controlled slow warming cycle allows the MDD to dry

vitreous ice at -150°C, which is below the vitreous/cubic recrystallisation temperature, so no ice damage artefacts are produced. Once dry, a fixative vapour was introduced into the vacuum. All unreacted fixative vapour was then pumped out of the chamber. Samples were removed, rapidly infiltrated in Spurr's resin, embedded under vacuum and polymerised at 60°C for 12h.

Method B: Metal mirror cryofixation followed by freeze substitution

This technique was carried out in the Electron Microscopy Unit, University of Plymouth. Small pieces (1mm diameter) of gill tissue were rapidly cryofixed by "slamming" onto a metal mirror (a polished copper block cooled with liquid nitrogen) as in the previous method (Method A). In addition, some of the tissue was lightly fixed in 1% glutaraldehyde for 5 mins and cryoprotected in 30% glycerol, prior to cryofixation. Following cryofixation, all tissue was transferred to liquid nitrogen and subsequently into methanol (at -80°C) in the processing chamber of a Reichert AFS freeze substitution unit. Freeze-substitution allows the slow replacement of ice, formed in the tissue during cryofixation, at low temperatures with an anhydrous, organic solvent. The tissue was infiltrated gradually with Lowicryl HM20 resin following a standard low temperature program (over 80h at -80°C and -50°C). Final tissue blocks were polymerised under U.V. light for 48h at -50°C and -30°C.

Method C: Plunge cooling followed by freeze drying and vacuum infiltration

This technique was performed at the Marine Biological Association, Citadel Hill, Plymouth. Small pieces (1mm diameter) of gill tissue were placed on a mounting pin, plunged rapidly into magnetically stirred liquid ethane at a temperature of -180°C (Ryan & Purse, 1984; Ryan *et al.*, 1987, 1990; Ryan, 1992) and transferred to liquid nitrogen. The tissue was subsequently freeze dried. Freeze drying is a well-established, versatile

technique for preparing tissue blocks; however, it is not always appropriate for elemental localization processes, as ice crystal damage often results, ion localization is often disrupted and the translocation of solutes during the subsequent resin infiltration phase causes potential artefact problems (Morgan, 1985). Gill tissue was freeze dried at -80°C, transferred rapidly to the vacuum chamber and embedded in Spurr's resin using vacuum infiltration techniques in an attempt to resolve these problems.

8.2.4 Transmission electron microscopy

Ultra-thin sections (0.1μm), cut with a glass knife using a Reichert ultramicrotome, were stained in uranyl acetate and Reynolds lead citrate. Sections were mounted on copper grids and examined with a Jeol 1200 transmission electron microscope, operated at 80kV.

8.2.5 X-ray microanalysis

X-ray microanalysis, using the transmission electron microscope, is a useful tool for biologists (Hall, 1974). However, X-ray microanalysis of biological tissue is still a developing and challenging field of research. Energy dispersive analysis (EDS) was utilised in this study as, unlike wavelength dispersive analysis (WDS), it can display simultaneously all mid-range (1-20keV) X-rays collected during a single analysis period. One particular problem with the analysis of biological material is that the sections have to remain unstained (to eliminate loss of ions to the staining medium) and have to be thicker than the ultra-thin sections prepared for transmission electron microscopy (to make X-ray detection simpler, especially if only small quantities of elements are expected). Therefore, maintenance of the structural integrity of the tissue throughout the preparation phases is extremely important and the validity of any data rests heavily on the success of sample preparation. Since electrons and X-rays are absorbed strongly by

air molecules, all samples are under high vacuum inside the operational electron microprobe. Tissue water of specimens used for X-ray microanalytical purposes must either be withdrawn (by chemical or physical methods) or immobilized (by freezing). Thus, dry, semi-thin sections (1μm) were cut with a glass knife using a Reichert ultramicrotome and collected on folding gold grids for X-ray microanalysis (gold grids were chosen since the X-ray peaks for gold are produced in the 9.5-11.5kEV region, away from the majority of X-ray peaks likely to be produced from biological tissue, thus, reducing peak interference). Unstained sections were examined with a Jeol CX transmission electron microscope, operated at 200kV, with a Link 860 energy dispersive X-ray analysis unit attached. To optimise count rates, specimens were tilted at a 35° angle towards the detector. Using the stationary spot of the scanning transmission system (STEM), X-ray micro-analytical spectra of various gill tissue were produced.

8.3 Results

Comparison of the ultrastructural integrity of the gill tissue following the three preparation methods showed that the appearance of ultra-thin (0.1μm) and semi-thin (1μm) sections of gill tissue varied in quality depending on the preparation technique used; 1μm sections were of a much poorer quality than 0.1μm sections. The ultrastructure of gill material prepared using Method A was poor. Ultra-thin (0.1μm) sections revealed distorted gill lamellae, the gill epithelial layer was barely visible and cells usually found within the haemolymph space (nephrocytes, glycocytes and haemocytes) were not recognisable. When 1μm sections of gill tissue prepared using Method A were examined, identification of gill cells proved to be impossible. Method B resulted in equally poor cell morphology; ultra-thin sections again contained distorted gill lamellae with little intralamellar content. However, when a cryoprotectant was used prior to cryofixation in Method B, the ultrastructural integrity of the gill lamellae was

retained and it was relatively easy to identify features such as the epithelial layer, gill cuticle and haemocytes circulating in the haemolymph space, in 0.1μm and 1μm sections. Method C also produced gill material of a morphological quality suitable for cell identification purposes. The ultrastructure of gill tissue prepared in this way did not match that observed in the cryoprotected material from Method B; however, individual cells were identifiable in both 0.1μm and 1μm sections.

Standard X-ray microanalytical spectra were acquired initially from an epithelial cell nucleus in the plunge-cooled gill material (Method C), to check elemental peak production. The nucleus produced a characteristic phosphorus peak, along with minor peaks for sodium, sulphur, potassium and calcium (Fig. 8.2a); a gold peak (from the grid bars) and a large chlorine peak (from Spurr's resin) were also produced (Fig. 8.2a). X-ray microanalytical spectra for each embedding resin revealed that Spurr's resin (Fig. 8.2b) had a different elemental composition to Lowicryl HM20 (Fig. 8.2c). Small copper peaks were also observed in Figures 8.2a & b and were probably derived from the microscope.

Figure 8.3 shows the typical ultrastructural appearance of granules within a smallgranule haemocyte in an ultra-thin (0.1μm) section of cryoprotected gill tissue prepared using Method B. It was evident from Figure 8.3 that two forms of granule were visible in the haemocyte cell cytoplasm. Some granules were completely amorphous and uniformly electron-dense (Fig. 8.3a), whilst others contained localised areas of intense electron density, often appearing as a rod-shaped crystalline structure in the middle of the granule (Fig. 8.3b & c). Haemocytes contained either the amorphous granule or a mixture of amorphous and structured granule types. Unfortunately, differentiation between granule forms was not possible in the 1μm sections used for X-ray microanalytical purposes, so specific spectra for each granule type could not be produced.

Analysis of the granules of the haemocyte in copper-exposed gills, processed by either Method B (with a cryoprotectant) or C, produced two different types of spectra. Firstly, many spectra contained peaks for sulphur and copper, with occasionally other small peaks, such as calcium present (Fig. 8.4a). Secondly, spectra with sulphur and copper peaks, and also relatively large phosphorus and calcium peaks, were produced (Figs 8.4b & c). Figure 8.4c shows that a small potassium peak was also produced (the origin of which was not known). Haemocyte granules from material prepared using Method C appeared to contain lower elemental peaks than cryofixed gill tissue. This difference was a result of the relatively large chlorine peak produced from Spurr's resin halting acquisition once 1000 X-ray counts had been acquired. The elemental composition of the granules was variable and not all contained large amounts of copper. In fact, spectra of some granules showed copper peaks no greater than background levels. Generally, X-ray microanalytical spectra showed that copper was always associated with sulphur in the granules and was also found frequently with phosphorus and calcium; this was true for gill tissue prepared by either method.

Since the ultrastructural integrity of gill tissue prepared using Methods B (with a cryoprotectant) and C was of a good quality, other areas of the gill were also analyzed. X-ray microanalytical spectra were produced from the epithelial cells of the gill processed using Method C. The elemental composition of the epithelial cells differed from that observed in the haemocyte granules. Peaks for sodium, magnesium, bromine, silicon, phosphorus and potassium were present in the epithelial cell (Fig. 8.5a). Small peaks for copper were also observed frequently, although these were often no greater than background levels (c.f. Figs 8.2b & 8.5a). The gill cuticle was also analyzed repeatedly and produced some interesting X-ray microanalytical spectra. The algal layer on the epicuticle of gill tissue prepared using Methods B (with a cryoprotectant) and C produced elemental peaks for bromine (Fig 8.5b & c). Bromine was present in

combination with sulphur, iron, copper and probably iodine and calcium in smaller amounts. The gold peaks were high in Figures 8.5b & c compared to other spectra, as the spectrum was produced from an area close to the grid bars.

8.4 Discussion

The provision of adequate structural information to enable cell identification in unstained, 1μm sections of gill tissue is difficult, but essential, in order to ensure identification and analysis of the correct cell type. In addition, X-ray microanalysis of metals in tissues requires special preparation techniques to ensure the retention of intracellular ions in the tissue. It was evident from this study that the different preparation techniques for X-ray microanalysis produced gill tissue of variable ultrastructural quality. The gill is a delicate organ, so retention of its ultrastructure is not always achieved during cryofixation techniques and mechanical disruption of the tissue is common. Some areas of the gill material, close to the "slammed" surface, were highly disrupted in tissue blocks from some preparations. At first, this was thought to be an artefact of the "slamming" technique, disrupting the outermost surface of the piece of gill tissue. However, serial sections showed that ultrastructural disruption was observed throughout the entire tissue block and not solely near the surface "slammed" directly onto the metal mirror. Overall, the most successful preparatory method was the metal mirror cryofixation of gill tissue, followed by low temperature freeze substitution. However, this technique produced material of an adequate structural quality for cell identification purposes, only when a cryoprotectant (in this case 30% glycerol) was used. Ultrastructural preservation was improved and ice crystal damage reduced when gill tissue was infiltrated with a cryoprotectant prior to freezing. It has been suggested that low molecular weight cryoprotectants, such as glycerol, are unsuitable for microanalytical purposes because they readily enter cells and cause ionic dislocation

(Morgan, 1985). There was no evidence for this in the present study. In fact, X-ray microanalytical results showed that the elemental composition of gill cells and organelles did not vary between gill tissue processed using Method B (with a cryoprotectant) and gill tissue processed using Method C (without a cryoprotectant). However, it was evident when comparing preparatory methods that the cryoprotectant enhanced greatly the structural integrity of the gill tissue, without the loss of ions during cryofixation techniques.

Metal mirror cryofixation (without a cryoprotectant) followed by either molecular distillation drying or freeze substitution produced gill tissue of poor ultrastructural quality, with disrupted gill lamellae and little intralamellar content. Structural disruption could have resulted from an unsuitable choice of "slamming" velocity during cryofixation of the tissue. The ultrastructure of the gill lamellae suggested the "slamming" velocity was probably too great and too much mechanical strength was conveyed to the tissue. Metal mirror cryofixation (without cryoprotection) may, therefore, produce gill tissue of a good ultrastructural quality in future experiments if the "slamming" velocity was modified. It is evident much experimentation with cryofixation techniques needs to be completed before tissue ultrastructural integrity is obtained using these methods. Unfortunately, time and resources did not permit a lengthy investigation into the effect of "slamming" velocity on the ultrastructural integrity of the *Carcinus maenas* gill lamellae. Tissue preparation prior to cryofixation must also be as simple as possible to ensure the specimen can be dissected, mounted and cryofixed with minimal delay; any delays may have caused ultrastructural damage and invoked problems such as cell shrinkage. The size of the tissue block may have also affected the ultrastructural integrity of the gill lamellae. The tissue must be of a minimal size to facilitate the fastest cooling rate, otherwise only the surface layer can be cooled fast enough to avoid intrusive ice crystal damage (Ryan, 1992). Avoiding ice crystal

damage was particularly important in this study as, in many instances, serial sections of the tissue block had to be cut before haemocytes were sited in the intralamellar space.

X-ray microanalytical spectra of haemocyte granules in the gill of *Carcinus maenas*, following copper exposure, showed that copper was always present in association with sulphur and, in some cases, with phosphorus and calcium. The variability in granule elemental composition was large, as far as the relative amounts of sulphur, phosphorus, calcium and copper were concerned; in some cases only small amounts of copper were recorded. Ultrastructural observations on the granular haemocytes revealed two forms of granule in the cytoplasm. Amorphous, unstructured granules and granules with a central rod-shaped, electron-dense area were identified. Differences in granule structure could be explained simply by variation in the plane of sectioning. Alternatively, the structural differences may imply that two distinct morphological types of granule were present within the haemocyte cytoplasm, possibly explaining, in part, the variability in the X-ray microanalytical spectra obtained. As stated previously, it was not possible to distinguish between these two granule types in the unstained, 1.0μm gill sections, therefore spectra for individual granule forms could not be produced. The different granule forms were not observed in the haemocytes in Chapters 4 to 7, when only the amorphous granule type was evident. This may suggest that the lengthy chemical fixation methods used in electron microscopy destroy the crystalline structure of certain granule types.

There is an extensive literature on the structure, composition and possible functions of the various types of intracellular granules in the organs of invertebrates (for reviews see Simkiss, 1976; Coombs & George, 1978; Icely & Nott, 1980; Mason & Nott, 1981; Brown, 1982). Intracellular granules have been identified in a wide range of invertebrates (*Asellus*: Brown, 1976; Coombs & George, 1978; *Balanus*: Walker, 1977;

Carcinus: Hopkin & Nott, 1979; Chassard-Bouchaud, 1982, 1983; *Corophium*: Icely & Nott, 1980; *Crangon*: Djangmah, 1970; *Littorina*: Mason *et al.*, 1984; Nott & Langston, 1989; *Mytilus*: George & Pirie, 1980; Marshall & Talbot, 1979; Chassard-Bouchaud *et al.*, 1989; *Ostrea*: George *et al.*, 1978; *Penaeus*: Al-Mohanna & Nott, 1982). The segregation of metals into granules of specific composition has been discussed extensively. It has been suggested that there are three principal granule types: copper-containing granules, which also contain sulphur and perhaps traces of calcium; calcium-containing granules, which may be of two types (one of high purity and the other with calcium in association with manganese, magnesium, phosphorus and perhaps other metals such as zinc, cadmium, lead and iron) and finally, iron-containing granules (Brown, 1982). In addition to composition, granules have also been classified based on their morphology. Copper granules are usually homogenous, spherical structures displaying no concentric structuring (Brown, 1982). Some variation in copper granules has been observed and, although they all tend to have a uniform electron density, some appear rounded, with multivesicular bodies (Icely and Nott, 1980) whilst others contain more granular inclusions (Brown, 1976). Thus, the haemocyte granules in this study may be of the copper granule type but with two slightly different morphological forms. Calcium granules have also been ascribed to two major classes, either sculptured, skeletal structures or spherical types with either dense, homogenous inclusions, needle-like microcrystals or layers of concretions (Simkiss, 1976).

Many studies have shown that the division of granule types based on composition or morphology is an oversimplification and not always applicable to each invertebrate species. For example, Nott & Langston (1989) found that the granules in the digestive epithelium of *Littorina littorea* contained cadmium associated with sulphur, and suggested that there could be an interaction between two different systems for binding metals, a sulphur and a phosphate system. Al-Mohanna & Nott (1982) found two

different types of granule in the hepatopancreas of *Penaeus semisculatus*, a homogenous dense granule in which metals were associated mostly with sulphur and a second type of granule containing concentric layers in which metals associated mainly with phosphorus. Walker (1977) also found zinc granules in *Semibalanus (Balanus) balanoides* made of concentric layers containing peaks for phosphorus and zinc, and copper granules that were homogenous in appearance and contained copper in association with sulphur. Chassard-Bouchaud *et al.* (1989) found that the granules of *Mytilus edulis* exposed to chromium, contained chromium in association with phosphorus and sulphur. Chassard-Bouchaud (1982) reported previously that granules in the digestive gland of *Carcinus maenas* contained cadmium in coexistence with sulphur, zinc and copper. Thus, the number of possible forms in which heavy metals can accumulate in the tissues of invertebrates is large and, for any one particular metal, it appears that more than one storage system may operate. The granule composition and form depends on the species of organism, the organ studied and the type of heavy metal to which the organism is exposed. Morphologically, it is generally agreed that granules can either form as amorphous, uniformly electron-dense structures or as structured, granular deposits. The composition and relative amounts of sulphur, phosphorus, calcium and heavy metal species varying in each granule.

Many studies have concentrated on the formation of granular deposits in organs such as the hepatopancreas and digestive gland, with little work on the gill or the granular haemocytes circulating in the gill. Some analysis of granular amoebocytes in invertebrate tissues following exposure to heavy metal has been carried out (George *et al.*, 1978; Marshall & Talbot, 1979; George & Pirie, 1980; Chassard-Bouchaud *et al.*, 1989, 1992). In agreement with present results, heavy metals (chromium, cadmium, copper and zinc) have been found associated mainly with sulphur and phosphorus (George *et al.*, 1978; Marshall & Talbot, 1979; George & Pirie, 1980; Chassard-

Bouchaud *et al.*, 1989, 1992). It has been suggested that sulphur in the granules is derived from sulphydryl rich metallothionein proteins; it is well known that soft, acid metals, such as copper, are frequently found in association with this protein type (Simkiss & Mason, 1983; Taylor & Simkiss, 1984; Langston & Zhou, 1986). Whether these proteins are present naturally in the granules, synthesised and form into granular deposits prior to copper immobilization, or combine with copper outside the haemocyte and are then phagocytosed into the haemocyte with the subsequent formation of granules, is not known. Ultrastructural observations on the granules in haemocytes of *Carcinus maenas* from cryofixed material showed that two granule forms were present. However, structured granules containing concentric whorls or needle-like material were not present in the haemocytes. Chapters 4 to 7 showed that nephrocytes in the gill of *C. maenas* contained needle-like microcrystal material in untreated and copper-exposed gills, thus, future work should include the X-ray microanalysis of this cell type, as well as the haemocytes, as changes in nephrocyte abundance and distribution were also observed in Chapters 6 & 7 following copper exposure. Unfortunately, nephrocytes were not easily identifiable in unstained, 1.0μm gill sections, and isolation and analysis of the needle-like microcrystals or ovoid elements seen in the central vacuole was not possible. Mason *et al.* (1984) did find that the kidney of *Littorina littorea* from Restronguet Creek contained nephrocytes with vacuoles which produced X-ray microanalytical spectra with peaks for iron, associated with minor peaks for calcium, sulphur, chlorine and phosphorus. Haemocytes and nephrocytes may, therefore, contain granular deposits with differing elemental compositions and may be involved jointly in the immobilization of heavy metal in the gill of *C. maenas*. Whether each cell is involved in the storage of all heavy metals, or different heavy metal elements are contained within separate cell types, is not known.

As stated previously, in gills from untreated crabs granular haemocytes could not be

analyzed successfully during this study. This failure was related to the production of material of a poor ultrastructural quality, and may also have been exacerbated by incorrect orientation of the gill tissue in the resin blocks and the absence of haemocytes in the gill lamellae of those tissue blocks successfully processed. It is pertinent to note that at 35‰ seawater, small and large-granule haemocytes were much fewer in untreated than copper-exposed gills of *Carcinus maenas* (c.f. Chapters 4 & 6). The very small pieces of tissue embedded for X-ray microanalysis purposes reduced the chance of finding lamellae containing granular haemocytes, and it proved impossible to find any granular haemocytes in gill lamellae that remained structurally intact after processing. This lack of comparison, unfortunately, means that quantitative elemental measurements cannot be made. Thus, it cannot be stated conclusively that haemocyte granules from copper-exposed gills contained elevated copper concentrations compared with untreated gills. It can only be said that granules from copper-exposed crabs did contain copper, however, whether this was present already prior to copper exposure or was derived from the copper-contaminated seawater is not known. Since the haemolymph of *C. maenas* contains haemocyanin, granules containing copper (and sulphur) may simply represent degradation products of haemocyanin, or alternatively a storage form of copper required for haemocyanin re-synthesis.

X-ray microanalytical spectra were also produced for the epithelial cells of the gill lamellae following exposure to copper. It was evident that these cells had a different elemental composition from the granular haemocytes, epithelial cells contained peaks for sodium, magnesium, bromine, silicon, phosphorus, sulphur, potassium and chlorine. In 1µm sections, chief cells could not be separated, based on ultrastructure, from striated cells. However, the spectra obtained from the epithelial cells did appear to reflect the increased ionic regulatory capabilities of this layer, compared with the granular haemocytes, with peaks for sodium, chlorine, magnesium and potassium clearly present.

Copper, also present in the epithelial cells, may have resulted from uptake via the epithelial layer into the gill from the seawater; however, in many cases copper levels in the epithelial cells were not greater than background levels in the resin. Bromine peaks in the epithelial layer were slightly unexpected but may be derived from the cuticle or algal layer, which is situated in close proximity to the epithelial layer. The diameter of the volume of the specimen sampled by the electron beam is usually larger than the beam diameter, due to the diffusion of the incident electron beam in the sample.

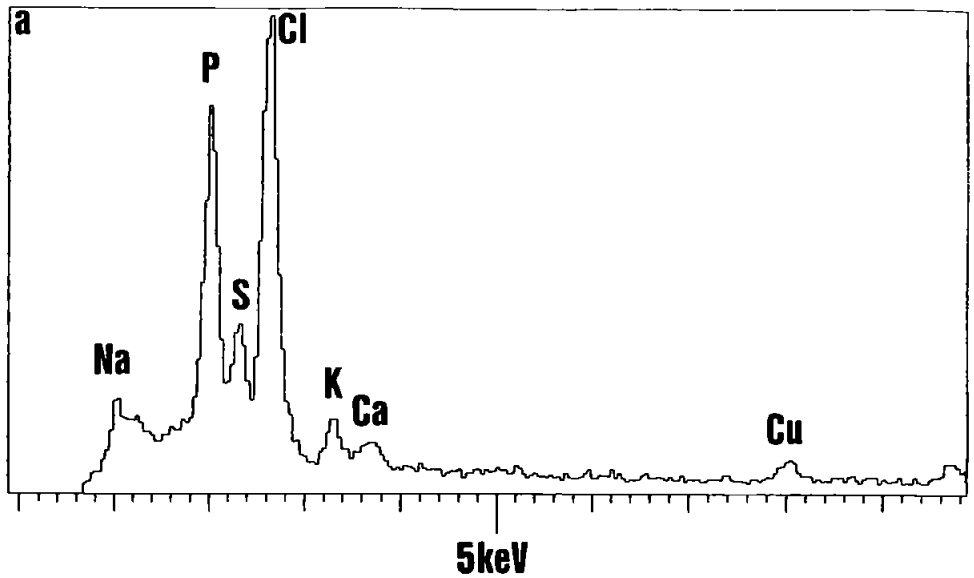
The algal layer, on the outside of the cuticle, produced X-ray microanalytical spectra containing major peaks for bromine, in the majority of cases in combination with sulphur, iron, copper and (probably) iodine and calcium. The presence of copper may imply that the cuticle is also involved in heavy metal sequestration in some way, however, it is highly likely that copper was microscopically derived as levels were not much greater than background levels observed in the resin. Bromine, iodine and sulphur peaks have been found previously in X-ray microanalytical spectra of red (Faulkner, 1977; Hofsten *et al.*, 1977; Pedersén *et al.*, 1979, 1980, 1981; Roomans, 1979; Westlund *et al.*, 1981; Codomier *et al.*, 1983) and brown (Pedersén & Roomans, 1983) algae. These analyses located bromine in the cell wall and chloroplasts. It has been suggested that bromine is present as bromophenol. Bromophenol is thought to be either a waste product that polymerizes in the cell wall (Pedersén *et al.*, 1979, 1980) or involved in the regulation of endo and epiphytes, since some are toxic to several unicellular marine algae (McLachlan & Craigie, 1966). Since algae are particularly effective concentrators of bromine and iodine, the presence of these elements confirms the layer on the outside of the epicuticle as algal material. Previous studies have revealed marked differences in the distribution of bromine compounds between different algal species. For example, Pedersén & Roomans (1983) suggested that in *Laminaria spp.* differences in distribution

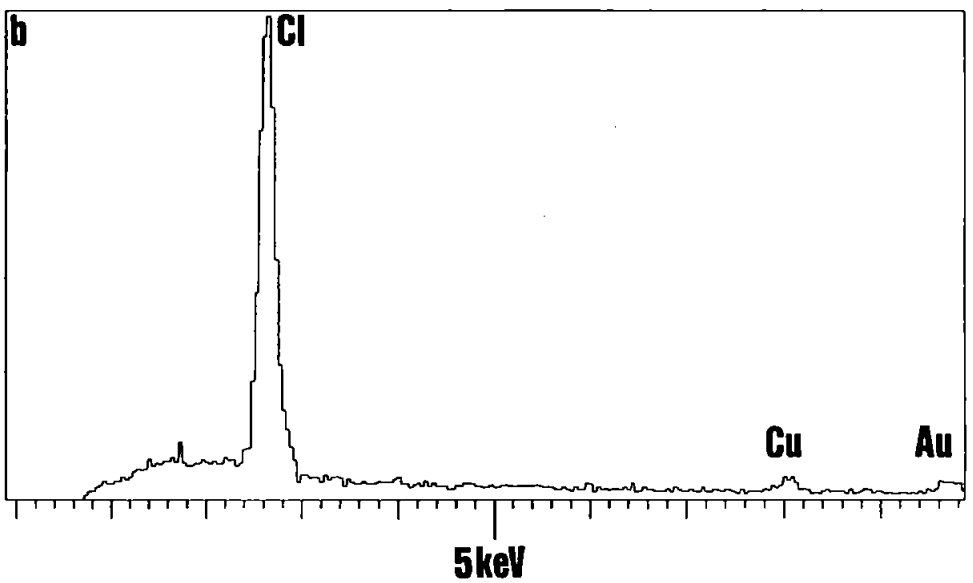
may be due to different mechanisms of accumulation of bromine and iodine, or differences in uptake, metabolism and transport of these elements. In future work it may be possible to identify the algal cells more precisely by using comparative X-ray microanalysis between several algal species.

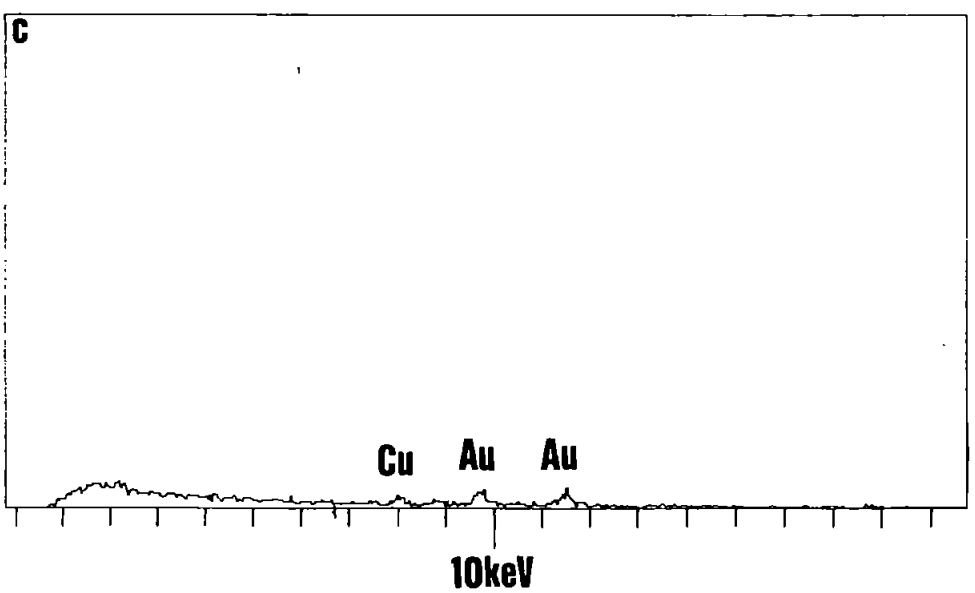
Although only a limited amount of X-ray microanalytical work has been completed in this study, it is evident that there is great scope for future work on the analysis of heavy metals in crustacean gill haemocytes, and possibly nephrocytes, following exposure to sublethal concentrations of trace metal. Intracellular granules have been considered previously as monitors of marine metal contamination (Walker *et al.*, 1975a,b; Carmichael *et al.*, 1979), although a greater understanding of the variation in chemical composition of haemocyte granules, and their capacity for loading and unloading different metals, is needed before this granule type could be used as a heavy metal monitoring system. If this is to be achieved, improvements in the rapid cooling of delicate biological specimens for X-ray microanalytical purposes is necessary. Until recently, the use of cryoprotectants was considered the only possibility to improve the preservation of tissue during cryofixation techniques (Skaer, 1982). More recent developments, however, include the use of microwaves to momentarily disrupt the transitory lattice organisation of water molecules immediately prior to freezing (Hanyu *et al.*, 1992). Another possibility for low level heavy metal detection in the gill includes secondary ion mass spectrometry (SIMS), which has a greater sensitivity than X-ray microanalysis and could be of greater use when observing the uptake of heavy metals into the gill cells when only sublethal exposure concentrations are used. X-ray mapping of elements is also a useful technique for interpreting unstained sections with poor contrast. For example, nuclei can be identified by the arrangement of phosphorus-rich chromatin, even though they may not be recognisable as discrete organelles under the microscope because of poor contrast (Hopkin, 1989).

Whether haemocytes are involved in the phagocytic clearance of copper from the gill is still not conclusively answered from the present X-ray microanalytical study. This work has shown, however, that granules do have a composition, based on previous literature, with the potential for heavy metal uptake. In ecotoxicological studies, it is clearly important to understand the intracellular distribution of heavy metals, and the involvement of detoxification systems, in order to interpret the toxicological consequences of tissue burdens. If on entry into the gill, heavy metals are stored immediately into an immobile form tissue burdens may not accurately reflect the amount of available heavy metal in the gill, capable of directly causing ultrastructural disruption. It is evident from previous X-ray microanalytical studies that not all metals are processed biologically in the same way. Within a species, age, sex, stage of breeding and feeding cycles and habitat can cause marked individual variation in the processing of heavy metals (Al-Mohanna & Nott, 1982). In addition, tissues within a single organism, and even granules within a single cell type, can accumulate metals to varying degrees. Thus, to create a more complete picture of the effects of copper on the gill of *Carcinus maenas*, a better understanding of the uptake, storage and detoxification systems, using X-ray microanalysis techniques, operating in the crab needs to be obtained.

Figure 8.2. X-ray microanalytical spectra produced at 200kV in the transmission electron microscope. Full vertical scale = 1000 X-ray counts; horizontal scale = X-ray energy. **(a)** Nucleus of an epithelial cell from gill tissue prepared using Method C: Plunge cooling followed by freeze drying and vacuum infiltration. **(b)** Spurr's resin. **(c)** Lowicryl HM20 resin.


a
Cl
P
S
Na
K
Ca
Cu
5keV


b
Cl
Cu
Au
5keV


c
Cu
Au
Au
10keV

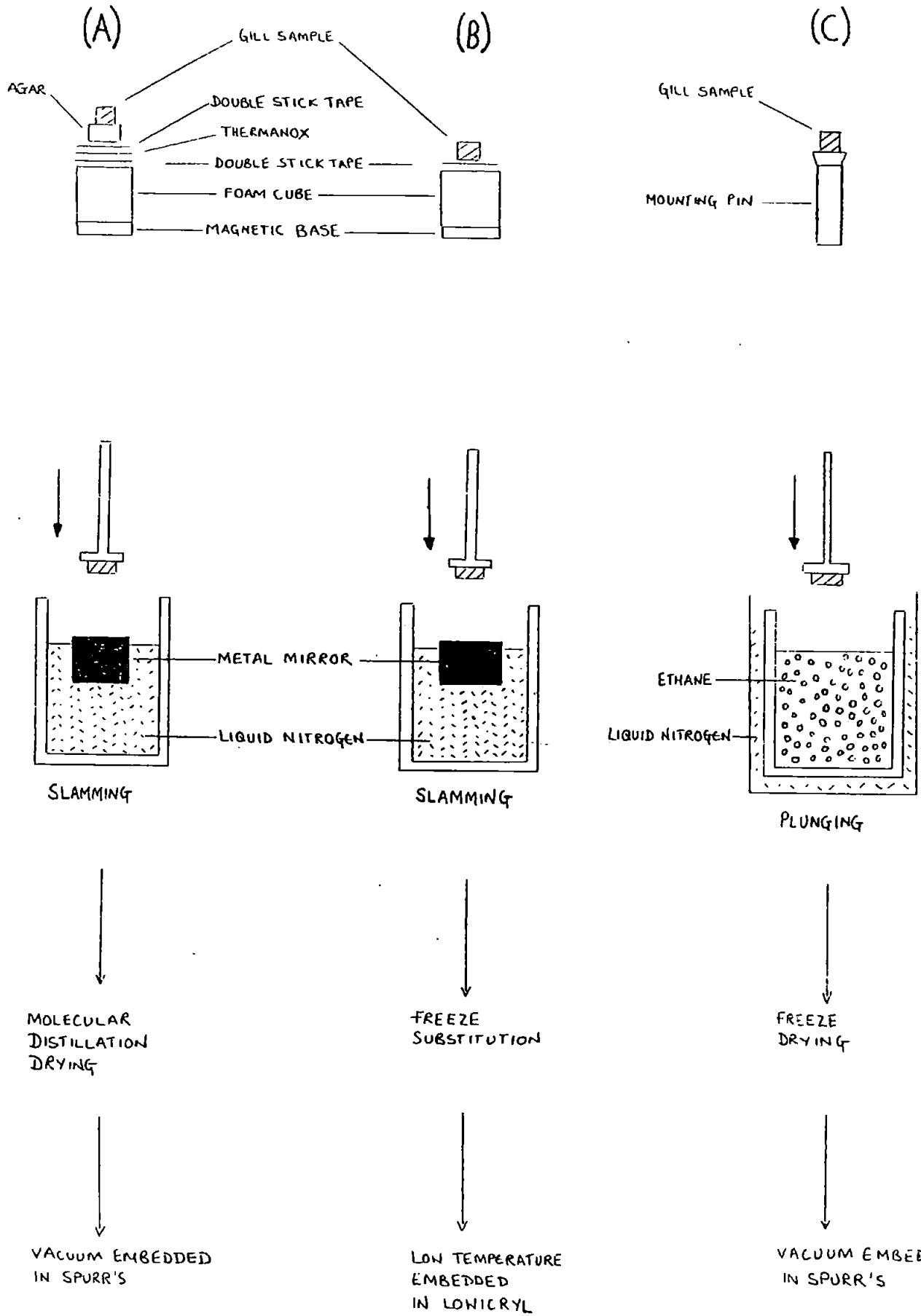

(A)
GILL SAMPLE
(B)
(C)
AGAR
DOUBLE STICK TAPE
THERMANOX
DOUBLE STICK TAPE
FOAM CUBE
MAGNETIC BASE
GILL SAMPLE
MOUNTING PIN
METAL MIRROR
LIQUID NITROGEN
SLAMMING
SLAMMING
ETHANE
LIQUID NITROGEN
PLUNGING
MOLECULAR
DISTILLATION
DRYING
FREEZE
SUBSTITUTION
FREEZE
DRYING
VACUUM EMBEDDED
IN SPURR'S
LOW TEMPERATURE
EMBEDDED
IN LONICRYL
VACUUM EMBEDD
IN SPURR'S

Figure 8.2. X-ray microanalytical spectra produced at 200kV in the transmission electron microscope. Full vertical scale = 1000 X-ray counts; horizontal scale = X-ray energy. **(a)** Nucleus of an epithelial cell from gill tissue prepared using Method C: Plunge cooling followed by freeze drying and vacuum infiltration. **(b)** Spurr's resin. **(c)** Lowicryl HM20 resin.

Figure 8.3. Transmission electron micrographs of the granules in the haemocytes, found circulating in the posterior gill of *Carcinus maenas*; tissue prepared using Method B: Metal mirror cryofixation followed by freeze substitution (with a cryoprotectant). **(a)** Amorphous granules. **(b)** and **(c)** Structured granules (ER: endoplasmic reticulum; G: glycogen; GR: granule; N: nucleus).

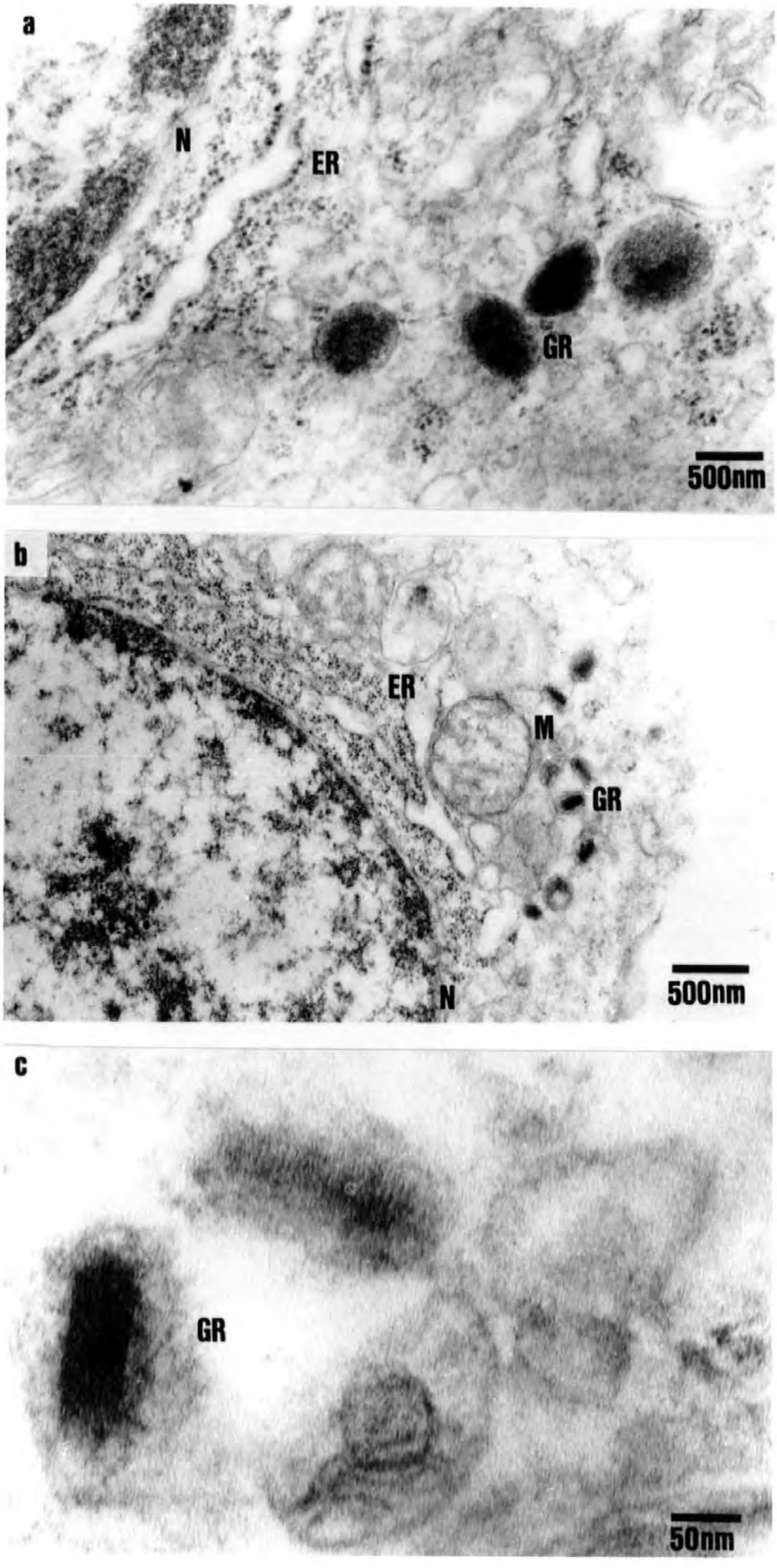
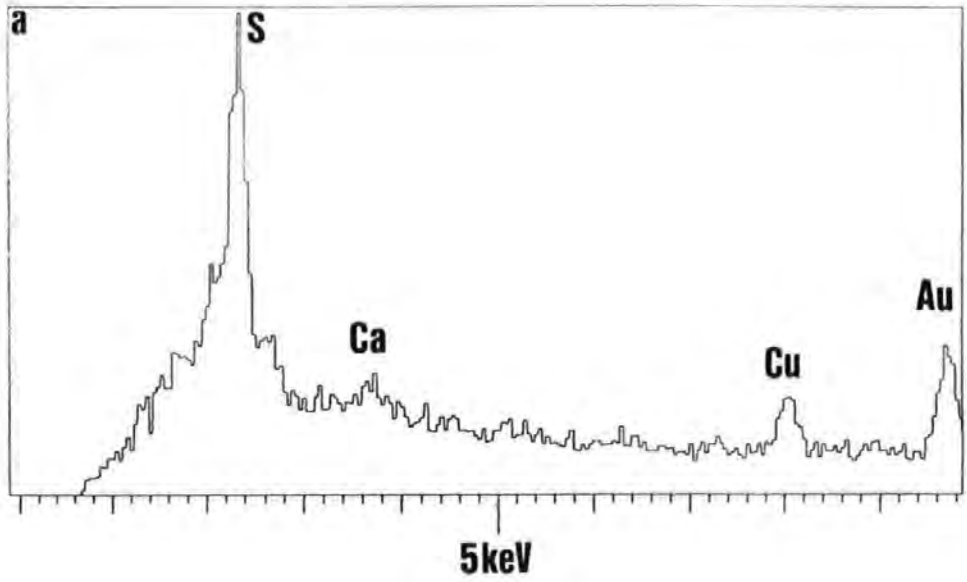
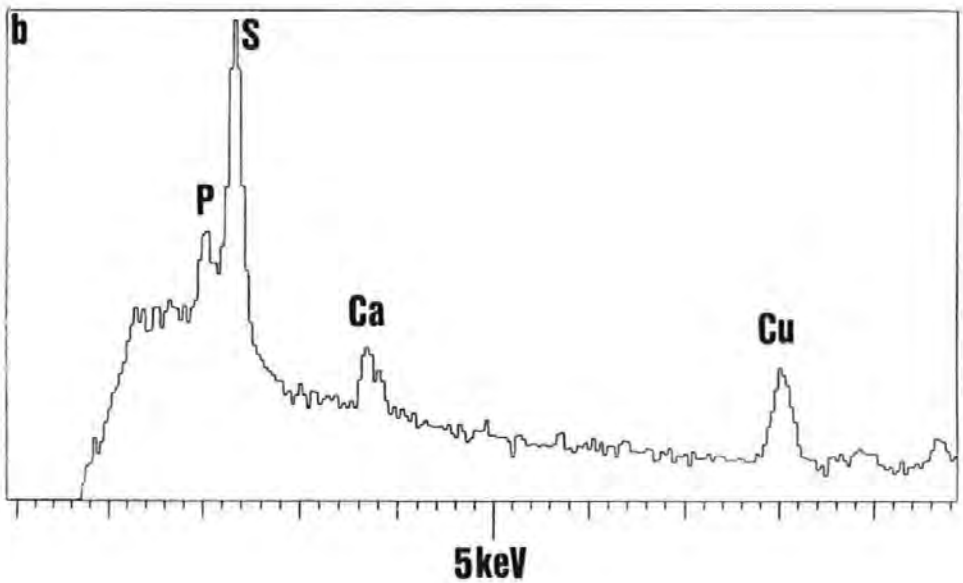

a
N
ER
GR
500nm
b
ER
M
GR
N
500nm
c
GR
50nm

Figure 8.4. X-ray microanalytical spectra produced at 200kV in the transmission electron microscope. Full vertical scale = 1000 X-ray counts; horizontal scale = X-ray energy. **(a)** and **(b)** Haemocyte granules from gill tissue prepared using Method B: Metal mirror cryofixation followed by freeze substitution (with a cryoprotectant). **(c)** Haemocyte granule from gill tissue prepared using Method C: Plunge cooling followed by freeze drying and vacuum infiltration.


a
S
Ca
Cu
Au
5keV


b
S
P
Ca
Cu
5keV

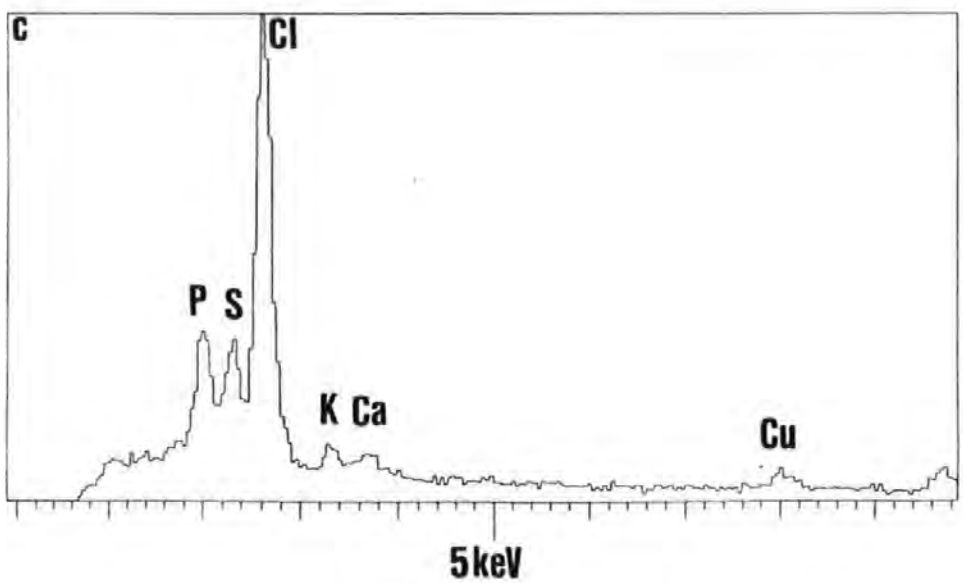
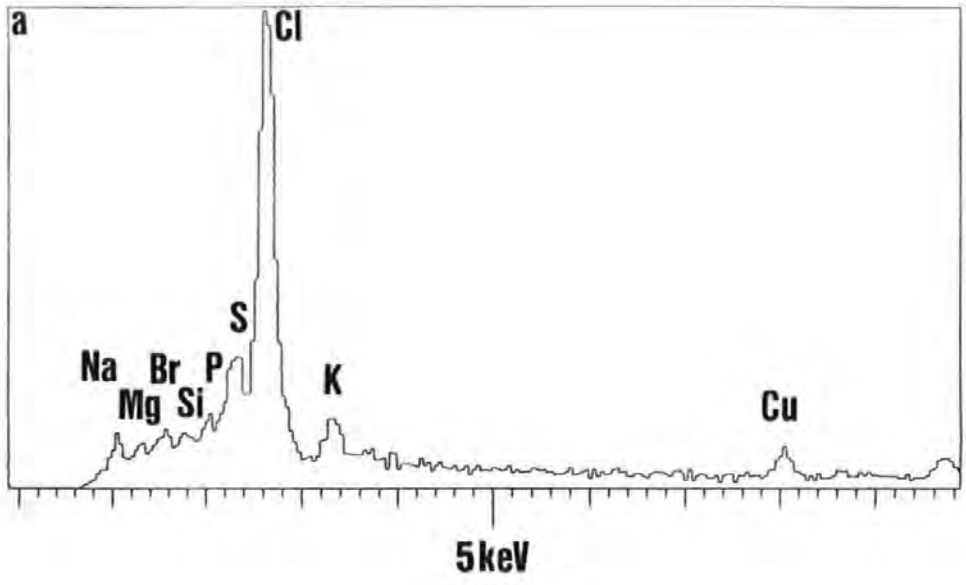
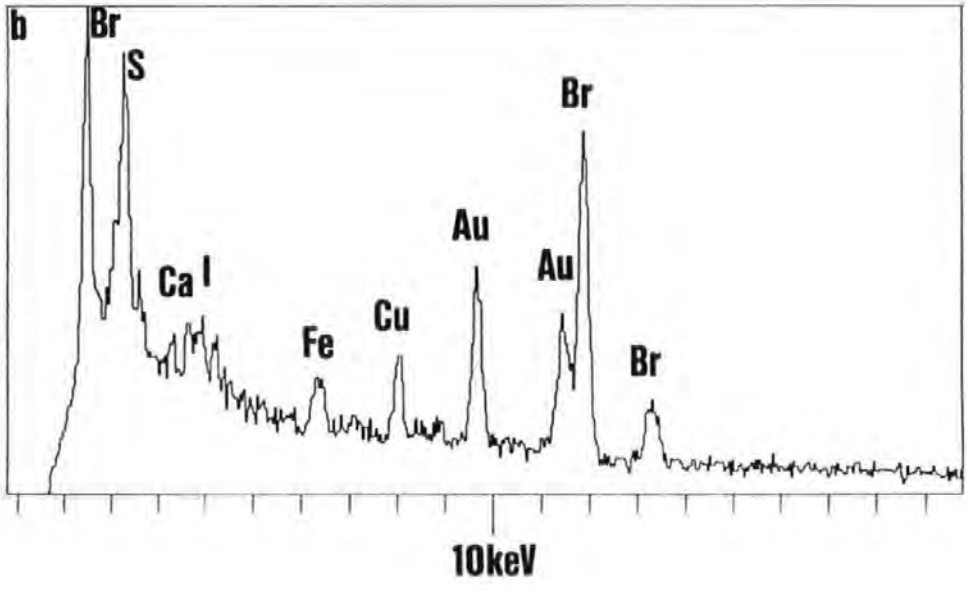
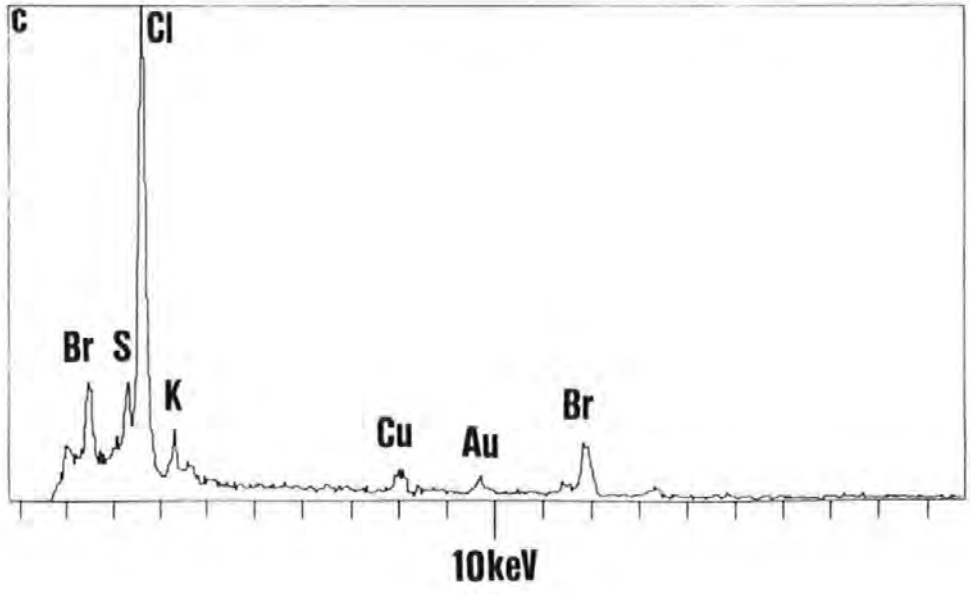

c
Cl
P S
K Ca
Cu
5keV

Figure 8.5. X-ray microanalytical spectra produced at 200kV in the transmission electron microscope. Full vertical scale = 1000 X-ray counts; horizontal scale = X-ray energy. **(a)** Epithelial cell from gill tissue prepared using Method C: Plunge cooling followed by freeze drying and vacuum infiltration. **(b)** Algal layer on the outside of the cuticle of gill tissue prepared using Method B: Metal mirror cryofixation followed by freeze substitution (with a cryoprotectant). **(c)** Algal layer on the outside of the cuticle of gill tissue prepared using Method C: Plunge cooling followed by freeze drying and vacuum infiltration.


a
Cl
S
Na
Br
P
Mg
Si
K
Cu
5keV


b
Br
S
Br
Au
Ca
I
Au
Fe
Cu
Br
10keV


c
Cl
Br
S
K
Br
Cu
Au
10keV

CHAPTER 9

GENERAL DISCUSSION

This study has contributed to the understanding of the effect of sublethal copper concentrations on the gross morphometry and ultrastructure of posterior gill number 8 of *Carcinus maenas*. A better insight into the possible mechanisms underlying the physiological and biochemical disruption in the gill following heavy metal exposure and/or acclimation to low salinity has been gained. The need to assess the effects of sublethal, as well as lethal, concentrations of heavy metal, the merits of moving towards the use of flow-through, and away from the use of static, seawater systems in ecotoxicological studies and the importance of assessing heavy metal effects at more than one salinity regime have also been emphasised in this study.

Primarily, this investigation showed, through image analysis measurements, that the gross morphometry of the gill of *Carcinus maenas* was not affected significantly by exposure to a sublethal copper concentration, except that a significant change in the size of the perimeter of the efferent haemolymph vessel was recorded in the distal gill region. The image analysis study only utilised a narrow size range of crabs and future image analysis studies should encompass a wider size range and a greater number of individuals within each size class to assess whether a change in gill morphometry following copper exposure is dependent on crab size. Marine invertebrates may be more sensitive to copper during moulting, due to the increased uptake of calcium and the subsequent increase in uptake of copper (Spear & Pierce, 1979), and such moulting stages need to be studied. It has also been demonstrated previously that smaller crustaceans contain higher amounts of metal relative to their size than larger individuals after heavy metal exposure (Rainbow & Moore, 1986, for instance). The relative gill surface area of crustaceans usually increases with decreasing body weight (Gray, 1957; Hughes, 1966, 1983; Johnson & Rees, 1988). Thus, since a decrease in gill size leads to a concomitant increase in the surface area to volume ratio, morphometrical considerations also suggest that smaller individuals of a given species would be more

sensitive to heavy metal than larger specimens. Smaller crustaceans may also have a higher oxygen consumption per unit body weight, accompanying the increased growth in these individuals. A higher oxygen consumption may equate to a relatively larger ventilation volume and larger quantities of water passing over the gills, subjecting smaller animals to a greater quantity of water-borne heavy metal over time than larger individuals.

The untreated gill ultrastructure of crabs kept in 35‰ and 10‰ seawater for three weeks was described in Chapters 4 and 5 respectively. Differences in gill cell ultrastructure and distribution were identified and described. The present study has emphasised the need to establish a clear, comprehensive picture of the untreated gill of *Carcinus maenas* before the effect of any environmental stress is investigated. The need to be precise and consistent in the regions sampled cannot be over-stressed, as ultrastructure varies markedly between different gill regions. Further ultrastructural work should also be completed on gill ultrastructural variation with other environmental parameters such as temperature and pH. This study dealt solely with the change in gill ultrastructure between two salinity regimes. An overview of the effects of varying salinity, pH and temperature, along a varied, more extensive, range than used in this study, for instance, on the gill ultrastructure of *C. maenas* (or any other crustacean for that matter) has yet to be produced. Further work also needs to be completed on the rate of epithelial cell turnover in the gills of crustaceans. It was evident from this study that the ultrastructure of the gill cells changes rapidly with a change in salinity. The rate of crustacean gill cell turnover is, as yet, an unexplored area of research. A study into the potential reversibility of ultrastructural change and whether any ultrastructural reversibility is dependent on the initial exposure concentration are also areas of research unresolved.

Gill cell ultrastructural and distributional changes resulted from exposure to copper at

each salinity. The degree of change, however, was greater in the gills of crabs exposed to copper at 35‰ compared to 10‰ seawater. One of the most plausible explanations for this decrease in effect at 10‰ seawater seems to be that there is a change in the apparent water permeability of the crab at the lower salinity. However, this is just one possible explanation, and it must be remembered that other factors such as the rate of urine production and the rate of removal of copper out of the gill may also play a role in the decreased ultrastructural change in the gill at 10‰ seawater.

Since the distribution of epithelial cell types was not uniform throughout the gill, it was not surprising that certain gill regions were altered to a greater extent than others. In the present study, the distal gill region, containing the greatest proportion of chief cells, appeared more ultrastructurally altered by exposure to copper than the proximal or mid gill regions. In addition, it was also shown that significant differences in gill haemolymph vessel size were recorded solely in the distal gill region. Following exposure to copper, nephrocyte and haemocyte (cells predominantly involved in cell defence mechanisms; Table 1) numbers also increased to the greatest extent in the distal gill region. This implies that cells, and the physiological processes ascribed to these cells, in the various gill regions are not uniformly affected by copper exposure. The differences in untreated and copper-exposed gill cell ultrastructure and distribution between different gill regions again emphasise the need to standardise sampling techniques in gill studies.

Chapter 8 showed that copper was found within the granules of the granular haemocytes. However, as stated previously, it could not be proven conclusively from this study that the granular copper was derived directly from the contaminated seawater, as granular haemocytes from untreated crab gills were not analyzed. If the copper present in the granules was derived from the contaminated seawater it could be suggested that granular haemocytes play an active role in either the direct detoxification

of copper or in the transportation of copper away from the gill to other areas of detoxification.

It must be emphasised that this study observed the effects of copper on a single, posterior gill. Future work should include a comparative study of the gross morphometrical and ultrastructural differences between untreated and copper-exposed posterior and anterior gills. It has already been indicated, for example, that the uptake of heavy metals differs between the anterior and posterior gills of *Carcinus maenas* (Clausen *et al.*, 1993). It has also been recognised that there are morphological and ultrastructural differences between the anterior and posterior gills of crabs, which have been correlated with functional differences. The anterior gills predominantly involved in respiratory gas exchange and the posterior gills having predominantly ionoregulatory and osmoregulatory roles (Copeland, 1968; Barra *et al.*, 1983; Gilles & Pequeux, 1985; Compere *et al.*, 1989).

In these investigations, only a single population of crabs was sampled from Portwrinkle, a coastal, unpolluted site in Cornwall. In future studies, it may be of interest to investigate whether similar ultrastructural effects, as observed in the present study, materialise when different populations of crabs are exposed to copper, such as estuarine *Carcinus maenas* populations, and crabs exposed continually to heavy metal pollutants in the field.

This particular investigation observed only the effect of a single heavy metal on the gill structure of *Carcinus maenas*. Future studies should compare the effects of other essential and non-essential heavy metals and assess whether the ultrastructural changes observed in this study were specific for copper or whether uniform changes in gill ultrastructure are observed following exposure to any heavy metal. Although groups of metals share the same metabolic pathways, for instance, when the intracellular distribution of these metals between and within organs and tissues is investigated,

differences are observed (Thurberg *et al.*, 1985). The effects of salinity on the uptake, accumulation and toxicity of heavy metals in marine organisms also often differs between metals (Phillips, 1980; McLusky *et al.*, 1986). Thus, changes in gill ultrastructure in this study cannot be extrapolated to predict the effect of heavy metals on the gill in general.

A major factor complicating studies on the toxicity of heavy metals is the complexity of their aqueous chemistry (Andrew *et al.*, 1977). The present study has shown that ecotoxicologists continually using static and recirculating seawater systems need to consider urgently the chemical behaviour of pollutants within the confines of their experimental vessels (Abel & Axiak, 1991). The differences in nominal and actual seawater copper concentrations when using recirculating seawater systems in this study highlighted this problem. The uptake and toxicity of copper in aquatic organisms is determined largely by the physical and chemical speciation of the metal in the environment, and is thought to be related to the concentration of the free cupric ion in solution (Sunda *et al.*, 1978; Engel & Fowler, 1979; Zamuda & Sunda, 1982; Borgmann, 1983; Florence, 1983; Campbell & Stokes, 1985; Wangersky, 1986; Nugegoda & Rainbow, 1988; Luoma, 1989). The combined effect of salinity change on copper toxicity was observed in this study; however, salinity is not the only environmental variable affecting copper speciation. Other variables such as pH (Fig. 1.1) will affect the speciation of copper in seawater and need to be studied.

It is very difficult to extrapolate from experimental laboratory results to the field situation, as an organism is never exposed to a single metal in the natural environment. Exposure occurs in both particulate and soluble forms; within each of these the organism is exposed to a variety of physicochemical forms of each metal, the accessibility into the organism of each form also probably varying (for a review see Luoma, 1983). It has also been shown that complexation of copper with organic ligands,

evident in the natural seawater environment, but largely absent from experimental seawater systems, decreases the uptake and toxicity of copper (Sunda & Guillard, 1976; Sunda *et al.*, 1978; Knezovich *et al.*, 1981; Zamuda & Sunda, 1982; Ahsanullah & Florence, 1984; Zamuda *et al.*, 1985; Blust *et al.*, 1986; Florence & Stauber, 1986). In addition, the results presented in this study are only applicable to specific concentrations of a specific copper salt. Further work should be completed on monitoring the effects on the gill structure of different types of copper salt and a wider range of sublethal copper concentrations.

In conclusion, this study has shown that sublethal concentrations of copper induce ultrastructural, rather than gross morphological, changes in the gill of *Carcinus maenas*. These ultrastructural changes being dependent on the experimental seawater salinity. In addition, the elemental composition of the gill granular haemocytes has suggested they play an important role in the immobilisation and removal of copper from the gill. The present study has highlighted the potential use of image analysis and X-ray microanalysis techniques in the study of heavy metal effects on gill structure. The scope for encompassing these techniques in future heavy metal investigations is immense. The way forward in developing a better understanding of heavy metal toxicity in marine crustaceans is the use of more sublethal, environmentally realistic, concentrations of heavy metal; administered in flow-through experimental seawater systems; with environmental variables, likely to be experienced in the field situation, encompassed into the experimental regime.

APPENDICES

Appendix I

Programme 1: Analysis of the whole gill

```
10 panel 0,0,80,79,1,14,3"":coltext 44: coltext 33: coltext 1
20 postext 12,20
30 print "Place disc in B drive then press space bar"
40 g$=inkey$:if g$="" then 40
50 test g$=" " then 60 else 40
60 input "What is the name of this gill section?  ",N$
70 open#1 "b:"+n$+".prn"
80 cls
90 binclear 5
100 binclear 2
110 binclear 6
120 qmenu 'image_setup'
130 qmenu 'calibrate'
140 qmenu 'image_setup'
150 acquire 1
160 qmenu 'detect'
170 riasettings "cal_value" k
180 mframe 19 22 470 490
190 iframe 0 0 512 508
200 binerode 1 2 0 25
210 build 1 2
220 BINMOVE 2 5
230 PAUSETEXT 2 "INPUT 5 OUTPUT 6"
240 qmenu 'bin_edit'
250 BINMOVE 6 2
260 PAUSETEXT 2 ""
270 fillholes 2 3
280 BINMOVE 3 5
290 setftrpar "1,2,3,13,4"
300 ftrgrey 0 : measfeat 5 0 0 0 : clraccept
310 acceptxfer 5 4
320 rfeatnum n(1)
330 for g=0 to n(1)-1
340 rfeatres g 1 a(1)
350 rfeatres g 13 b(1)
360 rfeatres g 4 c(1)
370 print #1:k*k*a(1),k*b(1),k*c(1)
380 next g
390 qmenu 'feature_results'
400 close #1
410 panel 0,0,80,79,1,14,3"":coltext 44: coltext 33: coltext 1
420 panel 25,18,30,5,5,4,2""
430 postext 20,29
440 coltext 5:coltext 32:coltext 45
450 coltext 0
460 end
```

Explanation

1-80 A file is opened with the name given in line 60
90-110 Binary images are cleared to accept newly created binary images
120 To allow operator to see the object as it will be acquired
130 To calibrate each pixel in millimetres
150 Image is stored as a grey image 1
160 Detection of the gill section to create a binary image 1
170 k = millimetres/pixel
180 + 190 To set the size of the frames within which the object is measured, it's FCP has to fall into the measure frame. FCP = feature point count which is the 'lowest' pixel on the screen.
200 + 210 Remove 'noise' -small objects
220-260 To allow the operator to tidy up the image if necessary
270 Fill in holes of detected binary image
290-380 Measurement of the area, perimeter and width of the whole gill and storage of the results to disk
400 Close the file

Appendix II

Programme 2: Analysis of the gill sections

```
10 panel 0,0,80,79,1,14,3"":coltext 44: coltext 33: coltext 1
20 postext 12,20
30 print "Place disc in B drive then press space bar"
40 g$=inkey$:if g$="" then 40
50 test g$=" " then 60 else 40
60 input "What is the name of this gill section?  ",N$
70 open#1 "b:"+n$+".prn"
80 cls
90 binclear 5
100 binclear 2
110 binclear 6
120 qmenu 'image_setup'
130 qmenu 'calibrate'
140 qmenu 'image_setup'
150 acquire 1
160 qmenu 'detect'
170 riasettings "cal_value" k
180 mframe 19 22 470 490
190 iframe 0 0 512 508
200 binerode 1 2 0 25
210 build 1 2
220 BINMOVE 2 5
230 PAUSETEXT 2 "INPUT 5 OUTPUT 6"
240 qmenu 'bin_edit'
250 BINMOVE 6 2
260 PAUSETEXT 2 ""
270 fillholes 2 3
280 BINMOVE 3 5
290 setftrpar "1,2,3,13,4"
300 ftrgrey 0 : measfeat 5 0 0 0 : clraccept
310 acceptxfer 5 4
320 rfeatnum n(1)
330 for g=0 to n(1)-1
340 rfeatres g 1 a(1)
350 rfeatres g 13 b(1)
360 rfeatres g 4 c(1)
370 print #1:k*k*a(1),k*b(1),k*c(1)
380 next g
390 qmenu 'feature_results'
400 BINMOVE 2 5
410 PAUSETEXT 2 "INPUT 5 OUTPUT 6"
420 qmenu 'bin_edit'
430 BINMOVE 6 2
440 PAUSETEXT 2 ""
450 fillholes 2 5
460 binx 2 5 5 3 0 0
470 setftrpar "1,2,3,13"
```

```
480 ftrgrey 0 : measfeat 5 0 0 0 : clraccept
490 rfeatnum n(1)
500 for f=0 to n(1)-1
510 rfeatres f 1 d(1)
520 rfeatres f 13 e(1)
530 acceptfeat 1 5 138889
540 acceptxfer 5 6
550 print #1:0,0,0,k*k*d(1),k*e(1)
560 next f
570 close #1
580 QMENU 'FEATURE_RESULTS'
590 panel 0,0,80,79,1,14,3"":coltext 44: coltext 33: coltext 1
600 panel 25,18,30,5,5,4,2""
610 postext 20,29
620 coltext 5:coltext 32:coltext 45
630 coltext 0
640 end
```

Explanation

1-80 A file is opened with the name given in line 60
90-110 Binary images are cleared to accept newly created binary images
120 To allow operator to see the object as it will be acquired
130 To calibrate each pixel in millimetres
150 Image is stored as a grey image 1
160 Detection of the gill section to create a binary image 1
170 k = millimetres/pixel
180 + 190 To set the size of the frames within which the object is measured, it's FCP has to fall into the measure frame. FCP = feature point count which is the 'lowest' pixel on the screen.
200 + 210 Remove 'noise' -small objects
220-260 To allow the operator to tidy up the image if necessary
270 Fill in holes of detected binary image
290-380 Measurement of the area, perimeter and width of the gill section and storage of the results to disk
420 A tidy up
450 + 460 Identify 'holes' ie gill vessels
480-570 Measurement of area and perimeter of gill vessels and save to disk in same file as gill section measurements.
580 Close the file

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