Physiological Ecology of the mud-burrowing shrimp, *Calocaris macandreae* Bell.

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A Thesis submitted for the degree of Doctor of Philosophy to the Faculty of Science at the University of Glasgow

May 1989

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Declaration.

I hereby declare that this thesis represents, except where a note is made to the contrary, work carried out by myself. It has not been previously submitted for any degree.

Stuart J. Anderson

24th May 1989.

To my wife, Susie

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. R.J.A. Atkinson and Dr. A.C. Taylor for their encouragement, constructive criticism and good example throughout the past three years.

This work was carried out at the Zoology Department, University of Glasgow and University Marine Biological Station, Millport. I gratefully acknowledge the facilities provided by Professor R.S. Phillips (Glasgow) and Professor J.A. Allen (Millport). The work was supported by an award from the Science and Engineering Research Council

I would also like to thank the skippers and crew of R.V. 'Aora' and R.V. 'Aplysia', and the diving staff at Millport (P. Lonsdale and K. Cameron) for their help. Cathy McLagan, June McMahon and Liz Denton at Glasgow helped with laboratory work and thesis production.

Many colleagues and friends contributed to both practical work and thought processes; I would especially like to thank Lois Calder, David Donnan, Tim Ferrero, Andrew Hill, Rohan Holt, Malcolm McGilp, Dave Morritt, Thom Nickell, Phil Smith and Chris Smith. A very grateful thanks to Dr. John Spicer, who stimulated much thought (and debate), and who also criticised the thesis.

My parents gave moral and financial support, and early encouragement to an interest in marine biology.

Finally, and most of all, thanks to my wife Susie, for her constant love and forbearance throughout.

ABSTRACT

Several species of thalassinid shrimp are found in the U.K., all of which construct burrows in muddy sediments of the intertidal or sublittoral. The most abundant species in Scottish west coast waters is the axiid ***Calocaris macandreae*** Bell, which burrows in soft mud from 10 to several hundred metres water depth. Previous studies have indicated that oxygen availability within the burrows of other burrowing decapods is limited.

A study was made of ***Calocaris macandreae***, in particular of the respiratory environment of the burrows, the behaviour associated with burrowing and of the respiratory physiology and metabolism of this species. In addition, literature information concerning other Thalassinidea was reviewed.

The structure and distribution of ***Calocaris*** burrows was investigated in the field using diving-based methods. Burrows were mapped and polyester resin-casts were made. Burrows constructed by ***Calocaris*** in sediment columns in laboratory aquaria were also examined. Oxygen tensions in burrows constructed by animals kept in laboratory aquaria were determined, and showed that respiratory conditions in burrows may be permanently and severely hypoxic, with oxygen tensions as low as 15 Torr recorded in the deeper levels of a burrow. The range of burrow forms constructed by thalassinid species was reviewed with regard to possible functions of a burrow.

The behaviour patterns shown by ***Calocaris macandreae*** in laboratory burrows were analysed using video recordings. In general, ***Calocaris*** has a behavioural regime characterised by low levels of activity. Burrow irrigation by pleopod beating was infrequent and of short duration in 'normal' conditions, but increased during experimental

depletion of oxygen tension in the overlying water column.

The branchial morphology of seven thalassinid species (from the U.K.) were compared using scanning electron microscopy. A range of gill formulae and gill anatomy was observed, with a trend towards simplification of the gill formula and a phyllobranchiate gill structure in ***Upogebia*** and ***Callianassa***. In addition, the surface area of the gills of ***Calocaris macandreae*** was determined to be relatively small compared to other, more active decapods.

In view of the hypoxic conditions encountered by ***Calocaris macandreae***, an investigation of respiratory physiology in this species was made. Open and closed respirometry techniques showed that the rate of oxygen consumption by ***Calocaris*** is low compared to that of most other decapods (but similar to those recorded for other thalassinids). However, oxygen consumption is maintained at a constant rate even at very low oxygen tensions (the 'Pc' varied between 10 and 20 Torr). Measurement of heart and scaphognathite rates using an impedance technique showed that hyperventilation is a consistent response to environmental hypoxia, although there was no response of heart rate.

Comparative data from the literature suggest that adaptive responses of the haemolymph oxygen transport system may be a major component of adaptation to hypoxia in Crustacea. The respiratory pigment of *Calocaris macandreae* (and other thalassinids reported in the literature) has a larger molecular weight haemocyanin than those of other Crustacea (as determined by gel filtration), although the sub-unit molecular weight is similar. The concentration of haemocyanin in the haemolymph is comparatively low, resulting in a low oxygen carrying capacity (thought to be related to low activity levels).

The oxygen affinity of the haemocyanin was studied using a diffusion chamber system. The haemocyanin oxygen affinity is exceptionally high in ***Calocaris macandreae*** (P50 as low as 1.8 Torr under *in vivo* conditions), probably resulting in efficient oxygen transport to the tissues even in severe hypoxia. However, there is little evidence for modulation of oxygen affinity. The functional significance of modulation of haemocyanin oxygen affinity in ***Calocaris*** and other decapods is discussed.

A preliminary investigation of carbon dioxide transport and acid-base balance in the haemolymph was made. Although there is no evidence for acid-base fluctuation in the environment of ***Calocaris***, there may be interactions of acid-base regulation with other respiratory processes (for example, hyperventilation in hypoxia).

The metabolic responses of ***Calocaris macandreae*** to prolonged hypoxia and anoxia were studied using enzymatic assays and high performance liquid chromatography. ***Calocaris*** is highly tolerant of anoxia (the LT_{50} was nearly 50 h) and accumulates L-lactate as the major end-product of anaerobic metabolism. Depletion of carbohydrate reserves during anoxia was not expected in this species (in contrast to most other decapods) and was not observed in experiments. Metabolic recovery of ***Calocaris*** from anoxia is relatively slow..

In conclusion, both comparative and functional approaches were used to interpret the ecological physiology of ***Calocaris macandreae***. An integrated suite of behavioural, physiological and metabolic characteristics represent adaptation to low activity levels and to the hypoxic burrow environment.

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CHAPTER 1. INTRODUCTION

1.1 General Introduction.

The interdependence of physiology and ecology have long been recognised. In a historical review of the development of ecological physiology, Jorgensen (1983) traced the development of both fields, and described the changing extent to which the value of integrating physiology with ecology has been recognised and achieved.

The development of animal physiology was historically the result of the use of animals as models for the study of human physiology. This approach developed into the discipline of comparative physiology, in which the aim is to derive an overview of fundamental physiological processes by investigating details of the particular physiology of species. This method has often emphasised similarities between species (e.g. Hill and Wyse, 1988). An alternative approach (adaptational physiology) is to examine the physiological mechanisms exhibited by animals living "where the environment seems to place insurmountable obstacles in their way" (Schmidt-Nielsen, 1979). This philosophy tends to exaggerate differences between species, and is prone to errors due to inadequate understanding of environmental constraints and selection pressures (the naive "adaptationist" concepts criticised by Gould and Lewontin, 1979).

Both comparative and adaptational physiology have, as a secondary aim, the elucidation of physiological evolution. Unfortunately, evolutionary patterns in physiological function remain obscure despite recent advances in understanding of evolutionary mechanisms (largely resulting from palaeontological and phylogenetic evidence; exemplified by the work of Gould (e.g. 1980) and Schram (e.g. 1981) respectively).

This is mainly because 'function' (as opposed to form) is not represented in the fossil record, and is not amenable to the quantitative analysis (cladistic, stochastic mosaical or otherwise) employed in the phylogenetic literature. Nevertheless, the importance of physiological function in evolution (and the influence of evolutionary history on current physiological characteristics) should be recognised.

An important theme in ecological physiology is the hierarchical system. Feibleman (1954) formulated a widely-quoted 'law of levels', "For an organisation at any given level, its mechanism lies at the level below and its purpose at the level above". Jorgensen (1983) interpreted this to mean "the use of physiology on problems whose purpose [function] is at the level of ecology". This theme is extended below to include characteristics at the ecological, behavioural, anatomical, physiological and metabolic levels of complexity.

Knowledge of ecological, behavioural and physiological processes in crustaceans (mainly decapods) has increased extremely rapidly since the review edited of Wolvekamp and Waterman (1960), largely as a result of developments in practical techniques. Much of the work on respiratory physiology in the late 1960's and early 1970's (e.g. Johansen *et al.*, 1970; Ansell, 1973; Spaargaren, 1973) followed the methodological lead given by studies of fish physiology (Hoar and Randall, 1970). Oxygen consumption and respiratory functions (branchial ventilation, perfusion etc.) were studied in crabs and lobsters under different conditions. A more comparative approach developed later (reviewed by McMahon and Wilkens, 1983). There have been many comparative studies of haemocyanin characteristics *in vitro* since the work reviewed by Redmond (1955), see Chapter 5. In particular, *in vitro* modulation of

haemocyanin has received a great deal of attention (reviewed in Chapter 5) although our understanding of *in vivo* function and the evolutionary significance of comparative differences is less developed. Finally, both comparative and functional studies of carbon dioxide transport and acid-base balance in decapod Crustacea are developing fields (Truchot, 1983; Cameron, 1986).

Although not recognised explicitly, both comparative and adaptational approaches have been used in studies of decapod physiology, and it is probably a fair statement that understanding of ecological function has lagged behind that of physiological mechanism. However, there have been more integrated approaches to the ecological physiology of crustaceans of a few ecological/habitat categories: rock-pool decapods (Taylor, 1988), terrestrial and semi-terrestrial land crabs (Burggren and McMahon, 1988) and terrestrial amphipods (Friend and Richardson, 1986; Spicer *et al.*, 1987).

Many species of decapod crustacean are known to construct permanent or semi-permanent burrows (as opposed to burying) in soft sediments (Atkinson and Taylor, 1988; and reviews below), including astacids, nephropids, alpheids and brachyurans. Probably the most adapted, however, (and possibly the most ecologically important; see below) are the thalassinids (infraorder Thalassinidea (Crustacea, Decapoda)). Although the ecology of mud-burrowing decapods is now being studied (Atkinson and Taylor, 1988; and further references below), the physiology of this group is not well-known. In the burrows of aquatic decapods (including those of thalassinids), oxygen availability is often limited (Atkinson and Taylor, 1988); thus the respiratory physiology of thalassinids is of particular interest. The

aim of this study was to examine the physiological ecology and ecological physiology of thalassinids, using a holistic approach comparable to those used for the select crustacean groups cited above. The axiid thalassinid ***Calocaris macandreae*** Bell (Fig. 1.1) was selected for the experimental work of the thesis since it is abundant in the Clyde Sea Area (and can be caught comparatively easily); some aspects of its general biology are well known (see below); and it is of considerable ecological significance (Buchanan, 1963; Buchanan and Warwick, 1974; Smith, 1988; further references and discussion below). (Hereafter, '***Calocaris***' in the text refers to ***Calocaris macandreae***.)

Following the precedents set in the previous crustacean literature, the discussions below follow both comparative and adaptational themes. It is intended, however, that the limitations inherent in both approaches will be made clear in the general discussion (Chapter 7). The evolutionary context of the ecological physiology of thalassinids will also be considered.

FIG. 1.1 ***Calocaris macandreae*** in burrows in the laboratory. Carapace length approximately 18 mm.

1.2 Aims and contents of thesis.

1. To collate and review the literature concerned with the ecology (with particular emphasis on the ecology of burrowing in soft sediments) and respiratory physiology of the Thalassinidea (remainder of Chapter 1, and following chapters). In order to provide an overview of thalassinid biology, the systematics, basic ecology and U.K. distribution of the Thalassinidea, and megafaunal burrowing ecology are briefly reviewed below.

2. To study a range of behavioural, ecological, morphological, physiological and metabolic characteristics of a selected thalassinid species (***Calocaris macandreae***). The results of studies of the burrow ecology (Chapter 2), branchial morphology (Chapter 3), respiratory physiology (Chapter 4), respiratory gas transport (Chapter 5) and anaerobic metabolism (Chapter 6) of this species are presented below.

3. To relate the ecology and physiology of ***Calocaris macandreae*** and other thalassinids to the burrowing habit (with particular emphasis on respiratory characteristics).

1.3 Thalassinid systematics

After more than a century of debate (see reviews of Borradaile, 1907; Calman, 1909; Gurney, 1942), the status of the thalassinid group is now generally accepted as intermediate between order and family (i.e. Infraorder Thalassinidea Latreille, 1831; Superfamily Thalassinoidea Latreille, 1831). The thalassinids have affinities with both the anomolans (i.e. the Anomura of Borradaile without the thalassinids) and the astacids (Gurney, 1942; Schram, 1986). The classification of the thalassinids used here (with example genera) is that proposed by Bowman and Abele (1982):

Infraorder Thalassinidea Latreille, 1831

Superfamily Thalassinoidea Latreille, 1831

Family Axianassidae Schmitt, 1924; ***Axianassa***

Axiidae Huxley 1879; ***Axius***, ***Calocaris***, ***Axiopsis***, etc.

Callianassidae Dana, 1852; ***Callianassa***, etc.

Callianideidae Kossmann, 1880; ***Callianidea***

Laomediidae Borradaile 1903; ***Naushonia***, ***Jaxea***, ***Laomedia***

Thalassinidae Latreille, 1831; ***Thalassina***

Upogebiidae Borradaile, 1903; ***Upogebia***

The Axiidae is the largest and most diverse family in the Thalassinidea with at least 200 species (Saint-Laurent and Le Loueff, 1979), although the systematics of the family are so confused that an estimate of the number of genera is impossible. It has been noted by several recent authors (e.g. Saint Laurent, 1972, 1979; Poore and Griffin, 1979; Kensley, 1980) that the taxonomy of the Axiidae is in urgent need of revision, especially with regard to generic status. The most complete description of the Axiidae is still the monograph of de Man (1925), although Poore and Griffin (1979) give a more recent catalogue of the

Australian species.

Borradaile (1903) classified the axiids into the following genera: ***Axius*** (subgenera ***Axius***, ***Iconaxiopsis***, and ***Eiconaxius***, ***Neaxius*** and ***Paraxius***), ***Axiopsis***, ***Calocaris*** (subgenera ***Calocaris*** and ***Calastacus***) and ***Scytoleptus***. De Man (1925) subsequently simplified ***Axius*** into the subgenera ***Axius (Axius)*** (with pleurobranchs), and ***Axius (Neaxius)*** (without pleurobranchs). Borradaile's genus ***Axiopsis*** (redefined by Sakai, 1986) is now divided into the subgenera ***A. (Axiopsis)*** (type-species ***A. (A.) serratifrons*** A. Milne Edwards, re-defined by Kensley, 1980) and ***A. (Paraxiopsis)*** (type-species ***A. (P.) brocki*** (de Man)).

Although de Man (1925) considered ***Calocaris***, ***Calastacus*** and ***Axiopsis*** as subgenera of ***Axius***, all three taxa are now usually recognised as distinct genera. Boesch and Smalley (1972) emphasised the similarities between ***Calastacus*** and ***Axiopsis (Axiopsis)***, while ***Calocaris*** and ***Calastacus*** were differentiated by Saint Laurent (1972). However, of the 11 species of ***Calastacus*** known in 1980, only 2 can be assigned to ***Calastacus*** as defined by Saint Laurent (Kensley and Gore, 1980). Clearly, taxonomic relationships within the major axiid groups ***Axius*** (***Axius/Neaxius***) and ***Axiopsis/Paraxiopsis/Calocaris/Calastacus*** remain confused. The position of ***Calocarides*** (Wollebaek, 1908) also remains unclear. The genus ***Coralaxius*** has affinities with the ***Axiopsis*** group but is distinctive in many characteristics (for example, the reduced branchial formula; Kensley and Gore, 1980). Similarly, ***Axiorygma*** is related to ***Axiopsis*** but has sexually dimorphic chelipeds, and no *appendix masculina* in the male (Kensley and Simmons, 1988). The genus ***Oxyrhynchaxius*** from Japan may be closely related (Sakai, 1986) to ***Calocaris/Calastacus***.

Hermaphroditism in axiids (see review below for ***Calocaris macandreae***) is restricted to the ***Axiopsis/Paraxiopsis/Calocaris/Calastacus*** group. However, most descriptions are confined to external sexual characteristics such as the presence of male *appendix masculina* on pleopod 2, female gonopores on pereiopod 3 and the condition of the first pleopod. The presence of these characters within the ***Axiopsis*** group is reviewed by Kensley and Gore (1980).

The Callianassidae is another large family, with at least 150 (probably now close to 200) described species (Saint-Laurent and Le Loueff, 1979). Saint-Laurent (1973) divided the Callianassidae into two sub-families, Callianassinae (the genera ***Callianassa***, ***Callichirus***, ***Gourretia***, ***Calliax***, ***Anacalliax***, ***Callianopsis***, ***Calliapagurops*** and ***Ctenocheles***) and Callianideinae (the single genus ***Callianidea***). Later additions to the Callianassinae include the genera ***Paracalliax*** (Saint-Laurent, 1979), ***Glypterus*** (redefined as a distinct genus by Manning and Felder, 1986), ***Corallianassa*** (Manning, 1987) and ***Neocallichirus*** (Sakai, 1988). At present, therefore, there are 12 genera in the Calianassinae. Bowman and Abele (1982) have restored the family Callianideidae to contain ***Callianidea***.

The Upogebiidae is usually considered monogeneric (Saint-Laurent and Le Loeuff (1979), although the genus ***Upogebia*** contains over 75 species. The Thalassinidae and Laomediidae are small, but generally recognised families with one and three genera respectively. The status of the Axianassidae is still unclear. However, pending a more complete revision of the Thalassinidea, the classification of Bowman and Abele (1982) is currently accepted.

The phyletic relationships between the families of the Thalassinidea remain unclear. It is generally accepted that there is a greater similarity between the Axiidae and Callianassidae than between the remaining families (Saint Laurent, 1973). This hypothesis is supported by evidence from larval development patterns (Gurney, 1942) and branchial formulae (Burkenroad, 1981). Although Saint Laurent (1979) established the superfamily Axioidea (to include the Axiidae, Callianideidae and Callianassidae), this classification has not been widely accepted (Manning, 1987).

The thalassinids are an ancient group within the decapod Crustacea, and are relatively well represented in the fossil record as a result of the burrowing habit (which is favourable to the probability of fossilisation; Schram, 1982). The earliest decapod fossil discovered so far dates from the Lower Devonian (approximately 350 million years before present; Schram *et al.*, 1978). Most of the major decapod families are present in the Jurassic (130 - 180 m.y.b.p.), implying that major radiation occurred between 250 and 200 million years ago. The Thalassinidea are first represented as Jurassic fossils (Schram, 1986). The axiid genera ***Etallonia***, ***Magila*** and ***Protaxius*** have been described from upper Jurassic deposits in Germany and England (Glaessner, 1969). The axiid ***Schlueteria*** and the callianassid ***Protocallianassa*** are more recent fossils from the upper Cretaceous (Glaessner, 1969). The ancestral phylogeny of the thalassinids is also discussed by Burkenroad (1963), who speculates that the common axiid/callianassid ancestor had two pairs of chelae and a *linea thalassinica*. Burkenroad also speculates that the thalassinids are closely related to the glypheids (infraorder Palinura).

1.4 Thalassinid Ecology

To some extent, the zoogeography of the thalassinids suggested by the literature reflects the distribution of marine laboratories throughout the world. The systematics of the thalassinids have been studied around the Soviet Union (Makarov, 1938); in the North-East Atlantic and Mediterranean (Balss, 1926; Gustafson, 1934; Poulsen, 1941; Holthuis and Gottlieb, 1958; Saint-Laurent and Bozic, 1976); the North-West Atlantic (Williams, 1984); the South-East Atlantic (Kensley, 1974; Saint-Laurent and Le Loeuff, 1979); the South-East Atlantic and Caribbean (Biffar, 1971; Rodrigues, 1971; Kensley, 1980; Kensley and Gore, 1980); the east Pacific (Williams, 1986); Japan (Sakai, 1962, 1967, 1969 etc; Ohshima, 1966); Australia (Poore and Griffin, 1979); and New Zealand (Wear and Yaldwyn, 1966). Further references to the taxonomy of thalassinids from these areas, and from South America and the Indo-Pacific region, are given in the sources cited above (particularly Saint-Laurent and Le Loeuff, 1979; Poore and Griffin, 1979).

There is little information concerning the bathymetric distribution of thalassinids. Almost all studies have used material collected from intertidal and shallow sublittoral (0 - 100 m) locations, in which thalassinids appear to be almost ubiquitous wherever soft sediments occur. Within fjordic systems and in the Skagerrak, the vertical distribution of the thalassinids (especially axiids) extends to at least 1000 m (Poulsen, 1941; Soot-Ryen, 1955). The callianassid genus *Ctenocheles* has a deep water distribution (100 - 800 m: Holthuis, 1967). (There is an interesting similarity between the unusual shape of the chelae of *Ctenocheles* and the deep water astacid *Thaumastocheles*.) Observations of burrows in the deep sea (e.g. Heezan and Hollister, 1971), which are probably due to astacid (nephropid or

thaumastochelid) or thalassinid crustaceans, suggest that the burrowing habit extends to very deep water.

All thalassinids so far studied (with respect to their ecology) occupy a secretive habitat, either burrowing in soft sediments (by far the majority of species), apparently boring in soft rocks (noted in ***Upogebia deltaura***; P.J. Schembri, pers. comm. to R.J.A. Atkinson), or boring and/or occupying crevices in sponges and scleractinian corals. The ecology and physiology of thalassinids which burrow in soft sediments are the major themes of this thesis. At least five species of ***Upogebia*** (Scott *et al.*, 1987) and at least one axiid (***Coralaxius abelei***: Kensley and Gore, 1980) have been described from coral cavities and siliceous sponges. There appear to be some morphological adaptations in the ***Upogebia*** species to coral-boring, including possible boring glands in the second pereiopods (Scott *et al.*, 1987).

1.5 British Thalassinids.

Nine species of the Infraorder Thalassinidea have been recorded from U.K. waters (Allen, 1967). Following more recent nomenclature these are:

Family **AXIIDAE** Huxley
Genus ***Axius*** Leach
Axius stirhynchus Leach
Genus ***Calocaris*** Bell
Calocaris macandreae Bell
Genus ***Calocarides*** Wollebaek
Calocarides coronatus (Trybom)
Family **LAOMEDIIDAE** Borradaile
Genus ***Jaxea*** Nardo
Jaxea nocturna Nardo
Family **CALLIANASSIDAE** Dana
Genus ***Callianassa*** Leach
Callianassa subterranea (Montagu)
Callianassa tyrrhena (Petagna)
(= ***C. laticauda*** Otto
= ***C. stebbingi*** Borradaile)
Family **UPOGEBIIDAE** Borradaile
Genus ***Upogebia*** Leach
Upogebia deltaura Leach
Upogebia stellata (Montagu)
Upogebia pusilla (Petagna)
(= ***U. littoralis*** (Risso))

Axius stirhynchus Leach occurs around Ireland (Selbie, 1914) in the Channel Islands (Sinel, 1906), along the south coast of England (Bell, 1858; Norman and Scott, 1906; Plymouth Marine Fauna, 1957;) and on both coasts of the Severn Estuary (Boyden *et al*, 1977; pers. obs.) where it constructs burrows in muddy sediments underneath and between boulders. As part of the present study, after several days of searching, only one specimen of ***A. stirhynchus*** was collected on a shore composed of large granite boulders overlying mixed muddy sediment near Porlock Weir, Somerset. Burrows were apparently more common, however, and were present from approximately mid-tide level downwards. Attempts to flush animals out of burrows were unsuccessful, as were attempts to resin-cast the burrows since the casts could not be removed from underneath the boulders. The single specimen was taken back to Glasgow University where it constructed a burrow in soft mud consisting of a vertical shaft about 40cm long, with subsequent horizontal development. The animal survived for several months. Another specimen of ***A. stirhynchus*** from the Severn Estuary (Sully Island, near Cardiff) was kept in an

aquarium where it constructed a U-shaped burrow 10cm deep (R.J.A. Atkinson, pers. comm.).

Axius stirhynchus has also been recorded subtidally from off the Norfolk coast (Hamond, 1971; Ellis and Baker, 1972); being collected in shrimp trawls in 1903, 1907, 1962 and 1971. Ellis and Baker (1972) kept two of the 1971 specimens for several months and reported the burrowing behaviour although the extent of burrow construction appears to have been limited by the conditions provided (sand underneath an irregularly shaped flint stone). They observed ***A. stirhynchus*** to line the burrow with soft brick pellets "balled with mucous secretions". No behaviour of this kind was noted in the Porlock specimen although burrow construction was more extensive.

Axius stirhynchus also occurs subtidally in the Clyde area although only one specimen has been captured (Allen, 1967). Larvae of ***A. stirhynchus*** have been recorded in plankton samples from around the Isle of Man (Bruce *et al*, 1963). Since abbreviated larval development is typical of the Axiidae (Gurney, 1942), this may imply the existence of unreported adult populations.

The distribution and ecology of ***Calocaris macandreae*** will be considered below. ***Calocarides coronatus*** is included in the British fauna (Allen, 1967) but appears to occur mainly in colder waters. Balss (1926) gives Scandinavian and USSR records.

The laomediid ***Jaxea nocturna*** appears to be present only in scattered locations around the south and west U.K. and Ireland (Selbie, 1914) although it is common in parts of the Adriatic (Pervesler and Dworschak, 1985). Rawlinson (1938) summarises the U.K. records of *J.*

nocturna to that date, all of which consist of single or very few specimens. The recorded distribution of *J. nocturna* larvae suggests that the distribution includes the Firth of Clyde (see Allen, 1967), the Irish Sea and the English South coast. In 1949-50 several specimens were reported from the Irish Sea south-west of Sellafield in mud at 25m (Bruce *et al*, 1963).

Jaxea nocturna has recently been discovered burrowing in soft muds in Loch Sween, Argyll (Atkinson, 1987; pers. obs.) although its presence was previously suspected from plankton samples (Kerr, 1912). The burrows constructed by *J. nocturna* at this site were different in some respects from the Adriatic burrows characterised by Pervesler and Dworschak (1985), and varied between different sites within the loch. *J. nocturna* were observed on the sediment surface during a period of low light intensity. *J. nocturna* has been found in the stomach contents of whiting (Plymouth Marine Fauna, 1957), gurnard and witch (Allen 1967) and sea scorpion (J.A. Allen and R.J.A. Atkinson, pers. comm.).

Rawlinson (1938) kept a living specimen of *Jaxea nocturna* in the laboratory for almost 4 months, although she did not provide adequate conditions for burrow formation, and no significant feeding was observed. To date, no live specimens have been captured from the Loch Sween population, although in 1986 an animal was taken from Loch Creran (C.J. Smith, pers. comm.).

The taxonomy of *Callianassa subterranea* has been somewhat confused in the past (Selbie, 1914; Makarov, 1938) leading to further confusion in the ecological literature. *C. subterranea* was first described by

Montagu (1808) burrowing in estuarine sand at a depth of nearly two feet. Bell (1858, quoting Thompson) records ***C. subterranea*** from fish stomach contents taken off County Down, Ireland. More recently ***C. subterranea*** has been recorded from the Plymouth area sublittoral and littoral, including ***Zostera*** beds (Plymouth Marine Fauna, 1957). In Brittany, a species of ***Callianassa*** is common in Zostera beds; (J.-P. Truchot, pers. comm.); this is most probably ***C. tyrrhena***. Selbie (1914) lists European records of ***C. tyrrhena*** (as ***C. stebbingi***), and considers the occurrence of ***C. subterranea*** doubtful north of the English south coast. Recent records, however, have been ascribed to ***C. subterranea*** (see Lutze, 1938; Saint-Laurent and Bozic, 1976).

It now seems likely that ***Callianassa subterranea*** is typically a sublittoral burrowing species (Lutze, 1938). ***C. subterranea*** is a fairly common component of the burrowing megafauna in several Scottish sea lochs and in the Clyde Sea Area (Farrow *et al*, 1979 (mounds); Nash *et al*, 1984; Atkinson, 1986). The burrow is highly characteristic with a vertical shaft (40-60 cm long) leading to a complex labyrinth of interconnecting tunnels (see also Chapter 2). The animal is rarely trapped during resin casting (R.J.A. Atkinson, pers. comm.) and, to date, attempts to catch specimens by coring, flushing burrows with a variety of solutions, and 'fishing' (as described by de Vaugelas, 1985) have failed. A single specimen was caught during the present study, using an anchor dredge at 25 m depth near the Lion Rock, Isle of Cumbrae (Firth of Clyde). Another specimen of ***C. subterranea*** has been taken from close to the Garroch Head sludge dumping ground, by Craib core, and was maintained in sand (where it promptly burrowed out of sight) for several months (B.J. Bett, pers. comm.). In addition, a small specimen was present in a mud tank at Millport although this was not realised until the burrow had been resin-cast along with those of

other species (R.J.A. Atkinson, pers. comm.).

Callianassa tyrrhena has been recorded from sandy mud around Lundy in the U.K. (Hoare and Wilson, 1976; Atkinson and Schembri, 1981). In addition, two right chelipeds of ***C. tyrrhena*** were taken from muddy sand around the Isle of Man, in 1912 and 1952 (Bruce *et al*, 1963). ***C. tyrrhena*** is also present in intertidal locations around the Channel Islands, and is the most abundant thalassinid in the Robertson collection from that area (curated at Millport). However, this species is mainly found in the Mediterranean (Saint-Laurent and Bozic, 1976; Saint-Laurent and Le Loeuff, 1979; Dworschak, 1987).

Upogebia stellata and ***U. deltaura*** have often been confused (Selbie, 1914). Although Montagu (1808) considered ***U. stellata*** to inhabit the subterraneous passages made by ***Solen vagina***, ***U. stellata*** was recorded by Leach (quoted by Bell, 1853), burrowing on the coast of Plymouth Sound, "under the mud of which it makes long winding horizontal passages, often of a hundred feet or more in length". The Plymouth Marine Fauna (MBA, 1957) records ***U. stellata*** and ***U. deltaura*** co-occurring in some locations (e.g. extreme low water springs, Salcombe harbour) although in other areas only one species occurs. This source also records ***U. stellata*** taken in a lobster pot from Plymouth Sound, and an exceptional case of 186 specimens of ***U. deltaura*** cast ashore after a heavy gale. Both ***Upogebia*** species have been recorded from a variety of fish stomachs (skate, dogfish and cod: Bruce *et al*, 1963; unnamed fish: Plymouth Marine Fauna, 1957; cod: Allen, 1967; Tunberg, 1986). The distribution of ***U. stellata*** and ***U. deltaura*** is summarised by Eales (1931) as North, East and South coasts. Selbie (1914) has recorded ***U. deltaura*** (but not ***U. stellata***) around the coast of Ireland.

The ecology of ***U. deltaura*** burrowing in sandy sediments off Sweden has been studied by Tunberg (1986).

Allen (1967) includes ***Upogebia pusilla*** (= ***U. littoralis***) in the British fauna, as it occurs rarely, on the south and south-east coasts of England. Makarov (1938) gives a distribution of the Black Sea, Adriatic, Mediterranean and Portuguese and SW French coasts, and states that the species is not found north of the English Channel. The species has been studied in the Adriatic by Ott *et al* (1976) and Dworschak (1981, 1983, 1987).

In conclusion, it is likely that thalassinids are much more common in U.K. waters than the distribution records would suggest. Most records are from intertidal habitats although it is possible that the main populations are sublittoral. The available evidence suggests that ***Axius stirhynchus***, ***Jaxea nocturna*** and ***Callianassa subterranea*** all construct very deep burrows and are unlikely to be caught by dredges and grabs employed in normal benthic sampling. In addition, the ***Upogebia*** species may inhabit areas of coarser sediment (possibly mixed with, or adjacent to mud) or maerl which have been little studied (Tunberg, 1986; R.J.A. Atkinson, pers.comm.).

Axius stirhynchus, ***Jaxea nocturna***, ***Callianassa subterranea*** and ***C. tyrrhena*** appear to be limited to southern and western coasts and sublittoral of the U.K., probably representing the northern limits of a Lusitanian distribution. In contrast ***Calocaris macandreae***, ***Calocarides coronatus***, ***Upogebia stellata*** and ***U. deltaura*** also occur on the northern and eastern coasts and are widely distributed throughout the North Sea, the Norwegian fjords, the Kattegat, Skagerrak, Jutland Peninsula and along the German coast (Wollebaek, 1908; Balss, 1926;

Gustafson, 1934; Poulsen, 1941). These distribution patterns are similar to those described for the U.K. brachyuran crab fauna (Ingle, 1980). Ekman (1953) includes ***Calocaris*** (and other components of the Scottish deep mud community, e.g. the sea-pen ***Funiculina quadrangularis***) in the relatively cold-water North Atlantic archibenthal fauna.

1.6 *Calocaris macandreae*

This species is almost certainly the most abundant thalassinid on the continental shelf around the U.K.. It is found at densities of 10 - 30 m^{-2} over large areas of soft mud sediment (Chapter 2). Buchanan and Warwick (1974) calculated that ***Calocaris*** accounted for >90% of the benthic productivity of an area off the Northumberland coast. ***Calocaris*** is usually present at low densities in the shallow (within range of SCUBA-diving) areas of Scottish West coast sea-lochs (Chapter 2, Nash *et al.*, 1984; Atkinson, 1986; R.J.A. Atkinson, C.J. Smith and S.J. Anderson, unpub. obs.). However, in deeper areas of the West coast, high density populations are present.

Some aspects of the biology of ***Calocaris macandreae*** have been studied previously. This species is unusual (for a decapod crustacean) in that it is a protandrous hermaphrodite (Wollabaek, 1908; Runnstrom, 1925; Carlisle, 1960; Buchanan, 1963; Calderon-Perez, 1981). The development of the testes and ovaries has been described by Runnstrom (1925) and Buchanan (1963) : both testes and ovaries are present in 1-year-old individuals and these grow at approximately equal rates for the first 3 years of life. During the 4th year the testes degenerate, leaving the vas deferens filled with spermatophores. The ovaries continue to mature, and the first eggs are laid in January - February of year 5.

Egg-laying is then biannual for the life of the individual (8 - 10 years in the Northumberland population: Buchanan, 1963). Calderon-Perez (1981) found that both development of the gonads, and life-span were shorter in the Irish Sea population. The reproductive behaviour of ***Calocaris*** has not been observed. Larval development is abbreviated (Bull, 1933; Gurney, 1942) and there is probably no significant pelagic phase (Buchanan, 1963).

The growth of the commensal ectoproct ***Triticella koreni*** on several crustaceans (mainly ***Calocaris*** and ***Nephrops norvegicus***) was noted by Eggleston (1971). The degree of ***Triticella*** coverage has been used as an indicator of the moult cycle. Nematode parasites of ***Calocaris*** have been studied by Calderon-Perez (1986).

The only study of the physiology of ***Calocaris macandreae*** is that of Dries (1975) who measured the rate of oxygen consumption (see Chapter 4).

1.7 Megafaunal burrower communities.

The megafaunal burrower communities of the Clyde Sea Area have been reviewed by Atkinson (1986); a description which is also applicable to most of the Scottish west coast sea-lochs. The somewhat different communities found in Loch Sween are described by Atkinson (1987, 1989), including work which forms part of this thesis. In most areas of the North-Eastern Atlantic and Mediterranean which have been studied, the dominant megafaunal burrowers are crustaceans; around the U.K. the main species are the lobster ***Nephrops norvegicus***; the crab ***Goneplax rhomboides***; the thalassinids ***Calocaris macandreae***, ***Callianassa subterranea***, ***Jaxea nocturna*** (and possibly ***Upogebia stellata*** and ***U.***

deltaura) and the amphipod ***Maera loveni***. The burrowing fish ***Cepola rubescens***, ***Lumpenus lampretaeformis*** and ***Lesueurigobius freisii*** and the echiurid ***Maxmuelleria lankesteri*** may also be present. The large isopod ***Natatolana (= Cirolana) borealis*** may also construct burrows (T.D. Nickell, pers. comm.).

The burrow structures of most of the above species are now relatively well known (Chapman and Rice, 1971; Rice and Chapman, 1971; Rice and Johnstone, 1972; Atkinson, 1974a; Atkinson, 1976; Atkinson *et al.*, 1977; Chapman, 1980; Nash, 1980; Atkinson *et al.*, 1982; Nash *et al.*, 1984; Smith, 1988). However, very little is known of the ecology and physiology of most of the crustacean species (with the exception of ***Nephrops***). There is a large (and increasing) literature concerning the ecology of ***Nephrops norvegicus***, a species which is of important economic value (e.g. review of Chapman, 1980). A limited amount is known about the ecology and physiology of burrowing crabs such as ***Goneplax rhomboides*** (Rice and Chapman, 1971; Atkinson, 1974a, b; 1975; Taylor *et al.*, 1985).

CHAPTER 2. BURROWS, BURROWING BEHAVIOUR AND ECOLOGY OF *CALOCARIS MACANDREAE*

2.1 INTRODUCTION

The experimental aims of the present study were to conduct an integrated investigation which would consider the physiological ecology of ***Calocaris macandreae*** at several levels of complexity (viz. habitat and behavioural ecology, branchial anatomy, respiratory physiology and metabolism). In order to achieve this end, a knowledge of the respiratory characteristics present in the burrow environment is necessary in order to interpret physiological and metabolic responses to supposed respiratory stress (i.e. it is necessary to define the environment, as a baseline for experimental manipulation).

The respiratory conditions within the burrow are a result of a dynamic equilibrium of oxygen flux into the burrow (mainly by mass flow of water) and oxygen uptake by the animal and burrow wall sediment. Oxygen flux into the burrow is therefore affected both by the physical structure of the burrow (i.e. morphology and characteristics of the surrounding sediment), and by behavioural responses of the animal (i.e. irrigation). Although a quantitative oxygen budget for the burrow was not constructed in the present study, the study was aimed at elucidating some of the parameters which affect oxygen conditions within the burrow (i.e. burrow morphology, and burrow effects on the surrounding sediment). The burrow structure of field populations of *Calocaris* has been described previously (Nash *et al.*, 1984; Atkinson, 1986), therefore little new work was necessary. This study concentrated on burrow development in the laboratory, and measurement of oxygen tensions within burrows. Aspects of the behaviour of ***Calocaris macandreae*** were also examined, with particular reference to effects on

respiratory conditions within the burrow.

The morphology of the burrows of thalassinids appears to be correlated with feeding strategy, categorised into three major groups by Suchanek (1985). The feeding strategy used by ***Calocaris*** has been studied previously (Buchanan, 1963; Calderon-Perez, 1981), with few firm conclusions, a further study was therefore made.

Finally, an understanding of the basic biology of an animal is required in order to interpret the results of physiological studies. Behavioural activity patterns are an important influence on physiology and metabolism, as are other characteristics of crustacean biology (e.g. moult and reproductive cycles). Some aspects of the ecology of ***Calocaris macandreae*** are therefore considered below.

2.2 MATERIALS AND METHODS.

2.2.1. Physico-chemical parameters of the burrow environment.

The distribution of ***Calocaris macandreae*** populations was observed at several locations, in particular in Loch Riddon (Firth of Clyde; lat.55°56'N, long.5°11'W), and in Loch Sween (Argyll; lat.56°2'N, long.5°36'W). These populations were all located within reasonable diving depth, at 20 - 30 metres (below chart datum), and all diving-based methods used standard SCUBA equipment. In collaboration with Dr R.J.A. Atkinson and Miss L.A. Calder, the megafaunal burrow openings at several sites in Loch Sween were mapped, using string grids (100 m X 2 m) deployed on the sea bed. This work is reported in Atkinson (1989), and (in part) in this thesis. Further information on the population density of ***Calocaris macandreae*** in Loch Sween (taken from Atkinson, 1989) was obtained from underwater TV videos (for methodology, see Atkinson, 1989). The population density of ***Calocaris*** was estimated from the density of burrow openings, by assigning each cluster of burrow openings to 1 individual. (N.B. in more dense thalassinid populations, clustering is not evident and assumptions of average number of openings per individual must be made.)

Polyester resin casts were made of burrows in the field using the method described by Atkinson and Chapman (1984). A shot-line (weighted either with concrete weights, or a 2m X 2m frame constructed from 'dexion') was lowered to the sea-bed and the sediment allowed to settle or be removed by the current. Marker lines were then placed by divers, along the sea-bed to groups of burrows which were marked with flags placed in the sediment. Funnels (made from plastic cups and buckets) were placed around individual openings. In the boat, low viscosity polyester resin (Strand Glass 471PA LV or Trylon SP701PA) was mixed

with catalyst (organic peroxide) to a final ratio of 1 - 2% (by volume). Mixed resin was then taken to the sea bed and slowly poured into the marked burrow openings. The resin was allowed to harden for 6 - 18 hours before being gently removed from the sediment (the casts were easier to remove while still slightly flexible since they become brittle when fully set). In addition, the burrow casts in the collection of R.J.A. Atkinson (mainly made by R.J.A.A., C.J. Chapman and R.D.M. Nash) were examined.

The behavioural patterns and burrow respiratory conditions of ***Calocaris macandreae*** were investigated in the laboratory. Animals were caught from 50 - 70 m depth in Loch Striven, Firth of Clyde by trawling with a ring-dredge or weighted Agassiz trawl. They were introduced to narrow aquaria (50 X 15 X 75(depth) cm) which were filled with muddy sediment (taken from the Firth of Clyde) to a depth > 40cm. The sea water temperature was between 8 and 12^{o}C. All sediment tanks were allowed to settle for at least 6 weeks before use, by which time the majority of sediment compaction had taken place. An apparent redox potential discontinuity (RPD) layer developed in most tanks (on the basis of visual appearance) before burrowing took place. In two tanks, thin (<5mm) layers of pale-coloured sand were interspersed with the mud, to allow a crude visual estimate of the rate of bioturbation. Any ***Calocaris*** which had not initiated a burrow within 48 hours were removed and replaced.

All observations on burrow conditions and behaviour were made on 'mature' burrows which had been established for a period of several months. One burrow was maintained by a single individual for over 2 years although most burrows were destroyed by experiments. The length

of time required for sediment settlement and burrow establishment was a limitation on the experiments which could be carried out during the project. In addition, it proved impossible to remove animals from burrows without damaging the burrow (see below for discussion); this factor also limited experimental design. On a few occasions animals died within burrows and other individuals were introduced. In general, this tactic was avoided for the following reasons:

1. The uncontrolled effects of a decaying ***Calocaris*** within the burrow.
2. The possible relationship between animal size and burrow diameter.
3. The possible behavioural effects of previous experience of the burrow.
4. The rapidity with which unoccupied burrows collapsed (possibly as a result of macrofaunal bioturbation).

The oxygen partial pressure (PO_2) of the burrow water in laboratory aquaria was measured using two different methods. Initially, lengths of cannula tubing were pushed through the sediment into the burrow lumen, in regions of the burrow which were visible through the aquarium glass. Burrow water was siphoned through the tubing until any sediment in the tubing had been cleared. Burrow water was then sampled with a syringe, or allowed to flow slowly past an oxygen electrode (Radiometer E5046) thermostatted at the appropriate water temperature. The main difficulty with this method of sampling was positioning the end of the tubing with sufficient accuracy, particularly in the deeper regions of the burrow. Oxygen electrodes were frequently calibrated, using a solution of sodium sulphite in 1% borax (PO_2 = 0 Torr) and aerated sea water (PO_2 = 155-160 Torr).

An alternative method used, was to drill holes directly through the plastic sides of the aquaria and glue hypodermic needles (gauge 21G) in

place using rapid Araldite adhesive. In most cases satisfactory sealing of the needles could be managed despite the hydrostatic pressure present at depth in the burrow. Both methods of burrow water sampling involved some degree of disturbance to the animal and burrow structure. Usually the animal 'investigated' the tubing or needle end and occasionally managed to block it with sediment. In general, however, the placement of a water sampler did not result in major changes in the burrow structure or obvious changes in animal behaviour. No measurements of burrow water parameters were made for several days following placement. In all cases the volume of water removed from the burrow was as small as possible, in order to avoid flushing the burrow artificially. In long-term experiments in which water was siphoned past a PO_2 electrode for several days, flow rates were < 10 $ml.h^{-1}$.

Two main patterns of PO_2 measurement were attempted. In the first, the aim was to establish the gradient of PO_2 throughout several complete burrows giving an estimate of the extremes of PO_2 which may be experienced by ***Calocaris***. In addition, PO_2 was measured from single points in a few burrows over time periods of several days, in order to assess the temporal variation in respiratory conditions within the burrow (i.e. whether respiratory conditions are 'steady-state' or actively regulated).

An additional experiment was carried out in which the surface openings to a lab burrow were destroyed by stirring the aquarium sediment to a depth of 2 cm, resulting in a decline in the burrow water PO_2. The PO_2 of the burrow water in the primary level was monitored until initial values had recovered.

Measurement of burrow PO_2's in field burrows was also attempted on several occasions. The method used was to introduce a length of cannula tubing down a burrow entrance as far as possible, and withdraw a sample (approximately 5 ml) into a syringe. The PO_2 of these samples was then measured as soon as possible by a thermostatted PO_2 electrode at the surface. This method was used successfully by Pullin *et al.* (1980) to measure PO_2 within the burrows of the fish ***Cepola rubescens*** in the field. However, ***Calocaris*** burrows are considerably smaller, and more complex, than those of ***Cepola*** and despite considerable effort on a number of occasions in Loch Sween and at the Creag Isles (Lynn of Lorne), no satisfactory samples of burrow water from ***Calocaris*** burrows were obtained. The main problem lay in preventing contamination of the sample with sediment from the burrow walls: due to the high biological and chemical oxygen demands (B.O.D. and C.O.D.'s) of the sediment, any contamination would deplete the PO_2 of the sample very rapidly.

A few measurements of the CO_2 partial pressure (PCO_2) were made on samples of burrow water taken from laboratory burrows. The method was similar to that used for single PO_2 measurements except that a thermostatted PCO_2 electrode (Radiometer E5037) connected to a Radiometer PHM 73 meter) was used. The meter was calibrated using sea water samples equilibrated to gas mixtures of known PCO_2, made using the Wösthoff precision gas mixing system (see Chapter 5).

Some physico-chemical properties of the sediment surrounding burrows were also investigated. Sediment samples were obtained from around field burrows by taking large cores, centred on a burrow openings. Cores were taken using lengths (50cm) of 25 cm diameter PVC drainpipe (area = 0.196 m^2), and also using stainless steel Senckenberg box cores (area = 112.5 cm^2) (Bouma, 1969), from depths of 15- 20 metres by

diving at several sites in Loch Sween. The cores were positioned to include one or more burrow openings which appeared from size and configuration to belong to ***Calocaris macandreae***. At the surface, the cores were sectioned and paired samples (2g) of sediment taken from areas of the section adjacent and distant to the burrow lumen. The sediment samples were immediately frozen.

Sediment samples were analysed for organic carbon content by wet chromic acid oxidation (Appendix 1), following the method of Walkley and Black (1934) as described by Buchanan (1984). This is a modification of the Schollenberger technique, in which the sample is digested with a chromic acid-sulphuric acid mixture and the excess of chromic acid not reduced by the sediment organic matter is titrated with a standard ferrous salt. Paired measurements were made of the redox potential (Eh) of the sediment adjacent and distant to the burrow at various depths in the core. A method similar to that described by Pearson and Stanley (1979) was used. The electrode (Russell pH Ltd.) was calibrated in a ferrocyanide-ferricyanide redox buffer (Zobell 1946) between readings. Readings were taken with a standard 5 mm penetration of the electrode tip into the sediment. The electrode was mounted in a Palmer stand, since it was found that the redox potential varied considerably over small distances in the sediment.

A series of redox profiles (at a vertical interval of 10 mm) were also measured through sediment columns (containing burrows) in the laboratory. The electrode, mounted in a Palmer stand, was wound down directly through the tank sediment, as close to the tank wall as possible. This allowed the redox measurements to be related to the electrode position relative to a burrow. This procedure also avoided

possible disturbance to the sediment as a result of coring, although an alternative source of error exists as the electrode may push a plug of sediment ahead of itself (J.B. Buchanan, pers. comm. to R.J.A. Atkinson).

2.2.2. Behavioural patterns of *Calocaris macandreae* within the burrow.

The behavioural patterns shown by ***Calocaris*** were observed in individuals which had established burrows in laboratory aquaria as described above. Only individuals which had been established for a period of several months were used.

Closed-circuit TV recordings were made of ***Calocaris*** using a low-light sensitive video camera (National CCTV WV1350) connected to a time-lapse VHS video recorder (National 5031). This system allowed up to 48 hours of continuous recording to be made. The pan, tilt and focal length (zoom) of the camera could be remotely controlled to minimise disturbance to the animal during recording.

The behaviour patterns recorded were analysed in terms of functional descriptions which were subjectively defined. The behavioural categories used were:

BURROWING: the animal shows behaviour obviously related to burrow construction and maintenance (i.e. movement of sediment).

LOCOMOTORY: the animal shows locomotory behaviour which is not obviously related to burrow maintenance. This often takes the form of consistently repeated 'patrolling' of the burrow, and results in periods when the animal is out of sight. (N.B. some 'piston-action' irrigation of the burrow may result from this activity).

PREENING: the animal makes grooming movements involving mainly the maxillipeds and 1st and 2nd pereiopods. (N.B. observations showed that

berried individuals may spend > 75% of the time grooming the pleopods and eggs. Berried individuals were therefore not used in further experiments. It is possible that preening has been confused with feeding activity to some extent. Definite feeding behaviour (i.e. manipulation of food by the mouthparts, and ingestion) could not be observed at the magnifications used in video recording).

IRRIGATION: The animal beats the pleopods in a characteristic, sustained manner. The uropods and telson are usually raised so that the abdomen assumes a horizontal posture.

STATIONARY: the animal makes no obvious movements.

The investigation of feeding behaviour in ***Calocaris*** required close examination of the animal, and was therefore based on visual observations from close to the aquarium wall, or video recordings using a longer focal length lens (with associated loss of field of view). Feeding time was therefore included in the 'stationary' or 'preening' categories described above.

There is a limitation to the approach outlined above in that the behavioural patterns may only be recorded and categorised while the animal is in a visible part of the burrow. The time during which recording is possible is not necessarily representative of the complete behavioural repertoire of the animal. Recordings were made of four individuals for periods of 24 - 48 hours during which the animals were visible for 60 - 75% of the time. Visual observations made while the animal was in parts of the burrow not visible to the camera, suggested that the video recordings represent as valid a sample of the total behaviour as was possible. It was felt that the alternatives, such as using very narrow tanks (forcing the burrow contruction to follow the

tank sides) or using transparent artificial burrows, would not result in 'natural' behavioural responses.

The irrigatory activities of ***Calocaris*** in burrows were of particular interest. The position in the burrow, and the direction of irrigatory flow were noted during bouts of pleopod beating in 'normal' conditions. Some attempts were made to measure the rate of burrow water flow using thermistor probes (as used for bivalve ventilatory flow (Brand and Taylor, 1974), and for burrow irrigation by the burrowing crab ***Corystes cassivelaunus*** (Bridges, 1979) and the snake blenny ***Lumpenus lampretaeformis*** (Atkinson *et al.*, 1987)). However, these proved susceptible to random fluctuations in water temperature and were not sufficiently sensitive to detect flow in this study. Further attempts were made to use tracer dyes (of various types), without success. The movement of dyes within the water column and burrow suggest that the rate of dye (and oxygen) diffusion may exceed that of active irrigation.

The behavioural response of ***Calocaris*** to a decline in the PO_2 of the water column was investigated by bubbling N_2 gas through the tank water. Burrow PO_2 was recorded at a burrow position in which the animal spent a relatively large amount of time. The behavioural patterns of two individuals were analysed (as described above) in response to this experimental stress.

2.2.3. General Biology.

During the course of this study, some observations on the general ecology of ***Calocaris macandreae*** in the Clyde Sea area were made. Since a regular sampling programme was not maintained, no attempt was made to record parameters such as carapace length, weight or catch size (which

may possibly be related to population density) or to make systematic observations on the reproductive and moult-cycle status of the population. These aspects of the biology of ***Calocaris*** have already been studied by Buchanan (1963) and Calderon-Perez (1981) in Northumberland and Irish Sea waters.

The feeding ecology of ***Calocaris*** is the subject of some debate (Elmhirst, 1935; Buchanan, 1963; Calderon-Perez 1981). A brief effort to collect additional data on the diet of ***Calocaris*** was made by examining the gut contents of 20 individuals.

2.3. RESULTS

2.3.1. Burrow distribution, structure and sediment effects.

A typical pattern of openings to burrows of ***Calocaris macandreae*** is shown in Fig. 2.1. Burrow openings take the form of circular holes in the sediment, leading to a short vertical shaft, as described by Nash *et al* (1984) and Atkinson (1986). The openings to ***Calocaris*** burrows often show a distinct pattern of groups of three, resulting from the mean number of openings/burrow and from the basic tripartite junction form of more complex burrows (described below). This pattern is shown in maps of megafaunal burrow openings at two sites in Loch Sween (Fig. 2.2; for site locations see Fig. 2.3).

The population density of ***Calocaris macandreae*** and the presence of other megafaunal burrower species, estimated from the number of burrow openings, at 15 sites in upper Loch Sween is shown are Table 1. The site locations are shown in Fig. 2.3; sediment parameters measured at several sites are given in Appendix 2.

Calocaris macandreae occurred only at comparatively low densities in Loch Sween (see below) and was absent at many sites. Most of the benthic communities of upper Loch Sween were dominated by the burrows of the thalassinids ***Jaxea nocturna*** and ***Callianassa subterranea***, and the echiurid ***Maxmuelleria lankesteri***. The possible reasons for this unusual burrowing megafaunal community are discussed in greater detail by Atkinson (1987, 1989).

A few populations of ***Calocaris macandreae*** were found in very shallow water, <10 m, during the course of the present study. In particular, a population with a burrow density of approximately 10 - 15 openings.m^{-2} was found in a restricted area of Loch Dunvegan, Isle of Skye

FIG. 2.1 Burrow openings of ***Calocaris macandreae*** in muddy sediment at approximately 80 m depth, Firth of Clyde. Scale bar 5 cm. (Photographed by Department of Agriculture and Fisheries for Scotland).

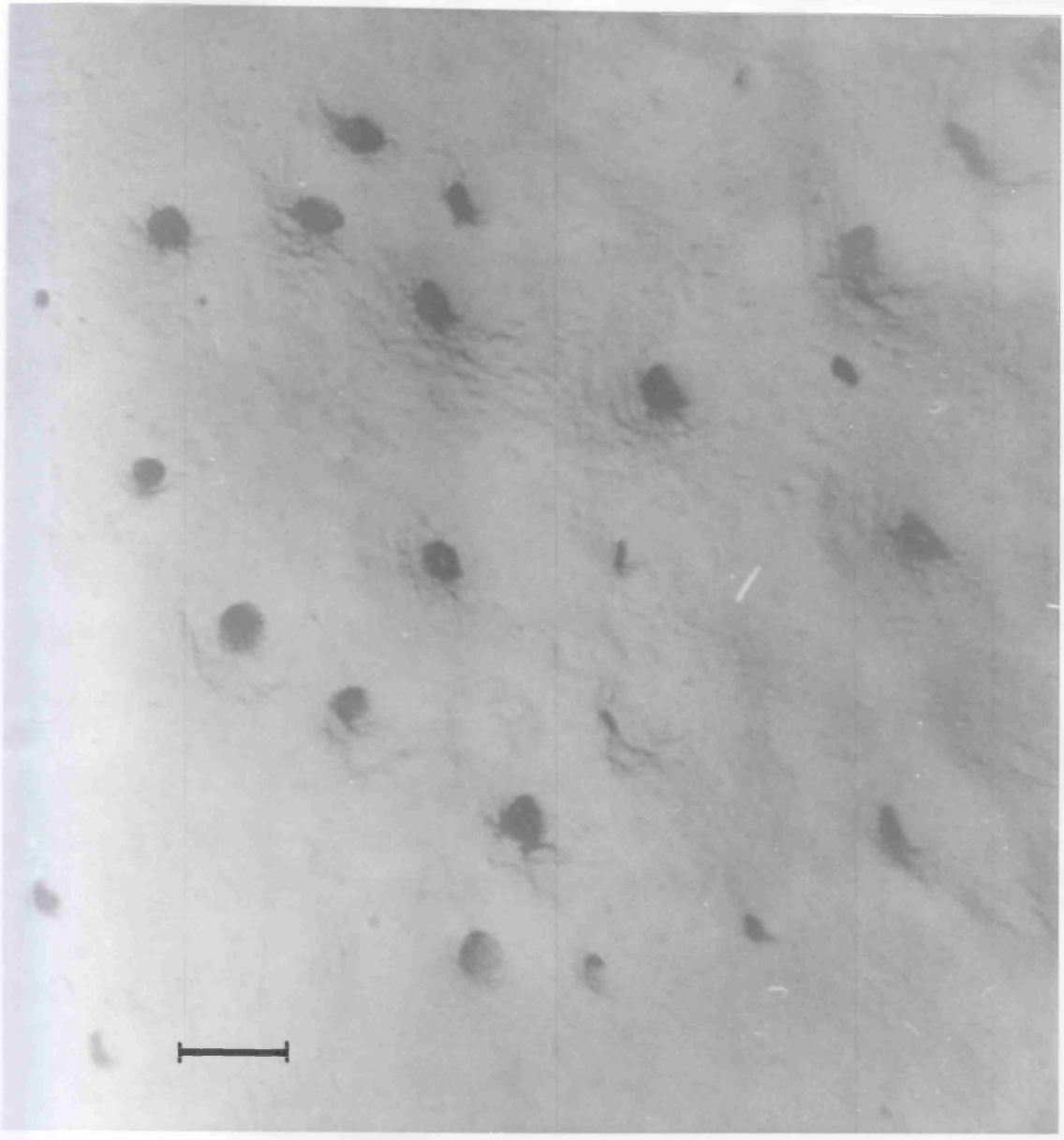
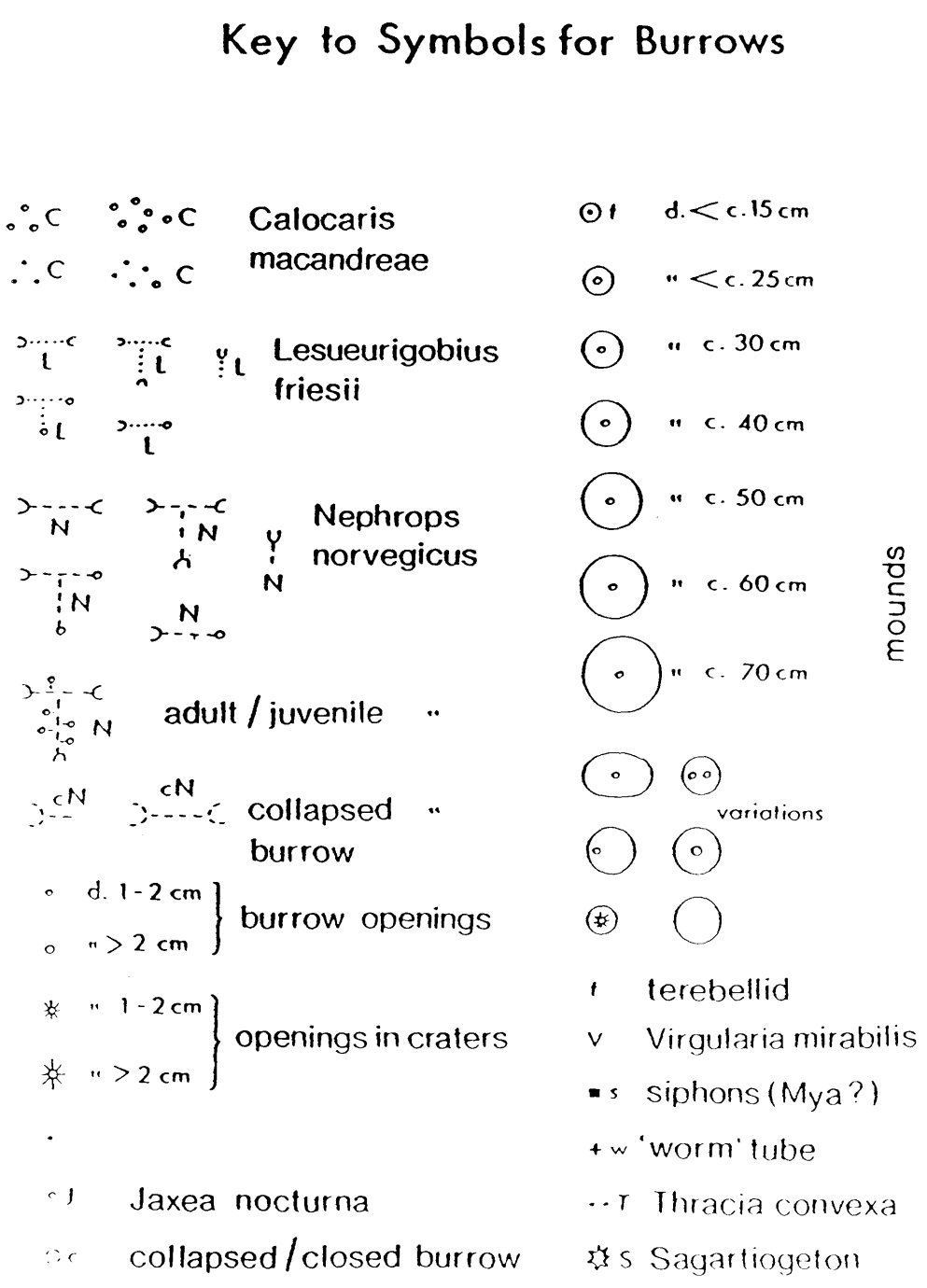
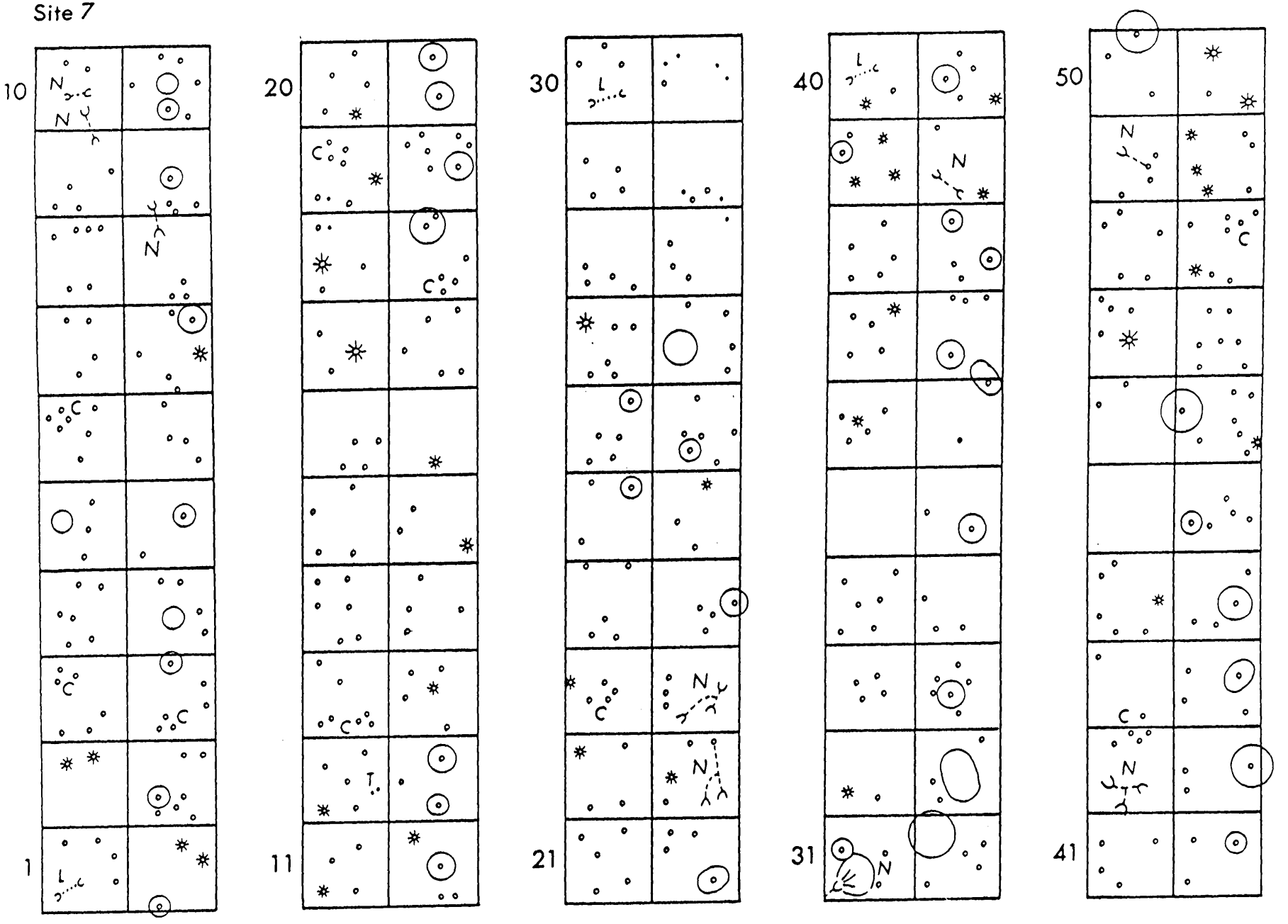


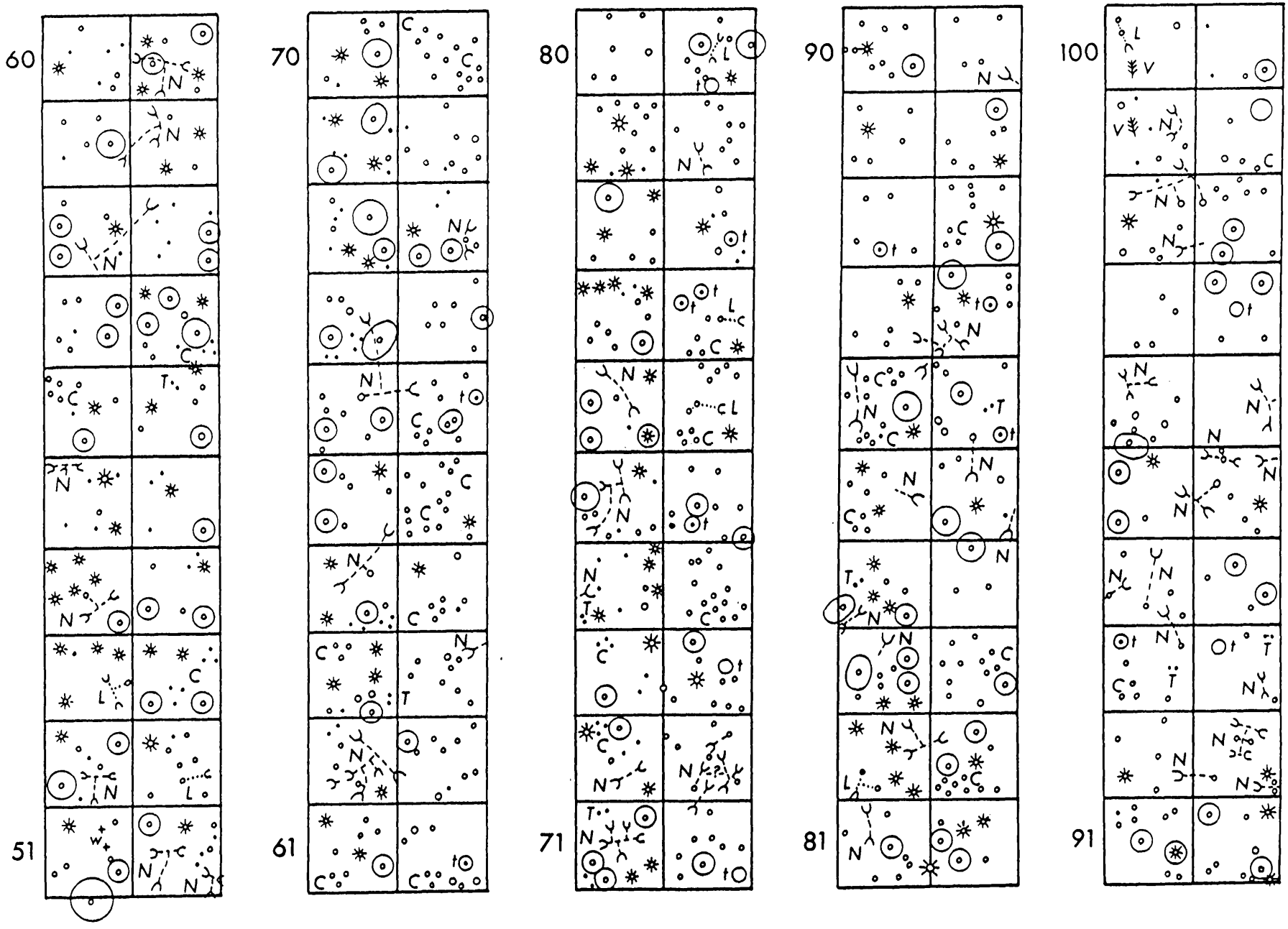
FIG. 2.2 Burrow distributions mapped by diving at site 7 (Achnamara Arm) and site 13 (Sailean Mhor) in Loch Sween. Each square is 1 m^2. Symbols are indicated in the key. The depths are c. 15 m and c. 19 m below chart datum respectively. Site locations are given in Fig. 2.3. (Taken from Atkinson, 1989).



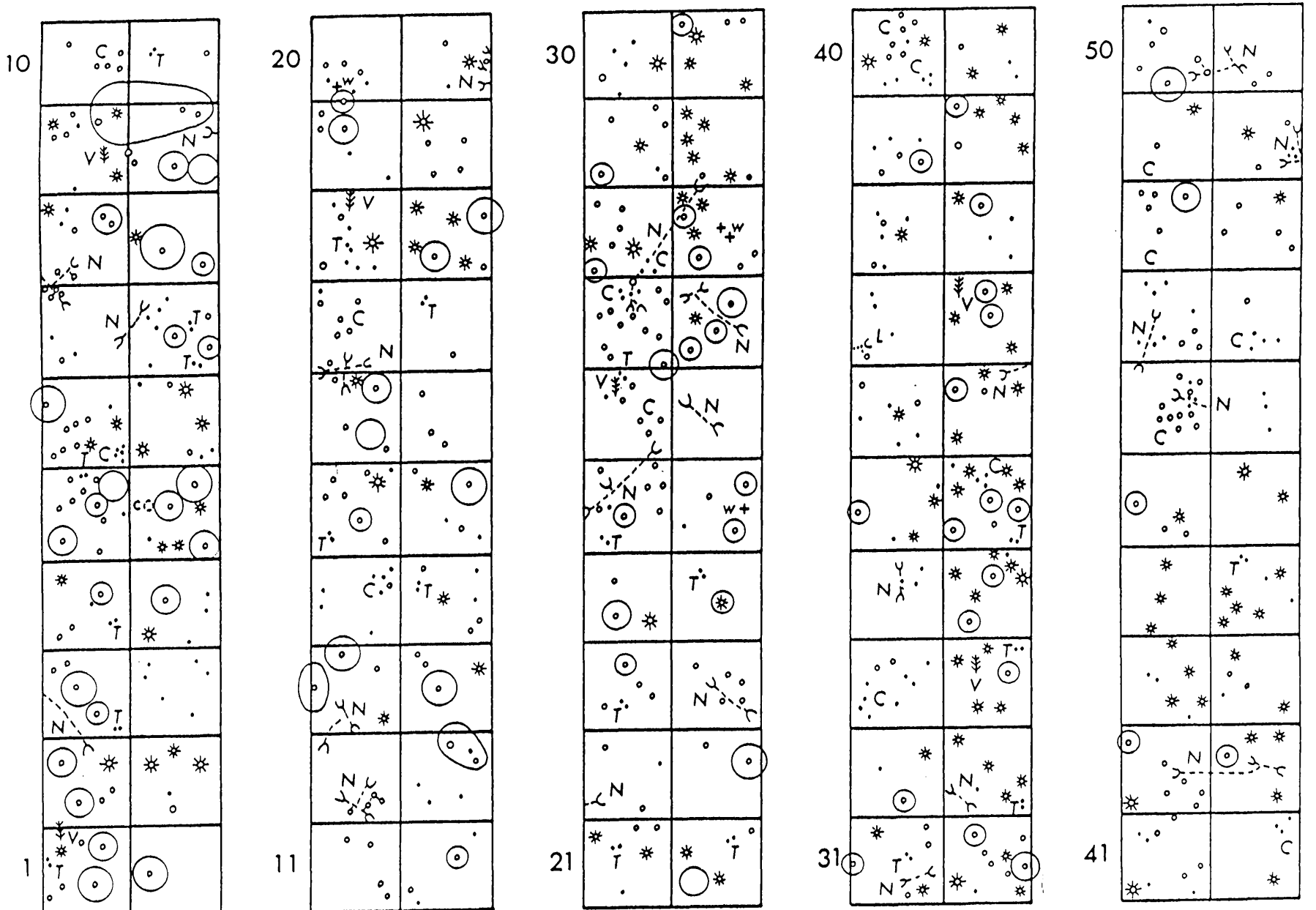
Site 7



Site 7



Site 13



Site 13

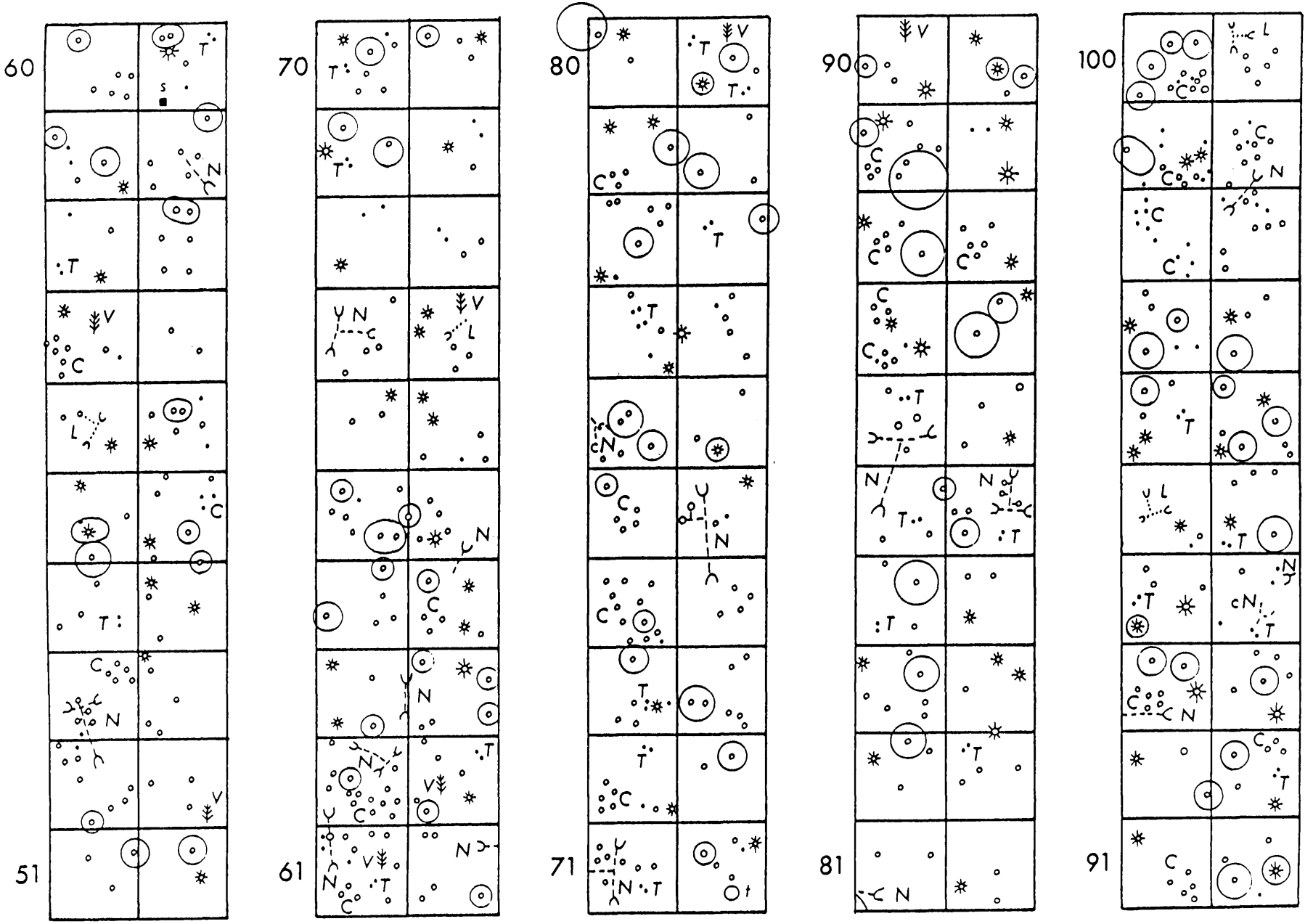


FIG. 2.3 Upper arms of Loch Sween showing the study sites (numbered solid circles). The dotted lines indicate Chart Datum, except in the case marked P where the dotted line delimits a sub-surface rock pinnacle (2.7 m below C.D.). For detailed bathymetric information refer to Admiralty Chart No. 2397. (Taken from Atkinson, 1986).

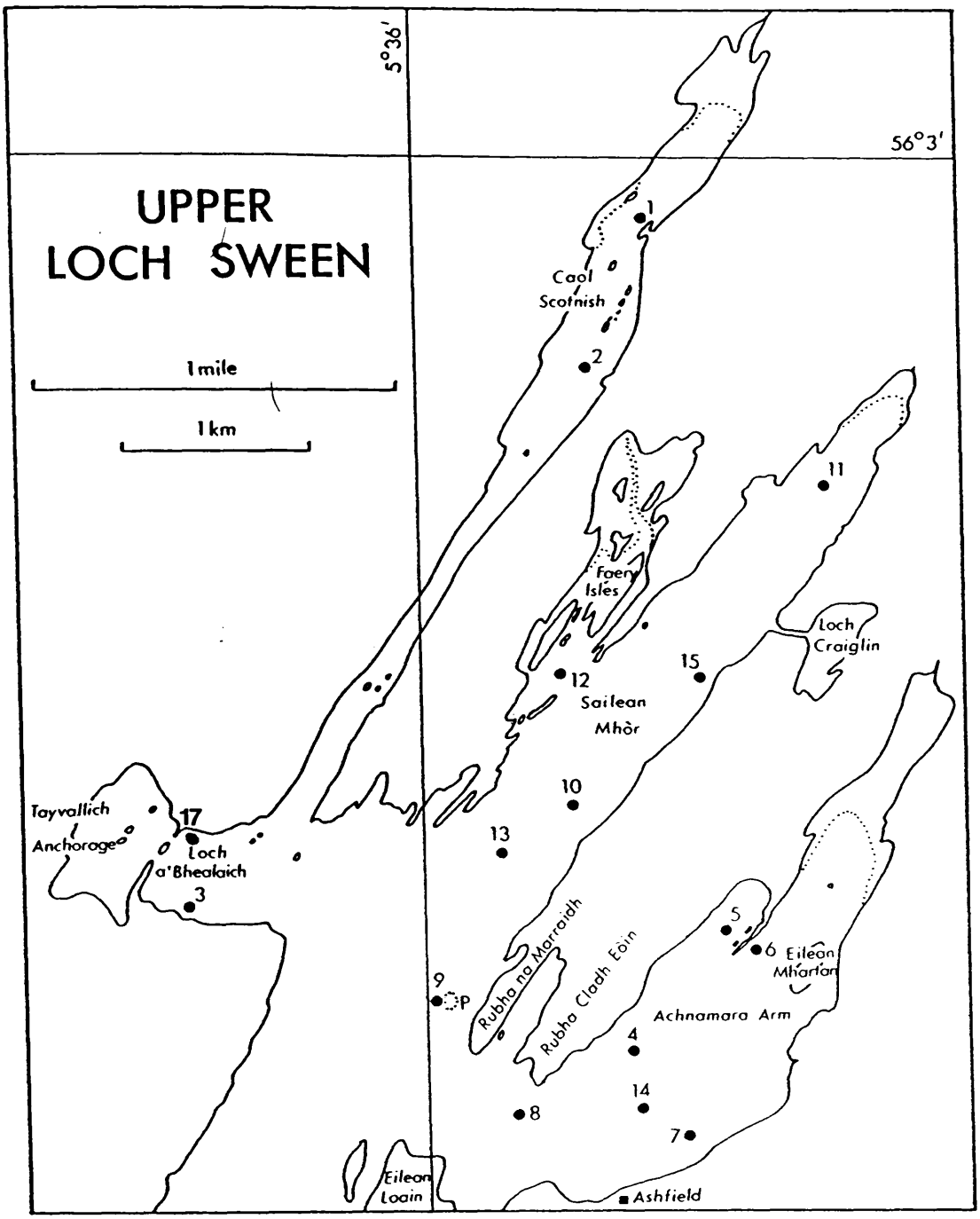

UPPER
LOCH SWEEN
5°36'
56°3'
1 mile
1 km
Caol
Scotnish
Faery
Isles
Loch
Craiglin
Sailean
Mhôr
Tayvallich
Anchorage
Loch
a'Bhealaich
Rubha na Marroidh
Rubha Cladh Eòin
Achnamara Arm
Eilean
Mhartan
Eilean
Loain
Ashfield
P
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
17

TABLE 2.1 Population densities (individuals. m^2) of *Calocaris macandreae*, *Nephrops norvegicus* and 'mound-builders' (*Callianassa subterranea*, *Jaxea nocturna* and *Maxmulleria lankesteri*) at 15 sites in Loch Sween. (For site numbers refer to Fig. 2.3). Population density estimated from observations made by diving (taken from Atkinson, 1986) and 200 m x 1 m TV transects (taken from Atkinson, 1989).

SITE	*Calocaris macandreae*	*Nephrops norvegicus*	mound-builders (see legend)
1	0	0	0
2	0	0	0.2-1
3	very low	0.1-0.2	5-6
3(TV)	0.02	0.03	0.13
4	0.2	0.1	2-3
4(TV)	0.18	0.19	0.74
5	0	0.3	1-2
6	0	0	0.2
6(TV)	0.005	0.06	0.12
7	very low	0.3	6 ***(Callianassa)***
7(TV)	0.17	0.20	0.86
8	0.1-0.2	1	6 ***(Callianassa)***
8(TV)	0.36	0.11	0.34
9	1	0.5	0
10	0	0.5	1-2 (***Callianassa***)
11	0	0.2-0.3	many (***Maxmulleria***)
11(TV)	0.07	0.1	0.84
12	0	0	0
13	0.2-0.3	0.5	2-3
13(TV)	0.15-0.19	0.17-0.18	0.74-0.8
14	very low	0.02	many (***J.*** and ***M.***)
14(TV)	0.18	0.13	0.48
15	0	0.3	5
15(TV)	0.11	0.21	0.67

(lat.57° 38.0'N, long.6° 26.5'W), at a depth of 7-8 m. Another such population was found, with a much lower density (< 1 entrance.m^{-2}) at a depth of 7 m in the Achnamara arm of Loch Sween.

An example of a ***Calocaris macandreae*** burrow resin-cast is shown in Fig. 2.4. Although there was considerable variation in the precise morphology of burrow casts, there was a general pattern of burrow form that conformed to the description of Nash *et al.* (1984). Using the terminology of Frey (1973), the burrows consisted of a complex of vertical shafts and horizontal tunnels. ***Calocaris*** burrows take the form of a primary level of a complex of U-shaped tunnels, opening to the sediment surface via vertical shafts (Fig. 2.4, 2.5). The tunnels are connected by characteristic tripartite junctions. In most field burrows, a deeper secondary level of development was present, consisting of slightly wider tunnels connected to the primary level by vertical shafts. In the most developed burrow casts, circular galleries are present in the secondary level (Fig. 2.5F). Typically, the primary burrow horizon was located at a depth of 10 - 15 cm sediment depth, with secondary development at 20 - 25 cm. All shafts and tunnels were approximately circular in cross-section.

Well-developed mounds (cf. ***Callianassa subterranea, Jaxea nocturna*** or ***Maxmuellleria lankesteri***) were never observed at the surface openings of ***Calocaris*** burrows although low mounds and piles of deposited sediment were sometimes present in close proximity to the openings.

The development of burrows by ***Calocaris*** in laboratory aquaria (Fig. 2.5A - D) usually followed the stereotyped pattern described by Nash *et al.* (1984). The initial burrow constructed took the form of a simple U-tunnel, constructed at the primary level. This was further

FIG. 2.4 Resin cast of a burrow of ***Calocaris macandreae***. Scale bar gradations in cm. (Burrow cast in collection of Dr R.J.A. Atkinson, Millport).

elaborated by the addition of horizontal tunnels and shafts to surface openings, joined to the initial tunnel by a series of tripartite junctions. In most cases, one shaft of the original 'U' was abandoned and collapsed relatively quickly, so that the oldest structure in most laboratory burrows consisted of a 'W' formed from an original and a slightly later vertical shaft (Fig. 2.5D, E). Development of complex secondary burrow systems did not occur in the laboratory (over a 1 year time scale), although a few examples were noted of the V-shaped dip (Fig. 2.5D, 2.6, 2.7) which appears to precede secondary development in field burrow casts.

The few long-term (› 6 months to 2 years) burrows which were observed suggest that the development of burrow structure is a continual process (Fig 2.6). All parts of the burrow may be abandoned, while new shafts and tunnels are continually constructed. The result may be a large increase (Fig. 2.6B) or decrease (Fig. 2.6A) in burrow complexity. Within the burrow, ***Calocaris*** carries out a large amount of sediment re-working and movement over short distances. This behaviour may be related to feeding activity involving the turn-over and renewal of the sediment lining the burrow, and will result in a large bioturbatory effect on the sediment surrounding the burrow.

A consistent observation was that non-maintained burrows (or parts of burrows) collapsed relatively quickly. Abandoned tunnels, or burrows in which the resident ***Calocaris*** had died, usually collapsed completely within 4 - 8 weeks, although the re-establishment of a highly stratified sediment with a well developed RPD usually took considerably longer (› 10 weeks; NB. this is longer than the time required for newly settled sediment to stabilise). In Figure 2.7,

FIG. 2.5 Stages in the development of burrows of ***Calocaris macandreae***. A - E: successive stages in the development of a single burrow in a laboratory aquarium. F: detail of secondary development in a resin cast of a burrow from Loch Brollum, Outer Hebrides (burrow cast by C.J. Chapman, DAFS). (Scale bars: A - B 5 cm; C - E 10 cm; F 5 cm).

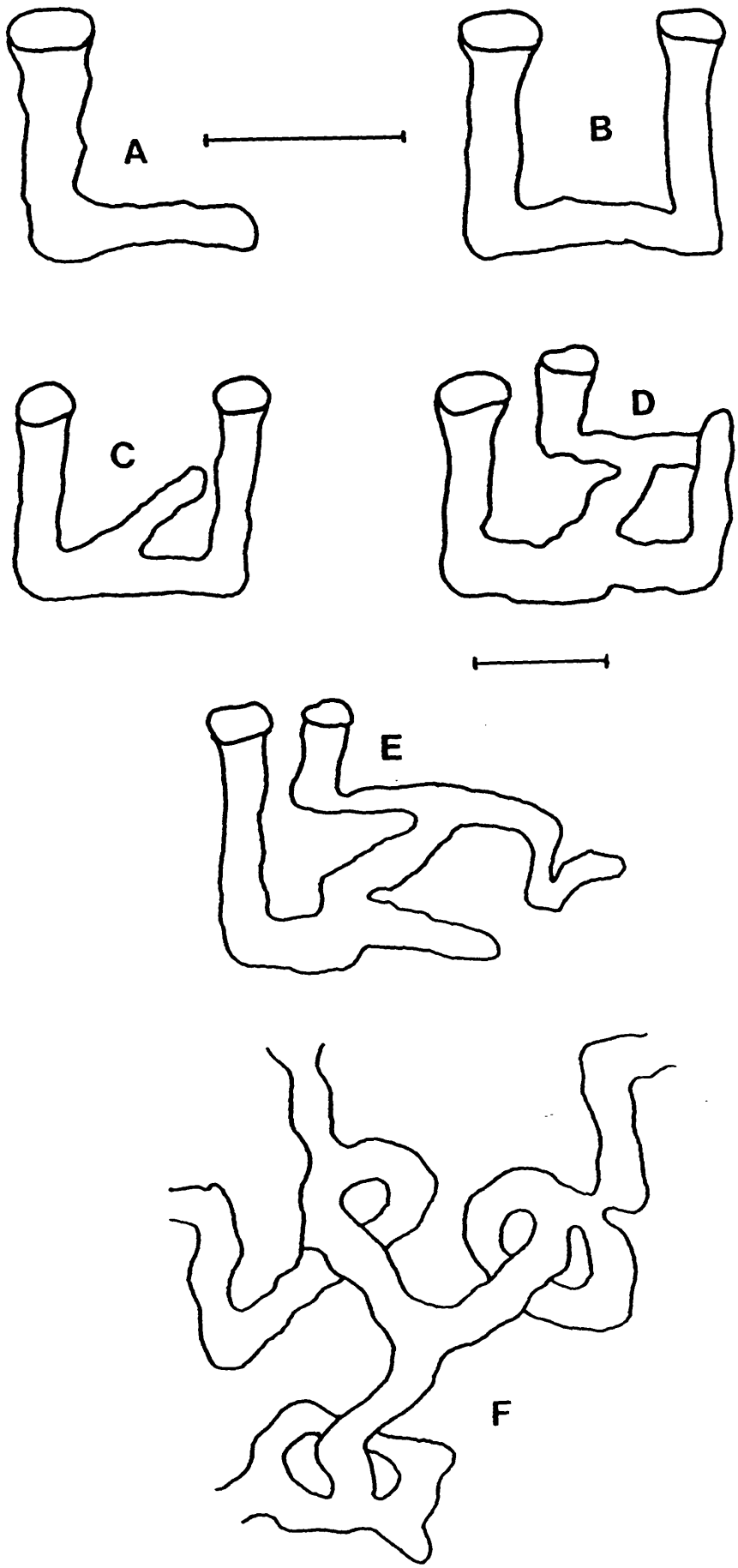
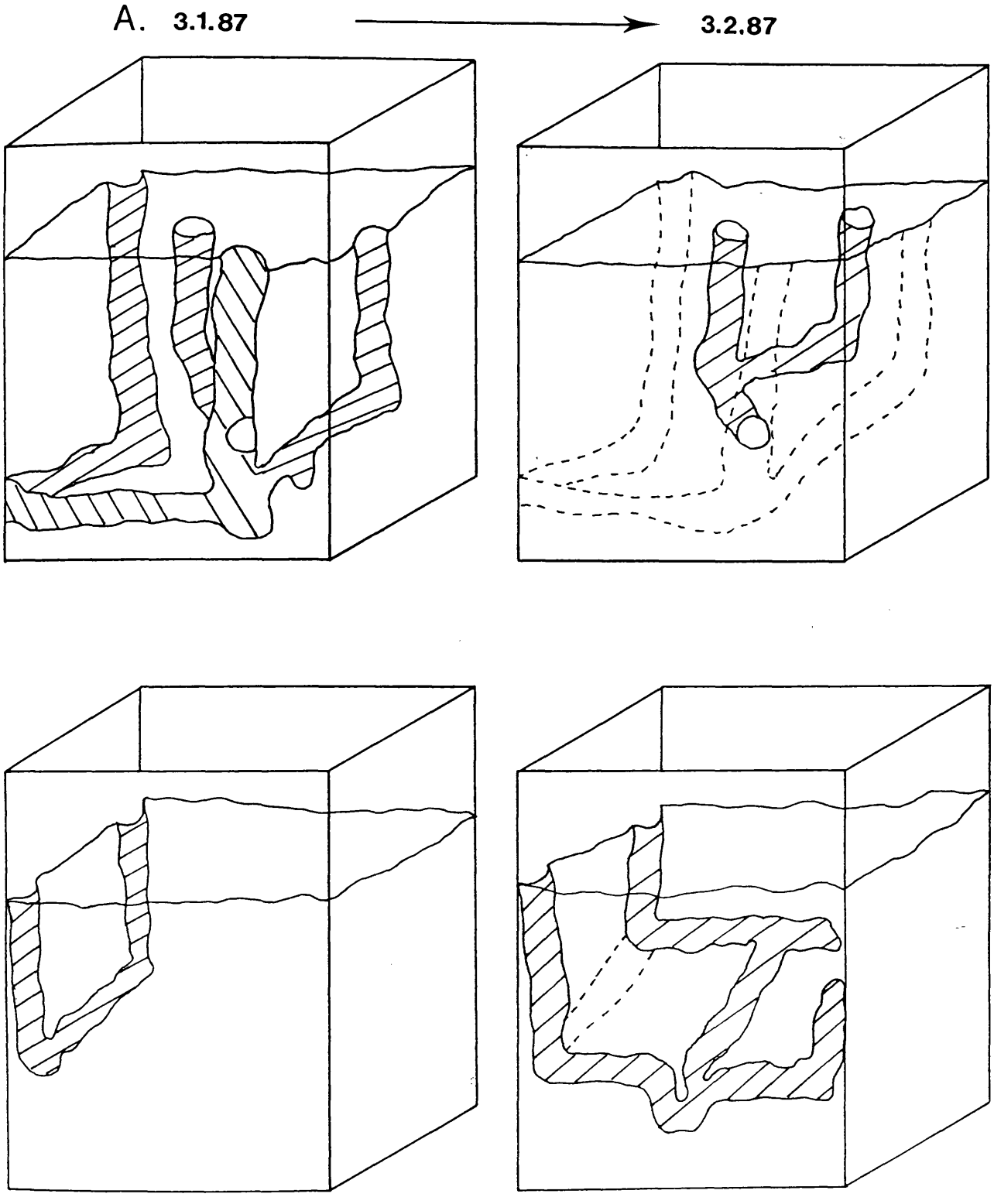

A
B
C
D
E
F

FIG. 2.6 Changes in structure of the burrows of ***Calocaris macandreae*** in aquaria. A) Simplification of a burrow over 31 days. B) Increase in complexity of a burrow over 28 days.

Burrow structure was inferred from parts of the burrow visible through the aquaria sides, and from the surface configuration of burrow openings. Collapsed burrows are shown as dashed lines. Aquarium width 50 cm.


A. 3.1.87
3.2.87
B. 4.2.87
4.3.87

lighter-coloured areas of sediment (other than the surface oxygenated layer) correspond to the position of previous burrows. The relative instability of abandoned burrow structures may also contribute to the high rate of bioturbation which results from megafaunal burrowing.

The bioturbatory effect suggested by visual observations of sediment 'patchiness' (due to the presence of areas of black, reduced sediment within the sediment column) was confirmed by observations of thin layers of sand positioned to act as markers of bioturbation. The break-down of a stable sediment stratification, indicated by a sharp redox potential discontinuity (RPD) layer (visible as an abrupt change in sediment colour and texture), was rapid in the proximity of a newly established burrow. Within several days of a burrow penetrating the RPD layer, a 'halo' of lighter-coloured sediment was visible around the burrow (Fig. 2.7). After several months of burrowing activity within a tank, the sediment column became a heterogeneous mixture of patches of light and dark (corresponding to oxygenated and reduced) mud. On the basis of visual observation through the tank walls, sediment heterogeneity was most pronounced on a scale of 1 - 5 cm. This scale may correspond to the effects of sequential burrow construction and collapse, although the effects of bioturbation by ***Calocaris*** were blurred by the high degree of macrofaunal (and presumably meiofaunal and microfloral) activity present. Similar effects were noted on the break-up of artificial sand strata in the sediment. However, in order to minimise the effects of layers of coarse sediment on the ability of ***Calocaris*** to burrow, the sand layers were made so thin (2 - 5 mm) that macrofaunal activity tended to mix the sand into the surrounding mud fairly rapidly (a few weeks).

Some measurements of redox potential and organic carbon content from

FIG. 2.7 Photographs of sediment columns in previously burrowed (A) and burrowed (B, C) sediment tanks. 'Oxygenated' sediment appears lighter in colour.

A

B

C

sediment surrounding *Calocaris* burrows (and from control areas) at Loch Sween are given in Table 2:

TABLE 2.2: FIELD BURROW SEDIMENT PHYSICO-CHEMICAL PARAMETERS.
(in collaboration with L. A. Calder)

Sample description	Redox. (mV)	% water	% org. C (dry wt)
CONTROL surface; site 9 (pinnacle)	+74	36.6	2.18
20cm; " "	+314	39.8	2.46
35cm; " "	+94	40.4	3.12
CONTROL surface; site 3 (L. a Bhealaich)	+136	40.4	3.66
surface; " "	+222	28.2	4.05
15cm; " "	+88	35.1	3.80
15cm; " "	+83	35.4	3.70
25cm; " "	+77	36.4	3.88
25cm; " "	+81	38.3	3.65
surface; site 17 (L. a Bhealaich)	+49	45.3	1.98
10cm burrow; " "	+128	52.8	1.98
10cm control; " "	+131	55.9	1.94
15cm burrow; " "	+255	51.8	2.07
15cm control; " "	+140	62.0	0.80
5cm burrow; site 17 (L. a Bhealaich)	+130	49.8	2.02
5cm control; " "	+73	50.4	1.95
15cm burrow; " "	+90	40.5	2.25
15cm control; " "	+81	49.7	2.04

The field samples from sites 9 and 3 in Loch Sween were taken in close proximity to *Calocaris* burrows, although the cores did not include any prominent burrow structures. At both sites the sediment column was well oxygenated, despite the very high organic carbon (chromic acid oxidation) content. These redox values are in close agreement with previous measurements at these sites (Atkinson, 1987), and probably reflect the high level of bioturbation which appears to be present.

The paired comparisons of burrow 'halo' sediment and sediment sampled

further from the burrows at Loch Sween site 17 suggest that the burrow 'halo' was not significantly more oxygenated than the surrounding sediment. Although sediment redox potential may not be directly related to oxygenation, the data suggest that the entire sediment column was relatively well oxygenated at least to a depth of 15 cm. Redox potential profiles measured through aquaria burrows (Table 3) followed a similar pattern, with no significant increase in redox potential in the burrow 'halo' relative to the surrounding sediment. Patchiness on the 1 - 5 cm scale was evident in most redox profiles from aquaria sediment columns, supporting the visual evidence described above.

TABLE 2.3: AQUARIUM BURROW REDOX PROFILES.

Depth	Burrow 1. Redox potential	Comments	Burrow 2. Redox potential	Comments
1cm water	+ 441 mV		+366 mV	
0cm	+ 452		+367	
1	+ 279	dark brown	+130	
2	+ 201	diatom film	+70	
3	+ 109		+61	mixed
4	+84		+55	brown/grey
5	+74		+65	diatom film
6	+76		+31	
7	+80		+48	
8	+85	grey	+55	
8.5	--		+53	
9	+93		+54	
9.5	+93		+53	burrow halo
10	+98		+50	
10.5	+95	burrow halo	+60	
11	+100		+60	burrow lumen
11.5	+98		+54	
12.5	+90	burrow lumen	+32	
13	+89		+23	
14	+69	burrow halo	+30	burrow halo
15	+67		+25	

Measurements of organic carbon contents of sediment samples from around field burrows, show only a slight (non-significant) elevation in carbon of sediment from the burrow walls. However, these samples were all taken from primary levels of burrows (deepest = 15 cm) and it is

possible that organic enrichment of the sediment surrounding the deeper parts of the burrow may occur.

2.3.2. Burrow Respiratory Conditions.

Respiratory conditions in the burrows of ***Calocaris macandreae*** were characterised by the presence of a pronounced gradient of oxygen tension from normoxic conditions (PO_2 = 150-160 Torr) at the burrow openings to severe hypoxia (PO_2 = 20 - 40 Torr) in the deepest parts of burrows in aquaria.

There was a high degree of consistency between measurements of PO_2 in burrows of similar morphology, in aquaria. Most burrows investigated contained water with a PO_2 of < 40 Torr in the deepest levels, with a relatively uniform gradient of oxygen partial pressure between the surface and the deepest levels. Therefore, results from only one burrow are shown in schematic form in Fig. 2.8.

Long-term measurement of the PO_2 of burrow water samples suggest that respiratory conditions at any one part of the burrow are likely to be relatively stable (Fig. 2.9). There was some evidence of variation and possibly regulation of PO_2 in 4 of the 10 burrows investigated, with changes (usually declines) of up to 30 Torr occurring over 5 - 7 hours followed by a more rapid recovery. However, no evidence was obtained of any effects of regular irrigation by the animal. Apparent 'cycling' of burrow PO_2 shown in part of Fig. 2.9 (approximately the middle of the trace; period approximately 50 minutes) reflects animal locomotion, with associated water movement. No pleopod irrigatory behaviour was observed during this time.

FIG. 2.8 Oxygen tensions (Torr) measured in a laboratory burrow of ***Calocaris macandreae***. Burrow diameter approximately 2 cm. The PO_2 of the overlying water was 157 Torr.

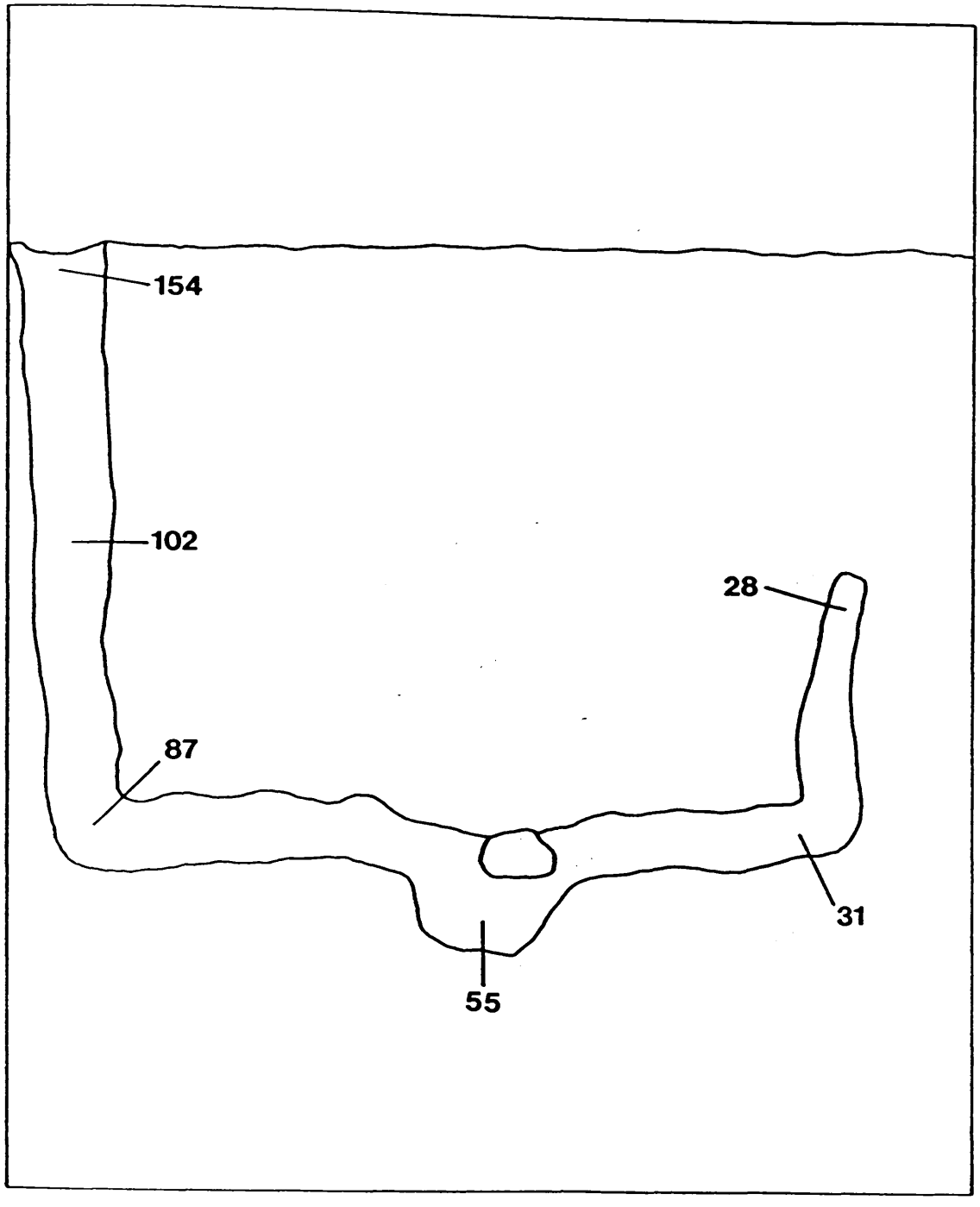

154
102
28
87
31
55

FIG. 2.9 Recording of oxygen tension in a laboratory burrow of ***Calocaris macandreae*** over 7 hours. The catheter was placed approximately 15 cm from the nearest burrow opening. No pleopod irrigation was observed over the duration of the recording.

FIG. 2.10 Recording of oxygen tension in a laboratory burrow of ***Calocaris macandreae*** in which the burrow openings were deliberately collapsed (see text for details).

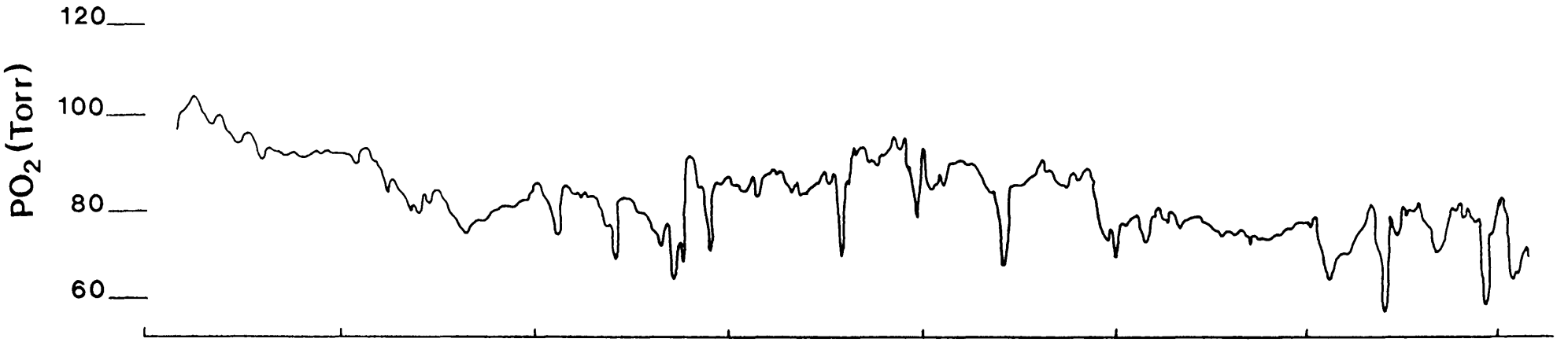


Fig. 2.9

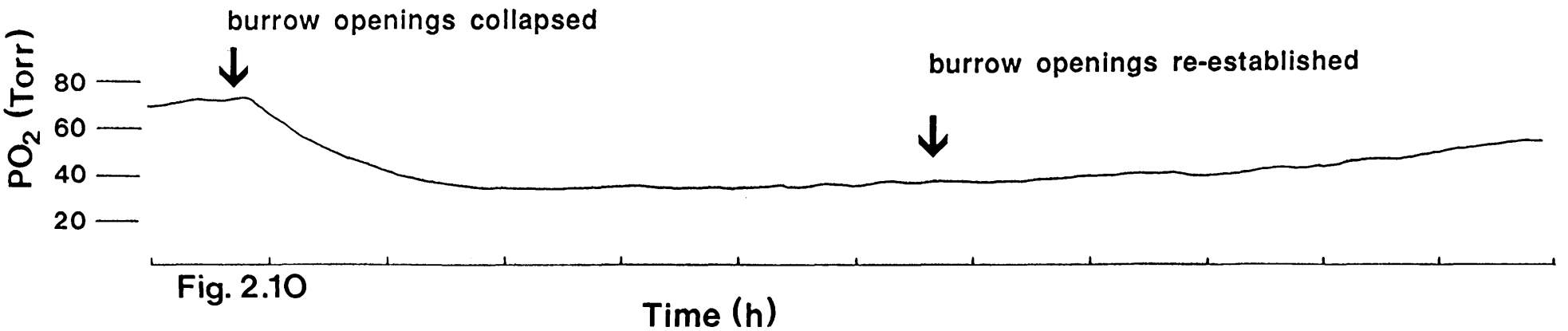


Fig. 2.10

As discussed above, no reliable values of PO_2 were obtained from field burrows.

Results from the experiment in which the burrow openings were deliberately collapsed are shown in Fig. 2.10. The burrow PO_2 (measured at 10 cm burrow depth) declined steadily over approximately 2 hours, before recovering to initial values over the following 3 - 4 hours. Visual inspection of the burrow confirmed that the start of recovery corresponded to the re-establishment of the major burrow entrance.

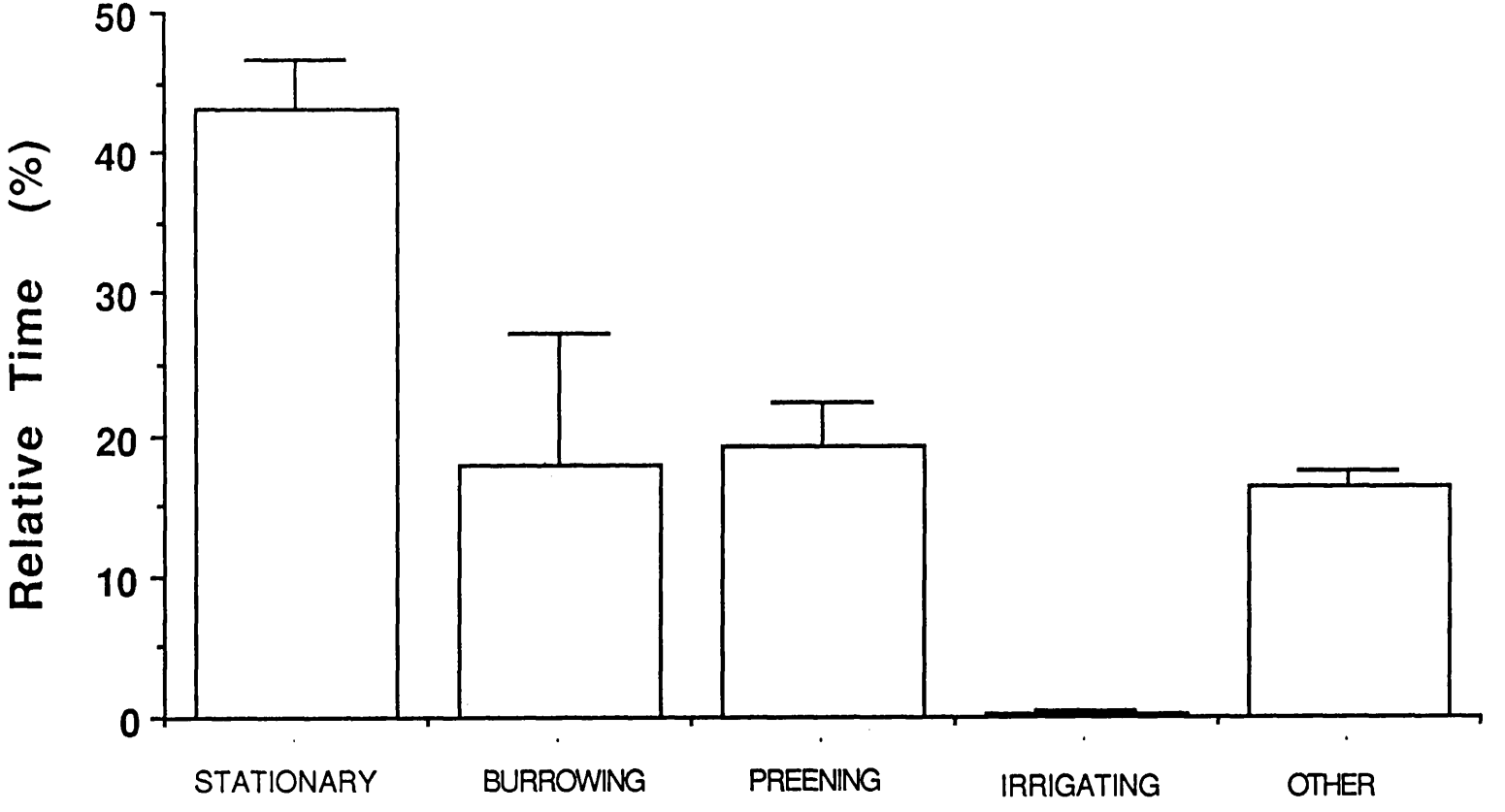
PCO_2 values proved difficult to measure reliably in burrow water samples, although the reasons for this are unclear. The few values which were obtained were in the range 0.2 - 0.3 Torr, not significantly different from air-equilibration values.

2.3.3. Behaviour patterns of *Calocaris macandreae*

The relative times spent in various behavioural activities by an individual *Calocaris* in 'normal' conditions are shown in Fig. 2.11. These conditions were defined as normoxia in the overlying water column and a water temperature of 10-12°C. Behavioural patterns were recorded and analysed for three individuals; Fig. 2.11 shows means and standard deviations of five periods of observation (each 6 hours) on one individual. The behaviour patterns shown by all non-ovigerous individuals were similar.

The most common activity pattern shown by *Calocaris* in terms of relative time was the apparently stationary type (43%). Approximately equal amounts of time (15 - 20%) were spent in burrowing, preening and locomotory behaviour (Fig. 2.11). One egg-carrying individual was observed to devote up to 60% of its time preening the pleopods, to

FIG. 2.11 The relative time spent in various activities (defined in the text) by an individual ***Calocaris macandreae*** under 'normal' conditions in a laboratory burrow. Error bars show standard deviation calculated from five 6-hour video recordings.


Relative Time (%)
50
40
30
20
10
0
STATIONARY
BURROWING
PREENING
IRRIGATING
OTHER

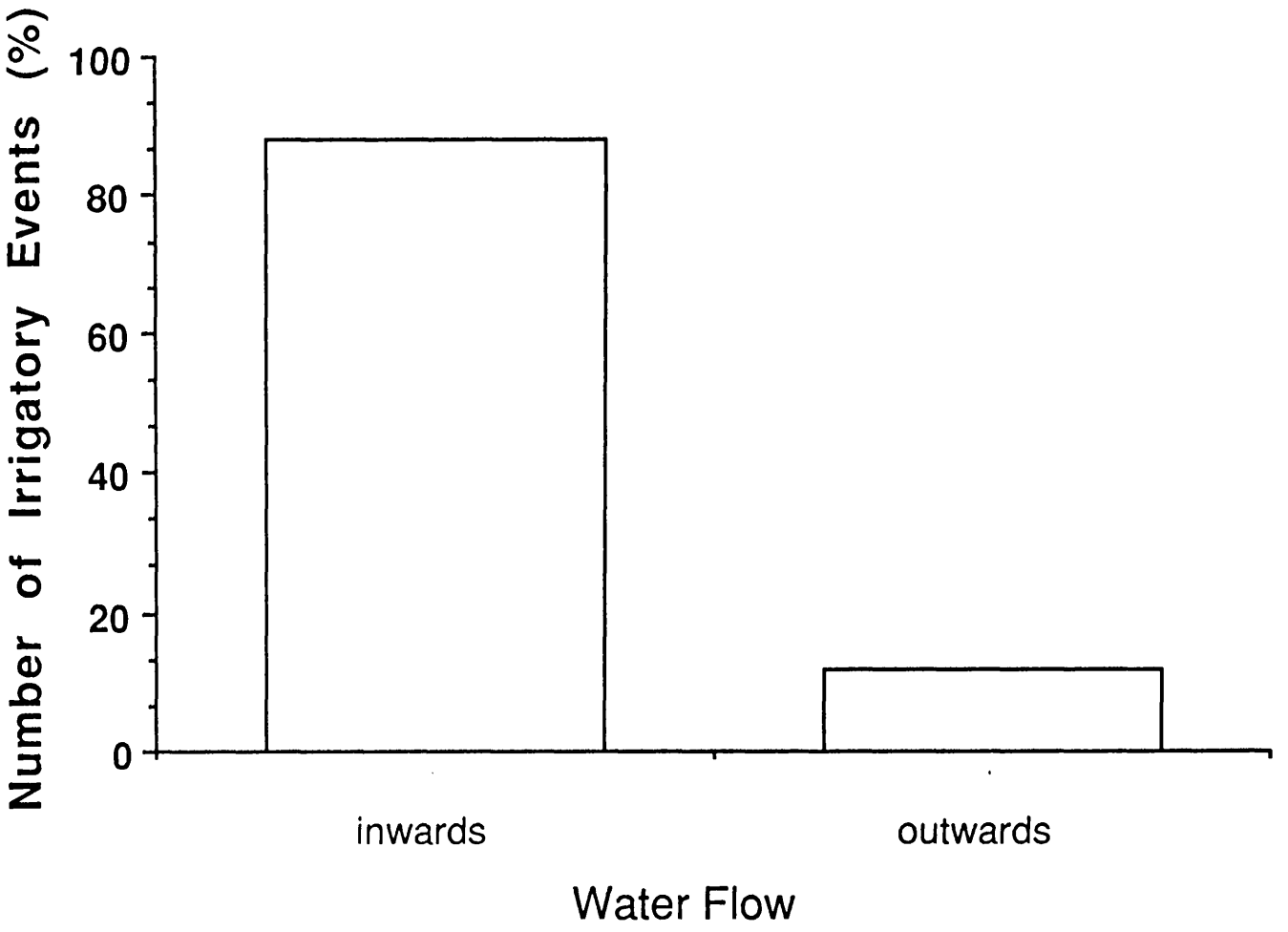
which the eggs are attached.

Comparatively little time (< 2% of each 6 hour period) was spent irrigating the burrow by pleopod beating in normal conditions (Fig 2.11). However, this activity usually occurred as several short episodes of 5 - 20 pleopod beats interspersed with 5 - 30 s stationary or locomotory periods. While irrigating, the animal was usually positioned at a particular position (or one of only a few positions), often at a tripartite junction or at the bottom of an entrance shaft. ***Calocaris*** irrigates with all four pairs of unspecialised pleopods (the first pair are specialised reproductive appendages), beating in a metachronal rhythm. The telson is usually raised, allowing water flow to be directed backwards.

The attitude of an individual ***Calocaris*** during episodes of burrow irrigation is shown in Fig. 2.12. As expected, there was a significant tendency for the animal to face the burrow entrance and pump exterior water into the burrow (with burrow water presumably expelled through other burrow openings).

The contribution of the 'piston effect' of locomotory activity to burrow water exchange is difficult to assess. A rough estimate suggests that ***Calocaris*** occupies 50 - 75% of the cross-sectional area of the burrow, although this varies with varying burrow diameter at different parts of the burrow. In addition, movement of undisturbed ***Calocaris*** within burrows was usually slow (approximately 1 - 5 cm s^{-1}) so that water shunting in front of a moving animal may be negligible. However, fluctuations in burrow water PO_2 (presumably due to mass flow of water) were evident in some traces (e.g. Fig. 2.9), and the most

FIG. 2.12 Relative number of occasions on which an individual ***Calocaris macandreae*** was observed to irrigate the burrow resulting in water flow out of, and into, the nearest burrow opening. Observations were made during conditions of 'normal' oxygen tension.


Number of Irrigatory Events (%)
100
80
60
40
20
0
inwards
outwards
Water Flow

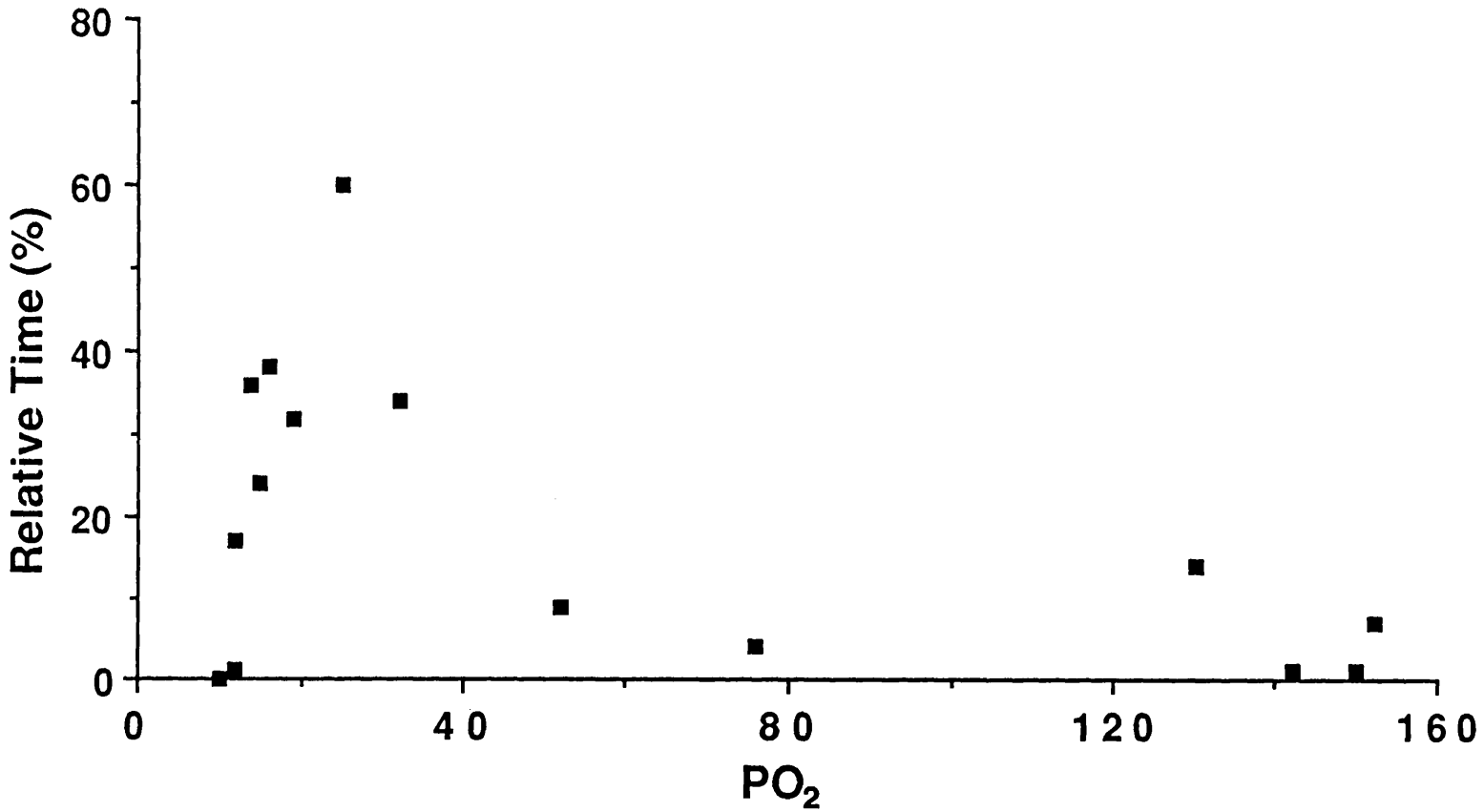
likely cause is animal movement.

Disturbed *Calocaris* are capable of rapid and sustained movement by tailflicking. Crude experiments with animals in open tanks suggest that velocities of 10 - 20 $cm.s^{-1}$ may be attained, and that the tailflick reaction can be sustained for 8 - 10 minutes given a gross mechanical stimulus (NB. see also Chapter 7: Metabolic Adaptations). Within burrows, spontaneous tailflicking was rarely if ever observed; and induced tailflicking was of short duration and distance and tended to cause damage to burrow walls. Although tailflicking may result in considerable movement of burrow water, it is unlikely to be of significant functional importance to burrow irrigation.

The behavioural response of *Calocaris* to induced environmental hypoxia is shown in Fig. 2.13. The incidence of irrigatory behaviour increased rapidly as surface water PO_2 decreased below 50 Torr. Below a PO_2 of approximately 20 Torr, the relative time spent irrigating decreased (eventually to zero) and the animals assumed a stationary, 'quiescent' behavioural pattern. It should be noted that these data were analysed in 10 minute periods and are therefore more variable than the 6 hour analyses described above.

No instances of *Calocaris* leaving the burrow as a result of environmental hypoxia were recorded. The only cases of animals leaving the burrow occurred during the initial stages of burrowing, and were of very short duration: 1 - 2 seconds. These cases involved animals removing sediment spoil from the burrow. During the later stages of burrowing, sediment was very rarely deposited outside the burrow, although field burrows occasionally showed evidence of recent activity in proximity to the openings. It remains a possibility, however, that

FIG 2.13 The relative time spent irrigating the burrow by an individual ***Calocaris macandreae*** during conditions of declining oxygen tension (measured in the overlying water column). The time course of oxygen depletion was 18 hours.


80
60
40
20
0
Relative Time (%)
0
40
80
120
160
PO2

animals may vacate the burrow during prolonged periods (> 24 hours) of enforced anoxia.

2.3.4. The General Biology of *Calocaris macandreae*.

A few observations on the general ecology of ***Calocaris macandreae*** are described below.

The stages of the moult cycle in ***Calocaris*** can easily be followed as a result of the growth of the polyzoan ***Triticella koreni*** on the cuticle (Eggleston, 1971). In the Loch Striven ***Calocaris*** population, approximately 80% of individuals in samples collected in May - August had ***Triticella*** growth. Recently moulted ***Calocaris*** were present in the population (as inferred from the condition of the exoskeleton) from mid-August - December. Egg-laying is apparently synchronised with moulting (Buchanan, 1963) and approximately 40% of individuals caught in November - January were berried. It therefore appears that the moult cycle in the Clyde population is similar to those observed in North and Irish Sea populations (Buchanan, 1963; Calderon-Perez, 1981). Although note was taken in all physiological experiments of the apparent stage in the moult/reproductive cycle of the experimental animal, no significant differences in physiological or metabolic parameters were observed (e.g. see Chapter 6).

The gut contents of 20 formalin-preserved ***Calocaris*** were examined. No identifiable remains were observed, although Buchanan's (1963) range of possible dietary remains was extended to include nematode spicules and dinoflagellate cysts (known to be common in sea-loch sediments; J. Lewis, pers. comm.). Several attempts were made to replicate the observations of Elmhirst (1935), Buchanan (1963) and Nash *et. al.*

(1984), who noted scavenging of macrofaunal material, and its retrieval into the burrow. However, such behaviour was not observed in the present study.

2.4 DISCUSSION

2.4.1. Burrow Structure of *Calocaris macandreae* and other thalassinids.

There is now a large amount of information (widely scattered in the geological, paleontological, ecological and taxonomic literature) concerning the burrow structures of thalassinids. Dworschak (1983) provided a review of most of the available literature to that date.

The burrow structure of ***Calocaris macandreae*** has been well described previously (Nash *et al.*, 1984; Atkinson and Nash, 1985; Atkinson, 1986; Smith, 1988), together with the stereotyped developmental sequence observed in the laboratory (Nash *et al.*, 1984). There appears to be some variation in structure (particularly with regard to complexity) between geographical locations. Smith (1988) reported a mean of 7 openings per burrow cast from a comparatively dense population at the Creag Isles (Lynn of Lorne), in contrast to the previous report (Nash *et al.*, 1984) of 4.7 openings per burrow at sites in Loch Riddon (Firth of Clyde), Camas Nathais (Lynn of Lorne) and Loch Brollum (Outer Hebrides). It is possible that burrow complexity (number of openings) is related to sediment type (as is population density: see below).

The burrows (of mud-burrowing) ***Upogebia*** species are comparatively simple, essentially Y-shaped structures (species: ***U. pusilla***, ***U. pugettensis***, ***U. major***, ***U. affinis*** and possibly ***U. deltaura***, with more limited data on ***U. africana***, ***U. carinicauda*** and ***U. wuhsienweni***). This also appears to be true of ***U. stellata***, whose burrowing behaviour is under current investigation (L.A. Calder, pers. comm.).

The burrows of ***Callianassa*** species may be considerably more complex than those of ***Upogebia***. In general, they consist of a long vertical shaft leading to a gallery complex at a relatively deep level (typified

by *C. acanthochirus*: Shinn, 1968; *C. kraussi*: Forbes, 1973; *C. pontica*: Dworschak, 1983). The burrows of *C. californiensis*, *C. tyrrhena*, *C. pontica*, *C. mirim* and *C. japonica* have a structure in the form of an interconnecting network of shafts and tunnels (Swinbanks and Murray, 1981; Ott *et al.*, 1976; Dworschak, 1983; Rodrigues, 1966; Ohshima, 1967). The burrows of *C. subterranea* from the North Sea have been reported to conform to a 'network' pattern (Schäfer, 1972) although the structure of over 50 casts from Scottish west coast sea lochs is consistently that of a vertical shaft/gallery (Lutze, 1938; Hertweck and Reineck, 1966; Atkinson and Nash, 1985; Atkinson, 1987; Smith, 1988; R.J.A. Atkinson, pers. comm.). The most probable explanation is that the cores that Schäfer examined, intersected the gallery system. Another form of *Callianassa* burrow was suggested by Dworschak (1983) to comprise a simple, deep vertical shaft (*C. major*: Pohl, 1946; *C. guassetinga*: Rodrigues, 1966) although it is possible that these may represent incomplete casts of the shaft /gallery type.

The structure of burrows of the laomediid *Jaxea nocturna* is well described for an Adriatic population (Hohenegger and Pervesler, 1985; Pervesler and Dworschak, 1985). The burrow structure in a population in Loch Sween appears to show similarities to that in the Adriatic (Atkinson, 1987), although there is considerable variation. The Adriatic burrows are similar in form to the *Callianassa* 'network' pattern, while some burrows from Scottish populations are unbranched, spiral tunnels.

The structure and function of the large burrows of *Thalassina anomola* are not well understood, however, some aspects have been described by Sankolli (1963), Bennett (1968) and Berry (1972).

There are few descriptions of axiid burrow structures in the literature (with the exception of ***Calocaris macandreae***). A good description of the burrows of an Indian Ocean ***Neaxius*** species (in calcareous lagoonal sediment) showed that several swollen chambers are linked in series by a succession of shafts (Farrow, 1971). ***Axiopsis (Axiopsis) serratifrons*** was confined to areas of coarse calcareous sands mixed with dead coral rubble and pieces of pavement rock in Belize; while in an aquarium, a pair of ***Axiopsis*** constructed a "branching burrow system" (Kensley, 1980). Incomplete casts of the burrows of ***Axiorygma nethertoni*** were obtained from subtidal (>27 m) sand flats off Key Largo by Kensley and Simmons (1988), and consisted of 15 cm long vertical shafts usually blocked by coral or calcareous algal debris. The temperate/sub-arctic axiid ***Axius serratus*** usually constructs extremely deep, relatively simple burrows at high densities in polluted muds (Pemberton *et al.*, 1976), although it is also present in (presumably) non-polluted areas of the Canadian shelf (Risk *et al.*, 1978). A single specimen of ***Axius stirhynchus*** was observed to construct a branched burrow (apparently similar to that of ***Calocaris***) in a mud-filled aquarium (S.J. Anderson, unpubl. obs.) although the burrow structure in the field habitat (gravelly mud between large granite boulders) could not be established. Another specimen was observed to construct a U-shaped burrow, with a third riser (R.J.A. Atkinson, pers. comm.).

In conclusion, there appears to be little correlation between taxonomic position and burrow structure in the Thalassinidea, with the exception of the comparatively uniform burrows of the upogebiids. Callianassids and axiids are found in a variety of substrate types from coral rubble to fluid muds. Burrows may be lined or unlined (see Dworschak, 1983 for discussion), relatively permanent or highly dynamic in structure,

and may be superficial in the substrate or up to 2.4 m (*Thalassina anomola*: Sankolli, 1963) or even 3 m (*Axius serratus*: Pemberton *et al.*, 1976) in depth. Burrow structure may also vary within species in response to sediment granulometry (Hertweck, 1972). The most likely functional implications of burrow morphology relate to feeding strategy (see below).

2.4.2. Population density of *Calocaris macandreae* and other thalassinids.

Estimates of population density obtained by diving observations, television and photography and grab sampling all give reliable results for megafaunal burrowers (Nash *et al.*, 1984; Atkinson, 1989). Low population densities (<0.3 m^{-2}) of *Calocaris macandreae* were recorded at the shallow locations in Loch Sween in the present study, although greater densities were present in the deeper areas examined by TV sledge (Atkinson, 1989). Atkinson (1986) reported densities of 0.8 - 14.0 m^{-2} in the Clyde Sea area, with the higher densities in the deeper water. Calderon-Perez (1981) found densities of 12.7 m^{-2} in the Irish Sea around the Isle of Man. *Calocaris* population densities of up to 20 m^{-2} have been reported in deep water (80 m) off the Northumberland coast (Buchanan, 1963), while densities reach 27 m^{-2} at 90 m in the Lynn of Lorne (C. Comely, pers. comm. in Nash *et al.*, 1984). The density of *Calocaris macandreae* populations appears to vary with depth (Nash *et al.*, 1984; Atkinson, 1986).

Reported population densities of thalassinids are often based on hole counts, with account taken of the average opening:burrow and burrow:occupant ratios. Reported densities vary widely; e.g. *Jaxea nocturna* up to 0.2 m^{-2} (Pervesler and Dworschak, 1985); *C. subterranea*

0.29 - 10 m^{-2} (Nash *et al.*, 1984; Atkinson, 1986); ***C. rathbunae*** 4:98 - 7.03 m^{-2} (Suchanek, 1983); ***U. deltaura*** 5 m^{-2} (Tunberg, 1986); ***Axius serratus*** 9 m^{-2} (Pemberton *et al.*, 1976); ***C. japonica*** 20 m^{-2} (Koike and Mukai, 1983); ***C. tyrrhena*** up to 60 m^{-2} (Dworschak, 1987); ***U. pusilla*** up to 416 m^{-2} (Ott *et al.*, 1976; Dworschak, 1981); ***C. californiensis*** over 500 m^{-2} (Posey, 1986). Extremely high densities are located on intertidal sand/mud flats (Ott *et al.*, 1976; Posey, 1986). Subtidal thalassinid population densities are usually lower than 10 m^{-2}.

The major non-stochastic factor which determines ***Calocaris macandreae*** population density may be the sediment granulometry (Buchanan, 1963; Nash *et al.*, 1984), in particular as it affects the sediment cohesiveness which may in turn affect burrow stability. In general, ***Calocaris macandreae*** populations are confined to areas where the silt+clay fraction (particles < 62 um) exceeds 20% of the sediment, and are most dense where silt+clay is above 50%. This is the case for most of upper Loch Sween, although a high density of ***Calocaris*** was found in a muddy sand (site 9; 69% sand, 13% silt and 18% clay: Atkinson, 1987). Further work (Atkinson, 1989) has shown that ***Calocaris macandreae*** is present in deeper water (> 25 m) throughout most of upper Loch Sween, in a more typical sediment habitat.

An alternative explanation for variation in the population density of ***Calocaris macandreae*** may involve interactions with other megafaunal burrower species (Smith, 1988), particularly ***Nephrops norvegicus*** which predate on ***Calocaris*** (Thomas and Davidson, 1962; Oakley, 1979; Bailey *et al.*, 1986; Smith, 1988). However, ***Nephrops*** and ***Calocaris*** do coexist at high densities in the field (Chapman, 1979). Possible interference interactions (amensalisms) between the burrowing species present in Loch Sween require investigation.

An interesting comparison of estimated increases in sediment surface area due to megafaunal burrows is possible. Koike and Makai (1983) calculated the increase due to a standing stock of 20 m^{-2} *C. japonica* to be 25%. Data from Dworschak (1981, 1983) suggest that a population density of *U. pusilla* of 87 m^{-2} results in an increase of only 3% (although at the population density recorded by Ott *et al* (1976) an increase of 16% can be calculated). Smith (1988) calculated average increases in surface area at four sites (due to total megafauna) to vary from 3.5 - 27.1%, with a maximum increase due to *Calocaris macandreae* of 13.6%. The megafaunal densities at the sites of low burrow surface area were thought to be limited by external factors (disturbance, excessive organic enrichment). For both intertidal and subtidal areas, therefore, the maximum megafaunal burrow surface areas recorded are in the range 15 - 25 area%. Whether megafaunal burrower density is limited by intra- or interspecific competition for burrow structural characteristics (total burrow surface area, internal volume, occupied volume) or by other factors (organic carbon supply, sediment structural effects) would be an interesting subject for research. It is certainly intuitively apparent that callianassid shaft/gallery burrow structure has responded to a selection pressure to maximise the surface area/occupied volume ratio.

2.4.3. The effects of *Calocaris macandreae* burrows on sediment oxygenation.

The consequences of megafaunal burrowing activity on physical sedimentary processes, were categorised by Smith (1988) to include: direct interference (i.e. 'bulldozing'), particle mixing, resuspension, sedimentation, grain size alterations, compaction, detritus

introduction, and oxygenation. Only oxygenation (and associated redox) effects were considered in the present study, since it was considered that oxygen conditions within the sediment might affect respiratory conditions within the burrow.

The results of the present study of burrows of ***Calocaris macandreae*** suggested that the effects on sediment oxygenation resulting from burrowing, may occur on two separate scales. Large-scale (i.e. >5-10 cm definition) oxygenation of the sediment column was observed to a depth of 15 cm in both field and laboratory systems, on the basis of redox potential (Eh) measurements compared with observations from non-burrowed areas (e.g. Pearson and Stanley, 1979). Effective control measurements for this type of study are difficult, however, since sites which are non-burrowed, but comparable in other respects, are limited. The 'control' cores used in this study probably represented heavily bioturbated sediment which did not contain a megafaunal burrow when sampled. Smith (1988) used control cores from sites without a major burrow for at least 1 m radius, finding no significant differences in mean redox potentials between these and cores taken through and around the burrows of ***Nephrops norvegicus*** (although variation was significantly greater in burrow cores).

Small-scale (< 1 cm definition) patterns of redox potential around the burrows of thalassinids have been investigated by Ott *et al* (1976), Dworschak (1983) and in the present study. Ott *et al* (1976) described redox potential profiles from sediment between burrows, and from burrow walls, of ***Upogebia pusilla*** (as ***U. litoralis***) and ***Callianassa tyrrhena*** (= ***C. stebbingi***), both in aquaria and from an intertidal location. In all cases, they stated that burrow walls were more oxidised than sediment between burrows, with the oxidative effect more pronounced in

aquarium systems. The latter difference was attributed to the decreased exchange of water across the sediment-water interface in aquaria (due to reduced wave action). The thickness of the oxygenated layer surrounding the burrows was approximately 10 mm. Ott *et al* (1976) also found that the vertical shafts of the burrows of ***U. pusilla*** were frequently packed with bundles of seagrass leaves in various stages of decomposition. The redox potential in one of these shafts was strongly reduced compared to the surrounding sediment. Dworschak (1983) has also described a similar increase in redox potential around the burrow walls of ***U. pusilla*** and also noted a distinctive colour difference between burrow wall and surrounding sediment.

The present study found that, in both field and aquarium systems, large differences in sediment Eh were present throughout the depth range measured, contributing to sediment heterogeneity ('patchiness') on the 1 - 5 cm scale. A statistically significant difference could not be demonstrated, however, in redox potential between paired measurements of burrow walls sediment and 'control' surrounding sediment.

The conclusions from both the field study of Smith (1988) and the present study are that megafaunal bioturbation in sublittoral systems produces a large scale oxygenation (see also Rhoads and Boyer, 1982; Flint and Kalke, 1986) and an increase in redox variation over a small scale. Highly localised Eh changes associated with the burrow structures are, however, less pronounced than in the intertidal studies described above. As noted above, interactions between bioturbators and sedimentary characteristics are not simple. In a mesocosm study, Smith (1988) found that introduction of an individual ***Nephrops norvegicus*** apparently induced the formation of a distinct redox discontinuity

layer (characteristic of non-bioturbated sediments: Pearson and Stanley, 1979). The most likely reason for this observation was thought to be inhibition of macrofaunal density and bioturbation. An additional cautionary note is that sediment redox potential is not simply correlated with sediment oxygenation (J. Parkes, pers. comm. to R.J.A. Atkinson), as is usually assumed.

2.4.4. Burrow irrigation and respiratory conditions.

The findings of this study were that the water contained in the burrows of ***Calocaris macandreae*** was consistently and chronically hypoxic. Burrow water PO_2's varied from 150 - 170 Torr at the burrow openings to a minimum of 20 - 40 Torr in the deepest parts of the burrow. There was little evidence of regular irrigation, or of pronounced changes in oxygen availability with time. Values for burrow water PO_2 for other thalassinid and burrowing species were reviewed by Atkinson and Taylor (1988); selected data are shown in Table 4:

TABLE 4: Oxygen tension in thalassinid and sublittoral decapod burrows.

Species	Burrow oxygen (Torr)	Comments	Reference
Nephrops norvegicus (Nephropid lobster)	80-130	Laboratory studies	A.C. Taylor (unpub. obs.)
Goneplax rhomboides (Brachyuran crab)	70-110	Laboratory studies	A.C. Taylor (unpub. obs.)
Callianassa japonica	19-70	Laboratory studies	Koike and Mukai (1983)
Callianassa affinis	0.5-3		Congleton (1974)
Callianassa californiensis	21-47	Low/high tide respectively	Torres *et al.*, (1977)
Callianassa californiensis	15-25		Thompson and Pritchard (1969)
Callianassa jamaicense	0-119	Depending on location	Felder (1979)
Upogebia major	45-64		Koike and Mukai (1983)
Upogebia pugettensis	15		Thompson and Pritchard (1969)
Calocaris macandreae	20-40	Laboratory studies	This study

There is a general trend for conditions within the burrows of the intertidal species (all ***Callianassa*** and ***Upogebia*** species above), measured during tidal emersion, to be severely hypoxic, when oxygen exchange with the external environment is limited. Conditions within these burrows during immersion are likely to be less extreme (e.g. Torres *et al.*, 1977), since most of these species are known to exhibit irrigatory activity. The PO_2 values measured in aquarium burrows of species from similar (sublittoral) habitats to that of ***Calocaris*** are moderately hypoxic. These burrows are larger, and less complex in structure than ***Calocaris*** burrows, and water exchange rates are probably greater. Alternatively, burrows of some species (notably ***Callianassa subterranea***) are likely to be even more restrictive of water flow, and of greater surface area (mean area 1556 cm^2: Smith, 1988). Burrows of these species may therefore contain more hypoxic water (N.B. active irrigation is presumed for ***C. subterranea*** on the basis of observations of ejected water 'plumes': R.J.A. Atkinson, pers. comm.; S.J. Anderson, pers. obs.).

Complete anoxia has not been recorded in the burrows of ***Calocaris macandreae***, even under conditions of deliberate collapse of burrow openings. The increased irrigation activity response noted in ***Calocaris*** (and in ***C. californiensis***: Torres *et al.*, 1977; and ***C. jamaicense***: Felder, 1979), under experimentally increased hypoxia would tend to ensure that anoxic conditions are avoided.

The irrigatory activity (expressed as % time) of ***Calocaris macandreae*** was observed to be comparatively low (< 2%) in conditions of 'normal' oxygen availability (i.e. normoxic overlying water column). In

contrast, Torres *et al* (1977) measured a mean relative irrigatory time of 40 % in ***Callianassa californiensis***, while Dworschak (1981) measured 28% in ***Upogebia pusilla***. The former study of ***C. californiensis*** was conducted using an artificial burrow system (a glass tube; at a PO_2 of 155 Torr) and may therefore be of limited relevence to real burrow conditions. The study of Dworschak (1981) used aquarium burrow systems similar to those of the present study. However, ***U. pusilla*** is a filter-feeding species and 'irrigation' may therefore represent feeding activity in addition to its respiratory function. Also, intertidal species such as these may show increased burrow irrigation when oxygenated water is available (in order to compensate for reduced oxygen availability during emersion of the burrow openings at low tide).

It was not possible, in this study, to determine the volume of water exchanged between the burrow of ***Calocaris*** and the overlying water column. Net water flow could not be detected close to the burrow openings using thermistors (see above). An overflow apparatus was not thought suitable because of the low irrigatory activity of the species (and the presumed low volume). Dye release experiments gave inconclusive results. It is suggested that as ***Calocaris*** can be induced to increase irrigatory activity by exposure to hypoxia (see above), it might be possible to measure pleopod beat volume in an artificial burrow system and extrapolate to observed pleopod beat rates in the burrow. However, this was not attempted.

Tentative conclusions from this study are that loss of oxygen from the burrow to the surrounding sediment is probably substantial; and that the mechanisms of oxygen flux into the burrow may be complex (including passive diffusion, active irrigation, and possibly viscous

entrainment). A more quantitative assessment of oxygen fluxes within the burrow system should be possible.

2.4.5 The functions of the burrow of *Calocaris macandreae*.

Possible functions of thalassinid burrows which have been postulated include protection from desiccation (in intertidal species); protection from predation; and a feeding function (MacGinitie and MacGinitie, 1949). While it is likely that protection from desiccation and predation is an important function, the presence of such pronounced differences in species-specific burrow structure suggests that there is strong selection pressure for particular burrow architecture, which differs between species. Suchanek (1985) has suggested a classification of thalassinid (primarily *Callianassa* and *Upogebia* spp.) feeding strategies, with associated types of burrow structure. Filter/suspension feeders (mostly *Upogebia* spp.) construct shallow, simple U- or Y-shaped burrows, and filter suspended particles from the 'irrigatory' current. Detritus/deposit feeders construct temporary, deep, complex burrows, and feed on sorted sediment material. Large amounts of sorted spoil are produced and either plumed (resulting in mound-formation around the burrow opening) or deposited within the burrow. Seagrass/algae harvesters capture detrital vegetable material and store it within the burrow, possibly harvesting the subsequently enriched sediment. Their burrows are usually long, straight and deep.

Although the burrow morphology of *Calocaris macandreae* does not conform closely with any of Suchanek's *Callianassa/Upogebia* ecological categories, some aspects of the burrow morphology (in particular the constant re-structuring and burrowing activity) suggest that *Calocaris* may be categorised in the detritus/deposit feeding group. *Calocaris*

differs from most deposit feeding callianassids in the lack of mound formation.

The feeding ecology of ***Calocaris macandreae*** has been the subject of some debate. Gut contents analysis (Buchanan, 1963; Calderon-Perez, 1981; this study) suggests that indiscriminate sediment ingestion is the major dietary source. Behavioural observations during this study showed only sediment ingestion, although considerable sorting of sediment particles appeared to occur. There are several observations, however, of scavenging and burial of macrofaunal material (Elmhirst, 1935; Buchanan, 1963; Nash *et al.*, 1984), so that there is a strong possibility that 'gardening' of the burrow sediment occurs (although consumption of enriched sediment has not been observed). Organic carbon content values for ***Calocaris*** burrow wall sediment samples were not significantly greater than control samples (this study). Preliminary estimates of meiofaunal abundance around ***Calocaris*** burrows *in situ* did not suggest significant enrichment of burrow wall sediment (T.J. Ferrero and S.J.Anderson, unpub. obs.). Calderon-Perez (1981) has also suggested (largely on anatomical grounds) that filter-feeding may occur in ***Calocaris***. Buchanan (1963) noted that the molar and incisor processes, and gastric mill were well-developed in ***Calocaris***, implying that more substantial food items may be ingested.

A further type of feeding was described by Devine (1966) for the New Zealand species ***Callianassa filholi***. Gut contents analysis suggested that diatoms (nearly all ***Chaetoceros armatus***) which had settled onto the sediment were resuspended by a flicking action of the second pereiopod. The suspended material is filtered by the third maxilliped and ingested. Devine (1966) suggested that the burrow habitat enabled this fine organic material to be exploited in the turbid surf zone,

since this feeding method requires still water. Observations on ***Calocaris macandreae*** suggested that sediment sorting and ingestion in this species does not involve resuspension, is similar to that described for ***Callianassa californiensis*** by MacGinitie (1934), and does not involve the pereiopods.

Although the generalisations about the geographical distributions of thalassinid feeding types are oversimplified (for example the temperate ***C. subterranea*** has a characteristic deposit feeding burrow structure), the analysis of Suchanek (1985) probably has general validity for callianassids and upogebiids. ***Axius serratus*** and ***Neaxius sp.*** were included by Suchanek in the seagrass harvester category on the basis of an eelgrass lining to the burrow (Pemberton *et al.*, 1976, and Farrow, 1971, respectively). As discussed above, it appears that a variety of feeding strategies (scavenging and burial of carrion; and selective and non-selective deposit feeding) are available to ***Calocaris macandreae***. Calderon-Perez (1981) suggests that predation and filter-feeding are also possible and might be used in an opportunistic manner (although neither Calderon-Perez, R.J.A. Atkinson (and co-workers), nor myself have observed these).

Protection from predation is undoubtedly provided by the burrows of ***Calocaris macandreae***. All studies of ***Calocaris*** have suggested that the animals very rarely leave the burrows. Excursions are always brief (1 - 5 s) and are only made in order to deposit burrow spoil. During observations from a submersible, Chapman *et al* (1970; cited in Nash *et al.*, 1984) observed that ***Calocaris*** forced to leave the burrow were preyed on by ***Nephrops norvegicus***; no ***Calocaris*** were observed to leave the burrow naturally.

Calocaris macandreae is an important prey species for the fish *Glyptocephalus cynoglossus*, *Rhinanemus cimbrius* and *Galeus melastomus* (Mattson, 1981). Armstrong (1980) estimated predation rates of *Calocaris* by *Trisopterus minutus* equivalent to 2 - 3 individuals $m^{-2}.year^{-1}$ (Calderon-Perez, 1981), with considerably lower rates by *Gadus morhua* and *Scyliorhinus caniculus*. Predation rates by *Nephrops norvegicus* (Oakley, 1978) were calculated to amount to 2 - 9 individuals $m^{-2}.year^{-1}$ by Calderon-Perez (1981); a total predation rate for the Irish Sea population of 4 - 12 individuals $m^{-2}.year^{-1}$. This contrasts with the population model for the Northumberland coast of Buchanan (1963), which found almost no predation.

In conclusion, the causal factors which produce a selective advantage in the burrowing mode of life, and which determine burrow morphology, remain unclear,and are probably not simple. One factor which has historically been neglected in hypotheses of crustacean phylogeny (and even more so in physiology) is chance. Recently, Schram (e.g. 1982, 1983) has advanced the theory that stochastic processes are a major causal factor in crustacean phylogeny (so-called 'stochastic mosaicism'). Similarly, the details of burrow morphology may not have profound implications for gene selection in thalassinids. In fact, the conservative characteristics noted in the comparative morphology of mud-burrowing animals may result from the conservative nature of the behavioural mechanism (in turn, presumably of genetic origin) which controls burrow formation.

The behaviour patterns shown by *Calocaris macandreae* suggested a relatively sedentary mode of life (43% of total time spent apparently

stationary). There is very little directly comparable information on crustacean activity patterns (in natural situations) in the literature. Diel emergence behaviour of the burrowing lobster ***Nephrops norvegicus*** has been studied, particularly in relation to commercial trawling (Atkinson and Naylor, 1976; Oakley, 1979; Chapman and Howard, 1979). Emergence from the burrow occurs mainly at dawn and dusk. Atkinson and Naylor (1974) also suggested a high level of nocturnal activity in the burrow. The general conclusion is probably warranted that comparative activity levels are lower in thalassinids than in other crustaceans (and that this either results from, or is reflected in physiological parameters).

As discussed below, sedentary behaviour is reflected in many aspects of the respiratory physiology of ***Calocaris macandreae***. The 'cause-and-effect' relationships between the selective pressures which have influenced behavioural patterns, respiratory physiology and metabolic adaptations are unclear. Presumably all the above characteristics developed together during the evolutionary history of the species. However, it is (at present) impossible to postulate a consistant evolutionary pathway for the thalassinids (to include ***Calocaris macandreae***), based on known current species and fossil record. This approach has been useful in integrating studies of other taxonomic/ecological groups (for example, land invasion by the talitrid amphipods: Friend and Richardson, 1986; Spicer *et al.*, 1987). It is interesting to note here that the Thalassinidea are a comparatively ancient group (see above), as also is the burrowing mode of life within the Crustacea.

An additional conclusion from this study and those of others, such as Dworschak (1981), and Smith (1988) is the validation of the use of

aquaria and mesocosm systems for the investigation of megafaunal ecology, behaviour and respiratory physiology. It appears that results obtained from these experimental systems are relevant to field conditions. In this respect, thalassinid shrimps are a particularly useful group in which to study the interactions between crustacean ecology and physiology since they occupy a defined, limited ecosystem which is amenable to replication and manipulation in the laboratory. The only problem is catching them!

CHAPTER 3. BRANCHIAL MORPHOLOGY IN *CALOCARIS MACANDREAE* AND OTHER THALASSINIDS.

3.1 INTRODUCTION

The behavioural adaptations of ***Calocaris macandreae*** to the mud-burrowing niche, and the physical characteristics of the burrow environment were investigated in previous chapters. The remainder of this thesis will examine some aspects of the morphology, physiology and metabolism of ***Calocaris*** and other thalassinids in this context.

The anatomy and functional morphology of branchae in decapod crustaceans has been the subject of considerable attention. Decapod gills have been classified according to their relationship with the thoracic appendages (pleurobranchs, arthrobranchs and podobranchs defined below, following McLaughlin, 1983; in turn following Huxley, 1880). Such characteristics as the presence and type of branchae, exopods and endopods of the maxillipeds and pereiopods (the 'gill-formula') have long been used as taxonomic features. In morphological terms, decapod gills have been classified as trichobranchs, phyllobranchs and dendrobranchs (also described below), although intermediate structural forms are found. The branchial morphology of the thalassinids is particularly interesting in this respect since they possess a gradation of gill types (as befits their uncertain taxonomic position). The branchial morphology of a number of thalassinid species (all found around the U.K.) was therefore examined, using scanning electron and light microscopy.

The surface area of the gills of decapods has been found to be comparable to those of fish (cf. Hughes *et al.*, 1969 with Hughes and Morgan, 1973), and to be correlated with habitat and activity (Gray,

1957). In view of the respiratory conditions encountered by thalassinids, the branchial surface area of ***Calocaris macandreae*** was estimated (together with preliminary estimates for ***Upogebia stellata*** and ***U. deltaura***).

Finally, the detailed structure of the trichobranchs of ***Calocaris macandreae*** was examined, together with the overall morphology and ventilatory flow patterns of the branchial chamber, as a preliminary functional investigation of branchial morphology.

3.2 MATERIALS AND METHODS.

Both fresh and preserved material were used for investigation of branchial structure. Fresh *Calocaris macandreae* (held in sea water) were killed by rapid cooling to 4° C (in a fridge). Preserved material was stored in 5-10 % formal saline or alcohol.

3.2.1 Branchial morphology in *Calocaris macandreae* and other thalassinids.

The branchial morphologies of *Calocaris* and several other thalassinid species were examined using Scanning Electron Microscopy (SEM). The species examined were: *Calocaris macandreae*, *Axius stirhynchus*, *Jaxea nocturna*, *Upogebia stellata*, *U. deltaura*, *Callianassa subterranea* and *Callianassa tyrrhena*. *A. stirhynchus* was collected from an intertidal location in Pembrokeshire by P.G. Moore; the specimen of *J. nocturna* was obtained from Loch Creran by C.J. Smith. *U. stellata* and *U. deltaura* were captured by dredging with a ring dredge in the Fairlie Channel just offshore from the Lion Rock (Isle of Cumbrae, Firth of Clyde). Specimens of both *Callianassa* species were obtained from the Robertson collection of the University Marine Biological Station, Millport. With the exceptions of *Calocaris macandreae* and *U. stellata*, only single, preserved specimens were available (the specimen of *Callianassa tyrrhena* dated from 1951). The quality of the specimens had therefore (in some cases) deteriorated to some extent. However, the type (and in most cases number) of gills present could be determined for each species.

The gills were described using the nomenclature of McLaughlin (1983). The term 'mastigobranch' is used here following the definition of McLaughlin (1983) as "a small lamellar structure arising at the base of the epipod". The term has been used ambiguously (to mean the complete

epipod, with an implied respiratory function) in previous literature (e.g. Calman, 1909). The derivation of the mastigobranch, setobranch, podobranch and anterior arthrobranch from the original epipod is discussed by Gurney (1942). In addition, the terms 'epipod' and 'endopod' are used (in preference to the earlier terms 'epipodite' and 'endopodite').

Whole animal material was prepared by dissecting off the abdomen and branchiostegite and fixing, staining and coating the specimen using the SEM preparative protocol given in Appendix 3.

Scaphognathites from several individuals of *Calocaris* were examined using a dissecting microscope (magnification X25) with eyepiece graticule.

3.2.2 Gill Anatomy in *Calocaris macandreae*.

Individual gills were removed from fresh *Calocaris* and were prepared for SEM using the protocol presented in Appendix 3.

Several gills were also dehydrated in alcohol, stained with iron haemotoxylin and eosin, and sectioned for light microscopy. These sections were examined and photographed using both Wild dissecting and Leitz compound microscopes.

In addition, several gills were silver-stained using the method of Holliday (1988). The dissected gills were rinsed three times in deionised water and immersed in 0.5 % $AgNO_3$ for 30 seconds. The gills were then washed in deionised water a further three times and developed for 30 s in undiluted Kodak D-19 developer. This method stains areas of the gills which are highly chloride-permeable; AgCl forms in these

areas and on developing, molecular silver (a black precipitate) is formed. The stained gills were examined and photographed using a Wild dissecting microscope fitted with an Olympus camera system.

3.2.3 Gill Formula in *Calocaris macandreae*.

During the course of gill surface area measurement, the gills of 25 ***Calocaris*** were dissected. This allowed some assessment of variation in the gill formula (i.e. the number and type of gills on each appendage) in ***Calocaris***.

3.2.4 Gill Surface Areas of *Calocaris macandreae* and *Upogebia stellata*.

After removal of the branchiostegite, individual gills were removed from fresh and preserved ***Calocaris*** and placed in sea water on a microscope slide. The following dimensions of the filamentous gills were measured (Fig. 3.1) using a dissecting microscope (magnifications X12 to X50; for terminology of gill structure see below):

(1) length of gill axial filament
(2) basal diameter of axial filament
(n) number of filaments
(3) length and (4) diameter of all side filaments of one side of the gill.

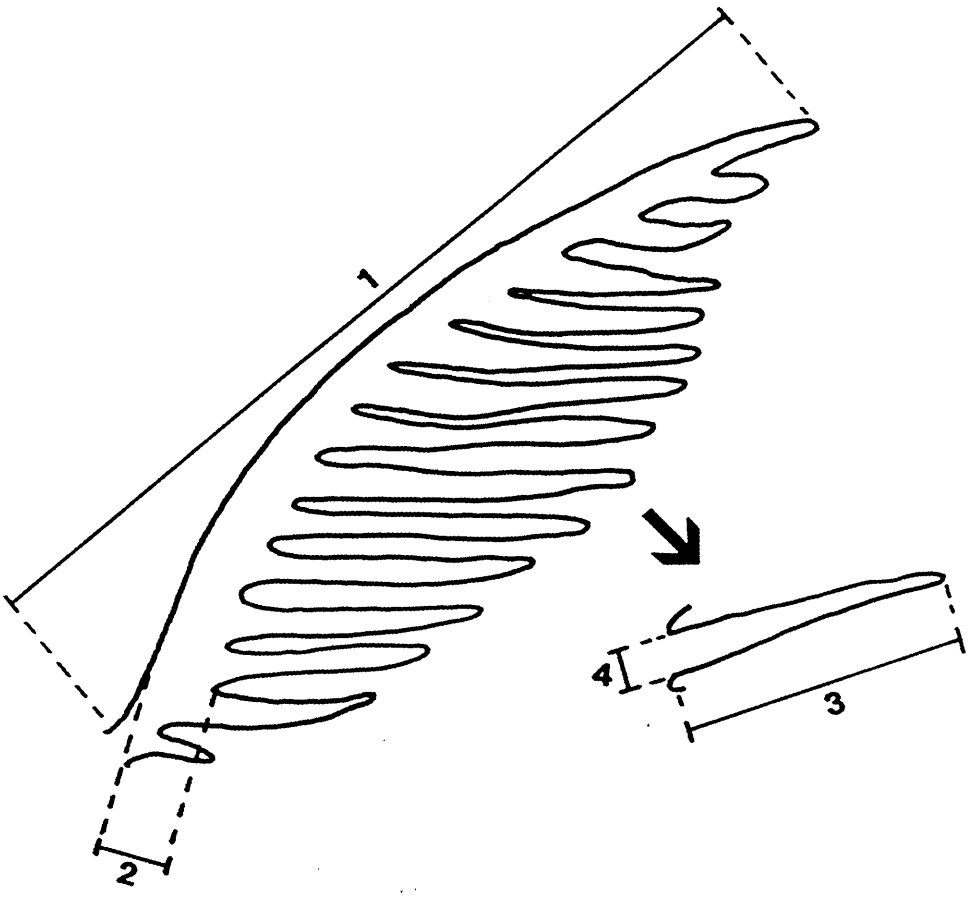
The length and width of the lamellae of the podobranchs were also measured (Fig. 3.1).

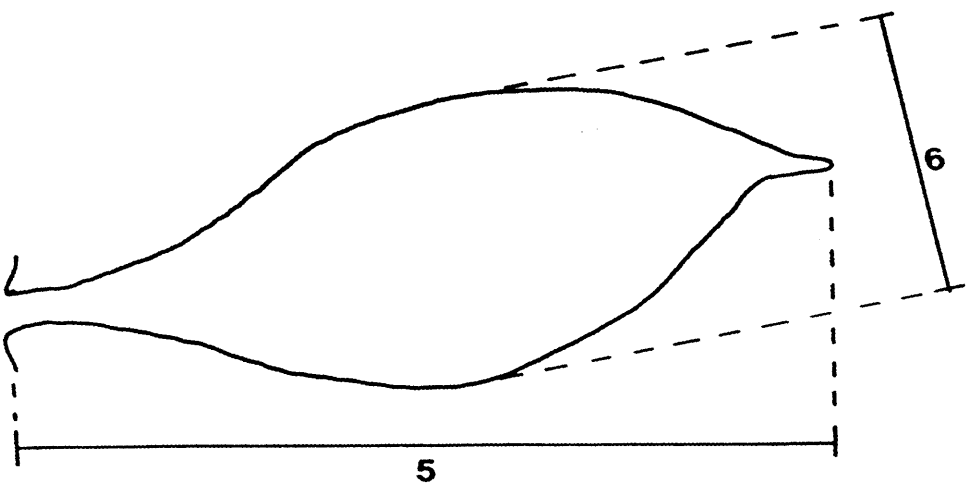
The surface area of each of the filamentous gills was calculated assuming that the axial filament was conical and the side filaments were cylindrical in shape. As preliminary observations confirmed that the branchial areas on each side of several individuals were identical,

FIG 3.1 The measurements used for calculation of gill surface area in ***Calocaris macandreae***.

Trichobranchs: 1) length of gill axis. 2) basal width of gill axis. 3) length of each gill filament. 4) basal width of each gill filament. The number of gill filaments was also recorded.

Mastigobranchs: 5) length of mastigobranch. 6) maximum width of mastigobranch.


1
2
3
4


5
6

only gills from the left side of specimens were measured. To allow comparison with data from the literature, gill areas are expressed as total gill area per g fresh body weight ('specific gill area', in $mm^2 . g^{-1}$).

The accuracy of the method described above was assessed using a 'pictorial' method (Gray, 1957) in which the individual gills were drawn using a dissecting microscope fitted with a camera lucida. The gills were placed on a slide and flattened with a cover slip before drawing. The areas of the drawings were measured using a digitising pad and image analysis software (written by Dr. M. B. Burns), run on a BBC microcomputer. Gills from one side of two individuals were measured using both methods for comparison.

The gill areas of two specimens of ***Upogebia stellata*** were also determined. The gills of this species were measured using the direct method for filamentous gills outlined above, with account made for the observation that ***Upogebia*** gills have four rows of filaments. There are no podobranchs in ***Upogebia*** spp. (see below).

3.2.5 Gill Ventilation in *Calocaris macandreae*

The ventilatory patterns of the branchial chamber were examined by releasing dye from a Pasteur pipette at several locations close to the inhalant openings of an animal, and observing the flow patterns under a dissecting microscope. Several dyes were tried before satisfactory results were obtained using blue pen ink.

3.3 RESULTS.

3.3.1 Branchial chamber morphology of thalassinid shrimps.

The general arrangements of the branchial chambers of seven thalassinid species found in the U.K. are shown in Figures 3 - 9. The gills are attached either to maxillipeds 2-3 and pereiopods 1-4, to the articulations between these appendages and the thoracic body wall, or to the body walls of each pleuron. The gills occupy most of the branchial chamber formed between the branchiostegite and the body wall. The gills are described following the nomenclature of McLaughlin (1983), as pleurobranchs (attached to the pleural wall of each somite), arthrobranchs (attached to the coxa of each thoracic appendage) and podobranchs (attached to the appendage-body wall articulation). McLaughlin (1983) also differentiates trichobranchs (rows of filaments attached to a gill axis) and phyllobranchs (pairs of gill platelets attached to an axis), although she notes that intermediate gill structures are typically found in the thalassinids. Mastigobranchs (branches of the podobranchs with a lamellar structure) are also present in the two axiid and the laomediid species.

Calocaris macandreae

In 25 specimens examined, the number and position of gills in *Calocaris* were found to be quite variable, with 30% of the animals examined lacking at least one gill. In particular, the gills on maxilliped 2 and pereiopod 4 were liable to be absent and also showed greatest variation in size. The following gill formula was consistent with most specimens (see also discussion):

FIG. 3.2 Scanning electron micrograph of the left branchial chamber of ***Calocaris macandreae***. Scale bar 5 mm.

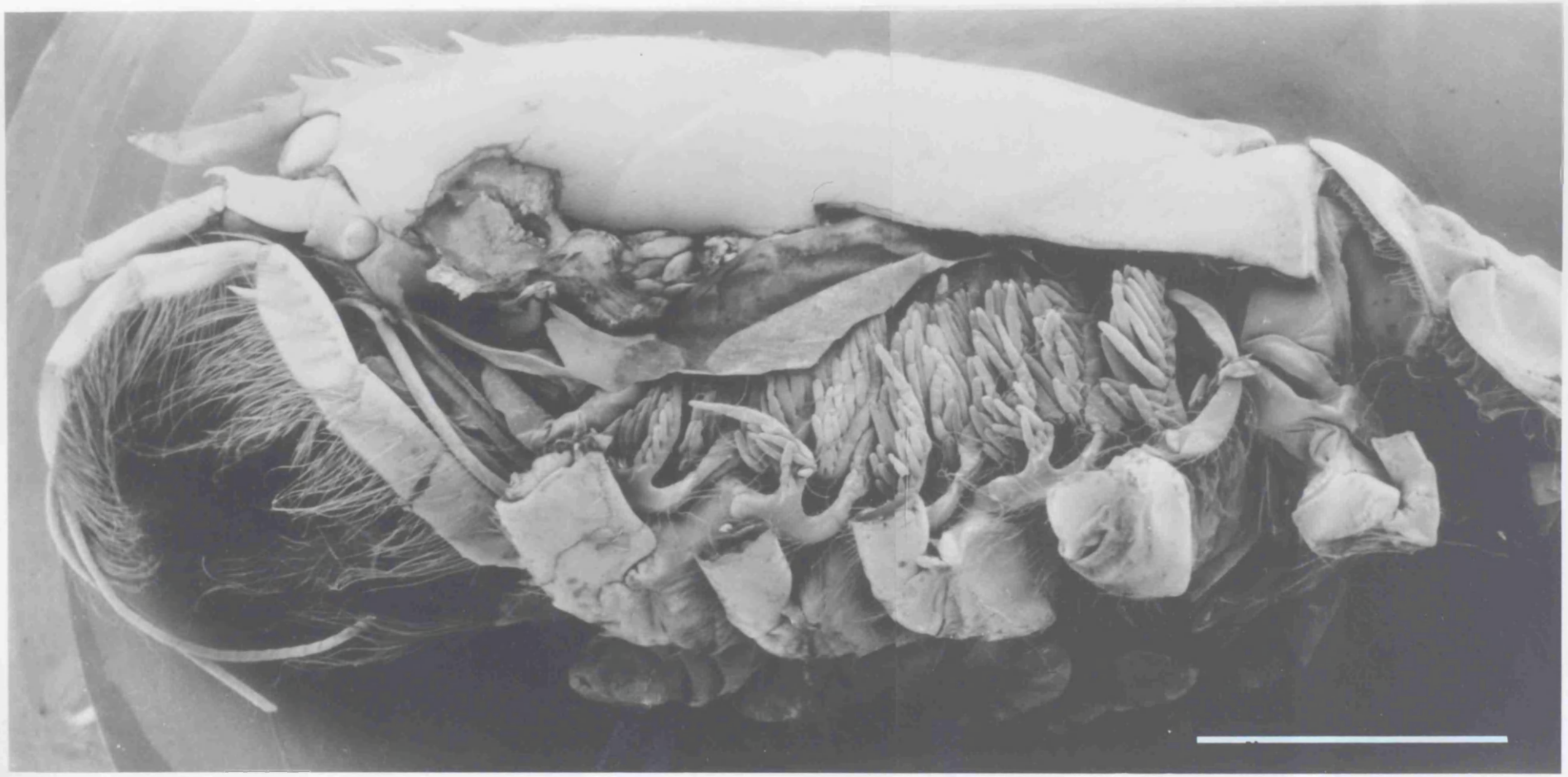


FIG. 3.3 Scanning electron micrograph of the left branchial chamber of ***Axius stirhynchus***. Scale bar 5 mm.

FIG. 3.4 Scanning electron micrograph of the left branchial chamber of ***Jaxea nocturna***. Scale bar 5 mm.

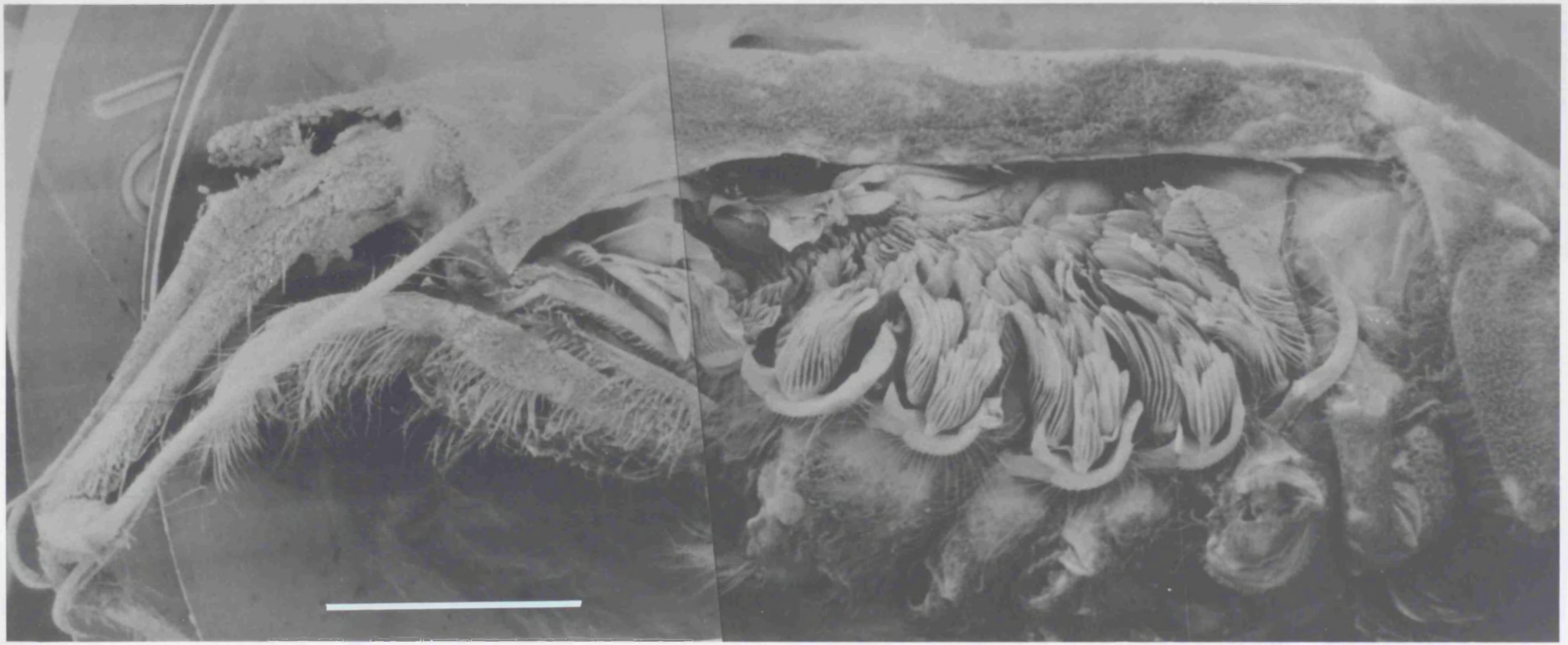


FIG. 3.5 Scanning electron micrograph of the left branchial chamber of ***Upogebia stellata***. Scale bar 5 mm.

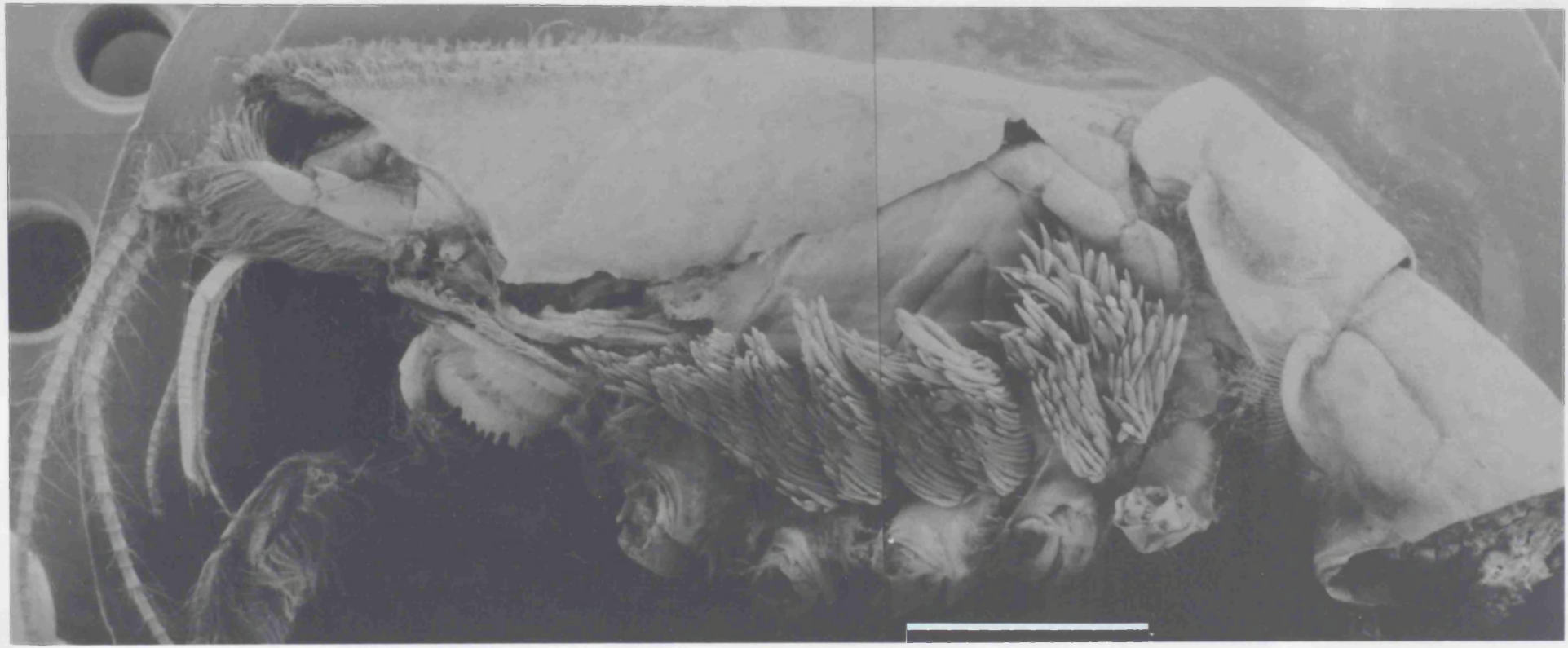


FIG. 3.6 Scanning electron micrograph of the left branchial chamber of ***Upogebia deltaura***. Scale bar 5 mm.

FIG. 3.7 Scanning electron micrograph of the left branchial chamber of ***Callianassa subterranea***. Scale bar 5 mm.

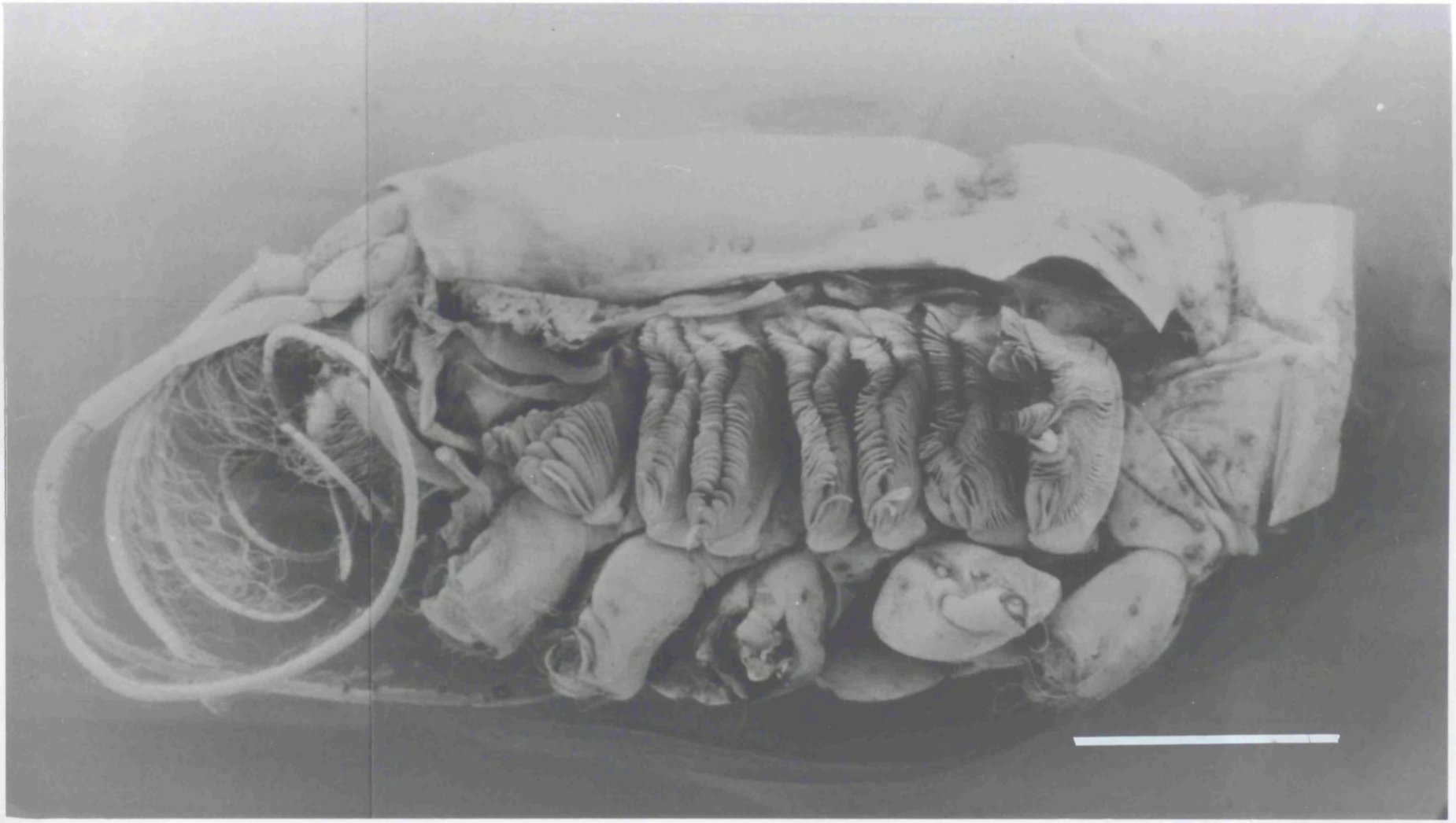


FIG 3.8 Scanning electron micrograph of the left branchial chamber of ***Callianassa tyrrhena***. Scale bar 5 mm.

	maxilliped			pereiopod				
	1	2	3	1	2	3	4	5
pleurobranchs	-	-	-	-	-	-	-	-
arthrobranchs	-	-	2	2	2	2	2	-
podobranchs: mastigobranch	-	1	1	1	1	1	1	-
trichobranch	-	1	1	1	1	1	-	-

As shown above, maxilliped 2 in each individual carried a branched podobranch (consisting of a mastigobranchiate branch (M) and a trichobranchiate branch (P)). Maxilliped 3 and pereiopods 1-3 each carried two trichobranchiate arthrobranchs (A1 and A2) and a branched podobranch. The two arthrobranchs were connected close to the point of attachment to the appendage. Pereiopod 4 usually had two arthrobranchs and a mastigobranchiate podobranch.

The trichobranchs in ***Calocaris*** consisted of two rows of paired filaments (approximately circular in cross-section) attached to the gill axis (see Figs. 3, 16). Thus each trichobranch carried between 30 - 60 filaments arranged in two rows.

The spatial arrangement of the gills within the branchial chamber in ***Calocaris*** was found to be highly consistent. The arthrobranchs and trichobranchiate podobranch of each appendage were positioned anterior to the mastigobranch , which was positioned with the lamellar surface facing forwards. This position is slightly disturbed in Figure 3, probably as a result of movement during the preparation. In all fresh specimens, however, the arrangement described above was observed.

The scaphognathite in ***Calocaris*** (and the other species examined) is a lamellar structure, roughly rectangular in shape, situated at the anterior end of the branchial chamber. It was positioned with the flat

surface horizontal such that the pumping action occurred in the vertical plane. A fringe of setae along the distal edge of the scaphognathite presumably provides a seal against the branchiostegite. An additional line of setae was present along the midline of the scaphognathite.

Axius stirhynchus: Fig. 3.3.

The branchial anatomy of ***Axius*** is distinctive amongst the U.K. thalassinids in that large, trichobranchiate pleurobranchs are present on pereiopods 2-4. This increases the number of trichobranchs to 4 on pereiopods 2-4, with a total of 18 (compared with 15 in ***Calocaris***). In other respects the branchial anatomy appeared to be similar to that of ***Calocaris***, with 2 arthrobranchs and a branched podobranch present on maxilliped 3 and pereiopods 1-4. The arrangement of the gills was also similar to that in ***Calocaris***, with the mastigobranchs positioned posterior to each cluster of arthrobranchs. The gills present on maxillipeds 1 and 2 were obscured in the single specimen available. The gill formula (with bracketed data for maxillipeds 1 and 2 taken from Calman, 1909 and Selbie, 1914) was:

('r' = rudimentary)	maxilliped 1	2	3	pereiopod 1	2	3	4	5
pleurobranchs	(-)	(-)	-	-	1	1	1	-
arthrobranchs	(-)	(r)	2	2	2	2	2	-
podobranchs: mastigobranch	(1)	(1)	1	1	1	1	1	-
trichobranch	(-)	(1)	1	1	1	1	1	-

Jaxea nocturna: Fig. 3.4.

The branchial formula of ***Jaxea*** was very similar to that of ***Calocaris***, with 2 arthrobranchs and a podobranch (trichobranch + mastigobranch) present on maxilliped 3 and pereiopods 1-3. Pereiopod 4, however, carries only two arthrobranchs and a mastigobranch. The trichobranchs

of *Jaxea*, however, are slightly different in structure, with two rows of flattened filaments arising from each trichobranch axis. The filaments remain rather narrow, so that the filament cross-section is approximately oval. The mastigobranchs on the single available specimen appear to be relatively smaller than those of *Calocaris* or *Axius*. The branchial formula (with bracketed data from Selbie, 1914) was:

	maxilliped			pereiopod				
	1	2	3	1	2	3	4	5
pleurobranchs	(-)	(-)	-	-	-	-	-	-
arthrobranchs	(-)	(1)	2	2	2	2	2	-
podobranchs: mastigobranch	(1)	(1)	1	1	1	1	1	-
trichobranch	(1)	(1)	1	1	1	1	-	-

Upogebia stellata: Fig. 3.5.

No pleurobranchs or podobranchs (including mastigobranchs) were observed in either species of *Upogebia*. Two trichobranchiate arthrobranchs were present on maxilliped 3 and pereiopods 1-4. Each trichobranch had four rows of filaments, approximately circular in cross-section. The gill formulae of *Upogebia stellata*, *U. deltaura* and the callianassid species were simpler than those of the axiid and laomediid species (bracketed data from Calman, 1909):

	maxilliped			pereiopod				
	1	2	3	1	2	3	4	5
pleurobranchs	(-)	-	-	-	-	-	-	-
arthrobranchs	(-)	-	2	2	2	2	2	-
podobranchs: mastigobranch	(-)	-	-	-	-	-	-	-
trichobranch	(-)	-	-	-	-	-	-	-

Upogebia deltaura: Fig. 3.6.

The branchial arrangement of *U. deltaura* appeared to be identical to that of *U. stellata*.

Callianassa subterranea: Fig. 3.7.

Callianassa subterranea had the same simplified gill formula as the two *Upogebia* species:

	maxilliped			pereiopod				
	1	2	3	1	2	3	4	5
pleurobranchs	*	-	-	-	-	-	-	-
arthrobranchs	*	-	2	2	2	2	2	-
podobranchs: mastigobranch	*	-	-	-	-	-	-	-
trichobranch	*	-	-	-	-	-	-	-

* Biffar (1971) states that no branchae are present on maxilliped 1 in the genus *Callianassa*.

The trichobranchs had a very different structure to those of the other thalassinids, however, being flattened to the extent of being intermediate in form between trichobranchiate and phyllobranchiate gills. Two rows of filaments/plates are present on each gill axis.

Callianassa tyrrhena: Fig. 3.8.

The branchial anatomy of *Callianassa tyrrhena* is similar to that of *C. subterranea*.

3.3.2 Gill Surface Area in *Calocaris macandreae* and *Upogebia stellata*.

There was a consistent difference between the gill area estimates produced by the pictorial and direct measurement methods for *Calocaris macandreae*. Gill area estimates for one individual are given below:

Table 3.1: Areas of each gill measured by two methods on a single *Calocaris macandreae* (mm^2). A1, A2, M and P = anterior and posterior arthrobranchs, mastigobranch and podobranch respectively.

	Max 2			Max 3				Per 1			
	A1	M	P	A1	A2	M	P	A1	A2	M	P
'pictorial'	2.20	1.16	1.82	8.54	8.28	2.86	2.08	9.62	9.03	2.84	2.46
'direct'	7.70	1.20	2.20	18.2	17.8	2.71	6.10	20.2	18.2	2.13	7.50
difference	5.5	0.04	0.38	9.6	9.5	-0.2	4.0	10.6	9.2	-0.7	5.0

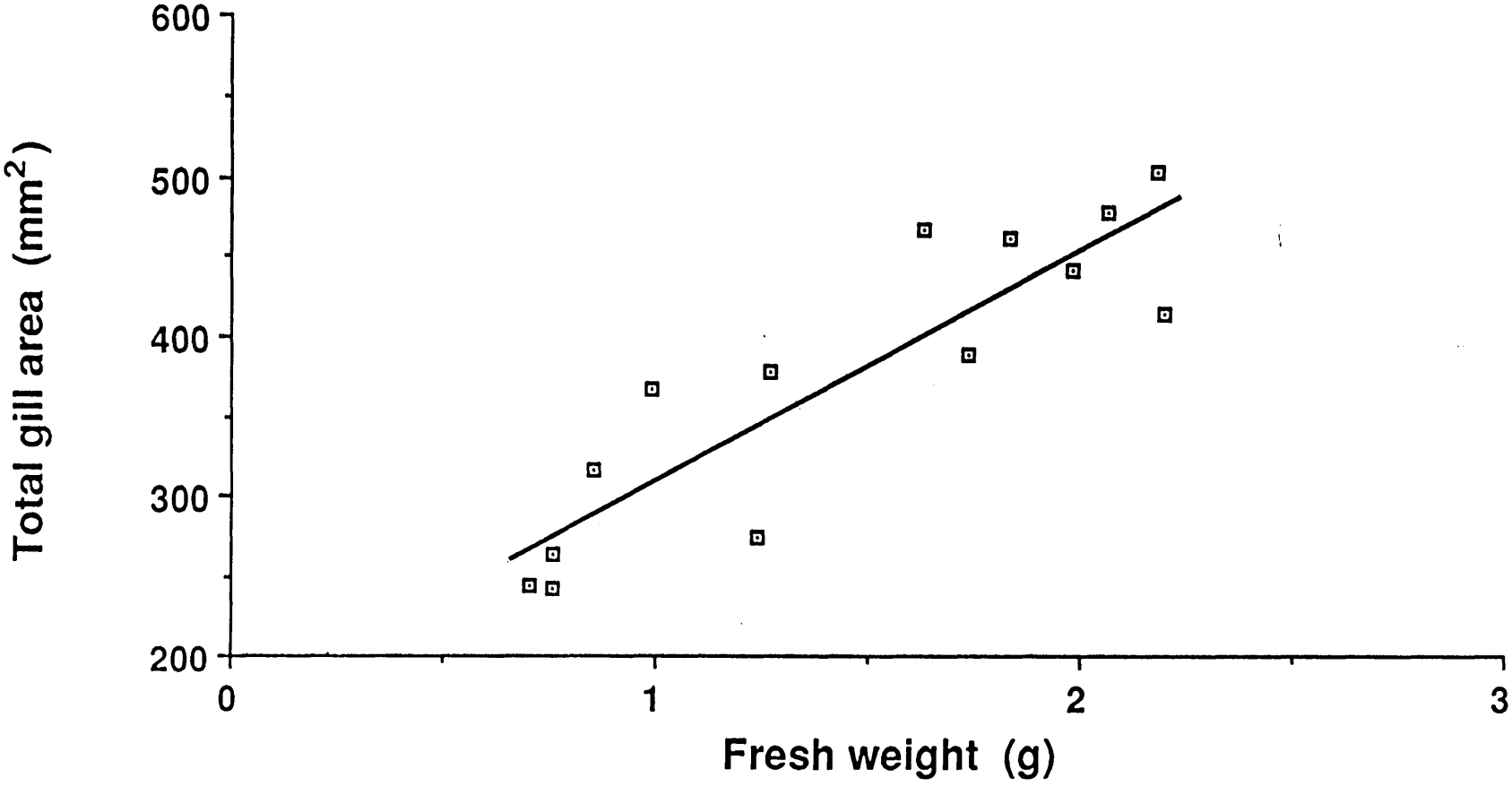
	Per 2				Per 3				Per 4		
	A1	A2	M	P	A1	A2	M	P	A1	A2	M
'pictorial'	12.1	7.68	2.66	4.96	14.7	12.1	2.50	3.02	12.2	6.72	3.32
'direct'	25.3	20.5	2.17	8.30	32.2	26.1	1.60	4.70	25.5	14.4	1.92
difference	13.2	12.8	-0.5	3.3	17.5	14.0	-0.9	1.7	13.3	7.7	

The areas of the trichobranchs, as measured by the pictorial method were significantly less (paired t-test, $P<0.001$) than those estimated by direct measurement. The gill areas as measured using the 'pictorial' method had a mean value of 48 $\pm$ 13 % of the areas as estimated by direct measurement. The reasons for this observed discrepancy between the two methods are considered in the discussion below. However, the direct measurements are considered to be more accurate. There was no significant difference (paired t-test, $0.05<P<0.1$) between the areas estimated by both methods for the mastigobranchs.

The relationship between total gill area (measured directly) and fresh body weight of 14 specimens of *Calocaris* is presented in Fig. 3.9. Within the size range 0.7 - 2.4 g (fresh weight) total gill area increased from approximately 240 to approximately 500 mm^2. There was little difference between fresh and preserved specimens. Specific gill

FIG. 3.9 The relationship between total gill area (mm^2) and fresh body weight (g) in 14 *Calocaris macandreae*.
The equation of the calculated regression line is:

$y = 167.5 + 144.0x$ ($r = 0.98$; $P<0.001$)


600
500
400
300
200
0
1
2
3
Total gill area (mm2)
Fresh weight (g)

area (s.g.a., i.e. total gill area / weight) decreased with size (Fig. 3.10) in an apparently linear relationship:

$$s.g.a. = 420 - 97.weight \quad (r=0.87;\ P<0.001).$$

For a 'standard' 1g animal, calculated gill area is 323 mm^2.

The increase in gill area with animal size (fresh body weight) resulted from an increase in the number of trichobranch filaments (Fig. 3.11) in addition to an increase in the dimensions of all the gills (expressed as filament length in Fig. 3.12).

Fig. 3.13 shows the relative areas of the gills on each appendage. The largest gills were located in the centre and posterior of the branchial chamber, with the greatest relative area attached to pereiopod 3.

The gill areas of two ***Upogebia stellata*** (1.74 and 1.51 g) and a single specimen of ***Upogebia deltaura*** (5.49 g) were measured using the 'direct' method. In both species the gills are all trichobranchs, with four rows of filaments. The measured weight-specific areas were: ***U. stellata***: 332 $mm^2.g^{-1}$, 356 $mm^{2}\cdot\cdot 1$; ***U. deltaura***: 122 $mm^2.g^{-1}$.

3.3.3 Detailed Gill Anatomy in *Calocaris macandreae.*

The two basic gill types found in ***Calocaris*** are trichobranchs (A1, A2 and P) and mastigobranchs (M). Typical trichobranchs from ***Calocaris*** are shown in Fig. 3.14A, B. As described above, each gill consists of an axis with two rows of filaments arising in pairs. Transverse sections of ***Calocaris*** trichobranchs (from an animal of about 2 g) showed that the gill axis contains two obvious blood channels which probably serve as afferent and efferent vessels (Fig. 3.15A, B). In contrast, the filaments contain only a single blood vessel. These

FIG. 3.10 The relationship between specific gill area ($mm^2.g^{-1}$) and fresh body weight (g) in 14 ***Calocaris macandreae***. The equation of the calculated regression line is:

$y = 420.0 - 97.1x$ ($r = 0.87$; $P<0.001$)

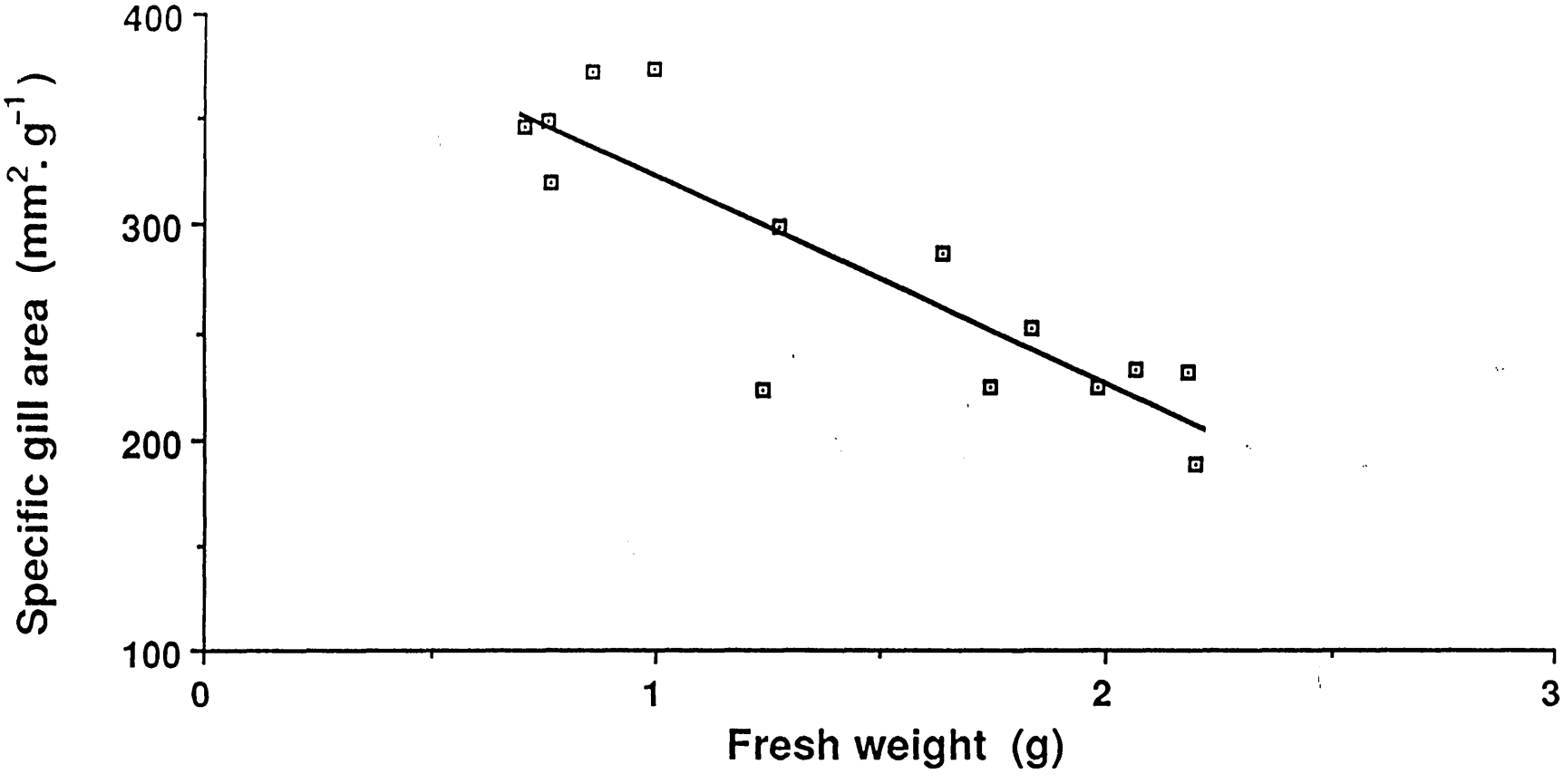

400
300
200
100
0
1
2
3
Specific gill area (mm^2. g^{-1})
Fresh weight (g)

FIG. 3.11 The relationship between total number of trichobranch filaments in the left branchial chambers and fresh body weight (g) in 14 ***Calocaris macandreae***. The equation of the calculated regression line is:

$y = 267.3 + 39.2x$ ($r = 0.63$; $P<0.05$)

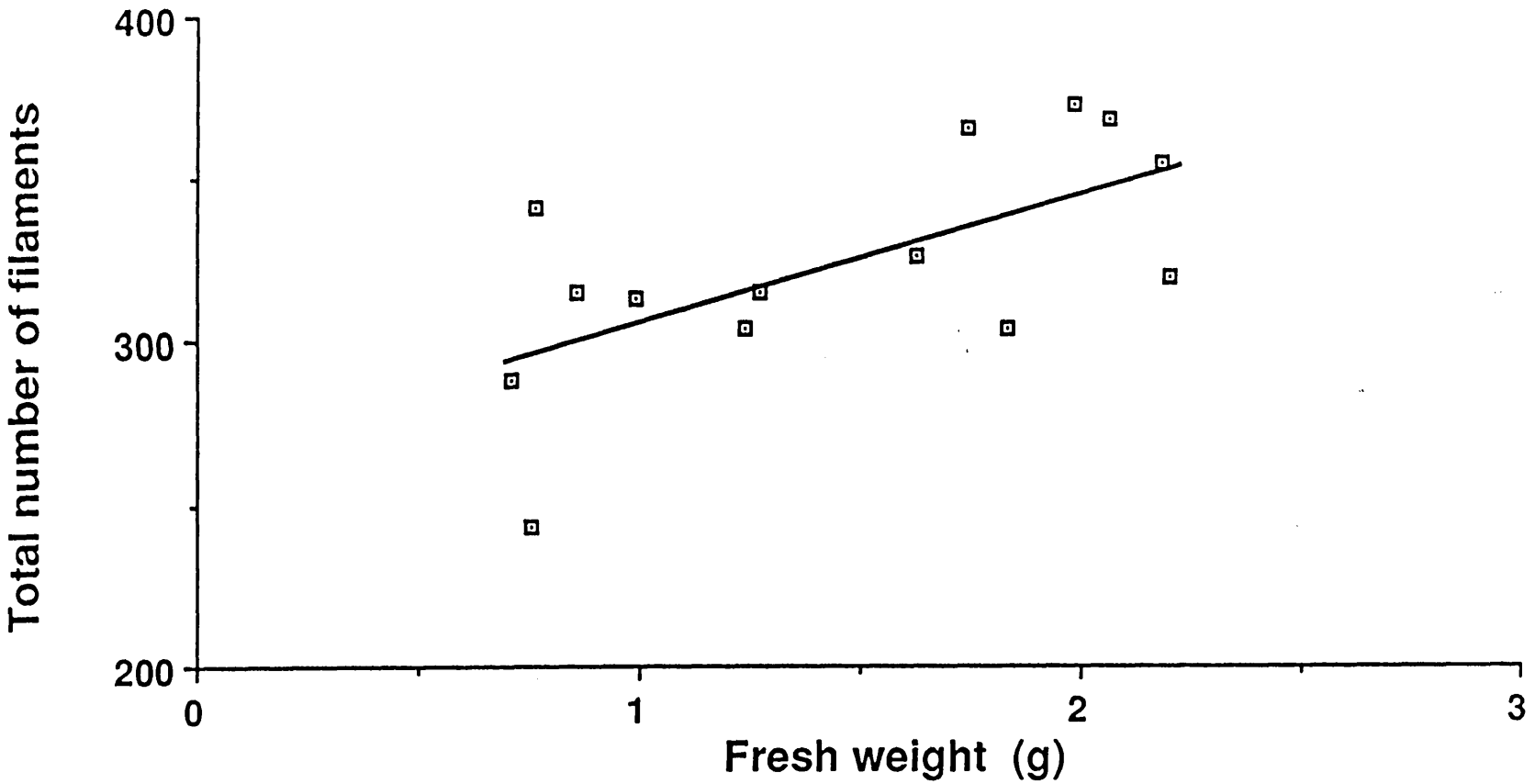

Total number of filaments
400
300
200
0
1
2
3
Fresh weight (g)

FIG. 3.12 The relationship between length of the longest trichobranch filament on left pereiopod 3 (mm) and fresh body weight (g) in 14 ***Calocaris macandreae***. The equation of the calculated regression line is:

$y = 2.39 + 0.74x$ ($r = 0.48$; $P<0.1$)

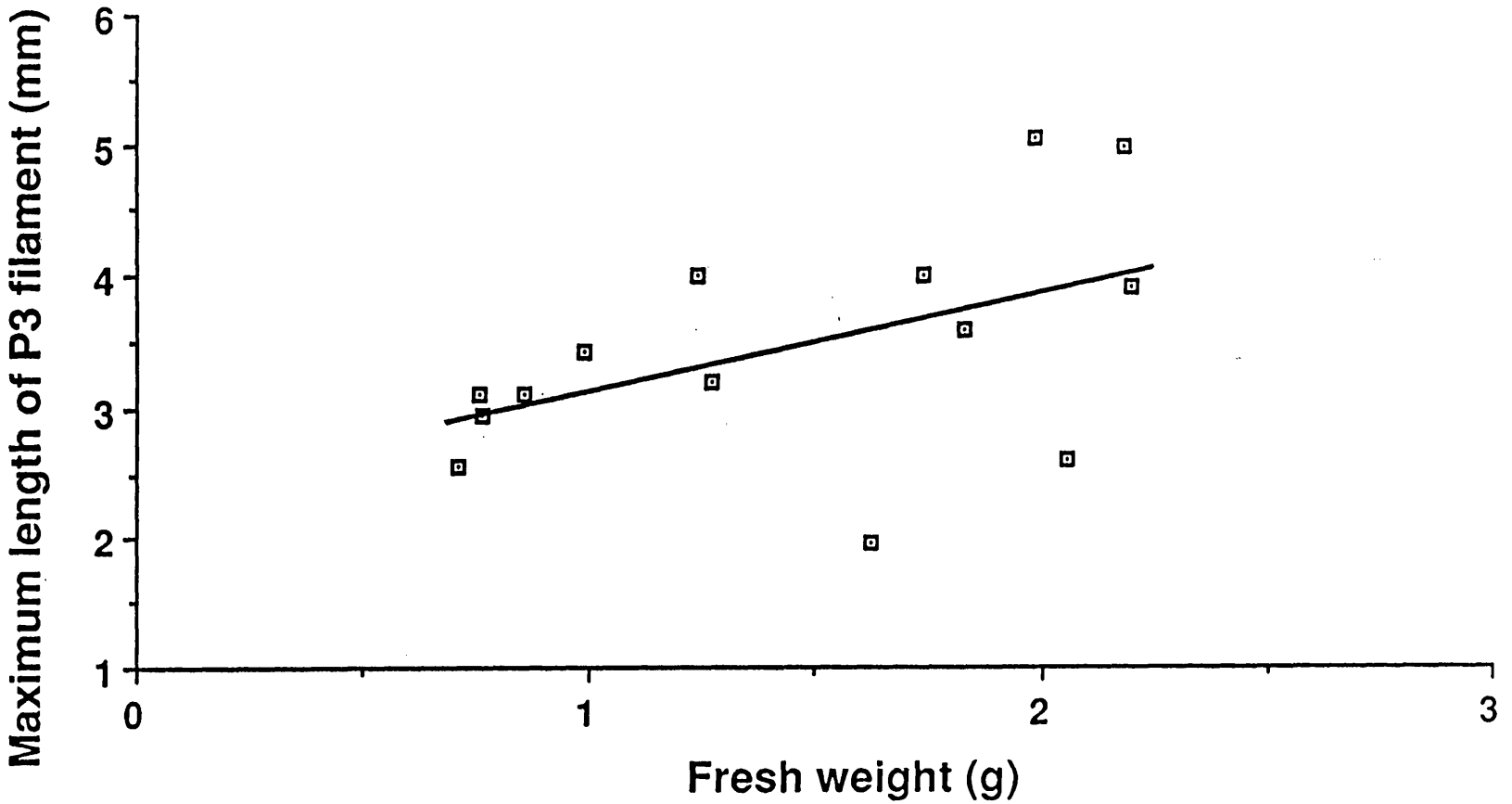
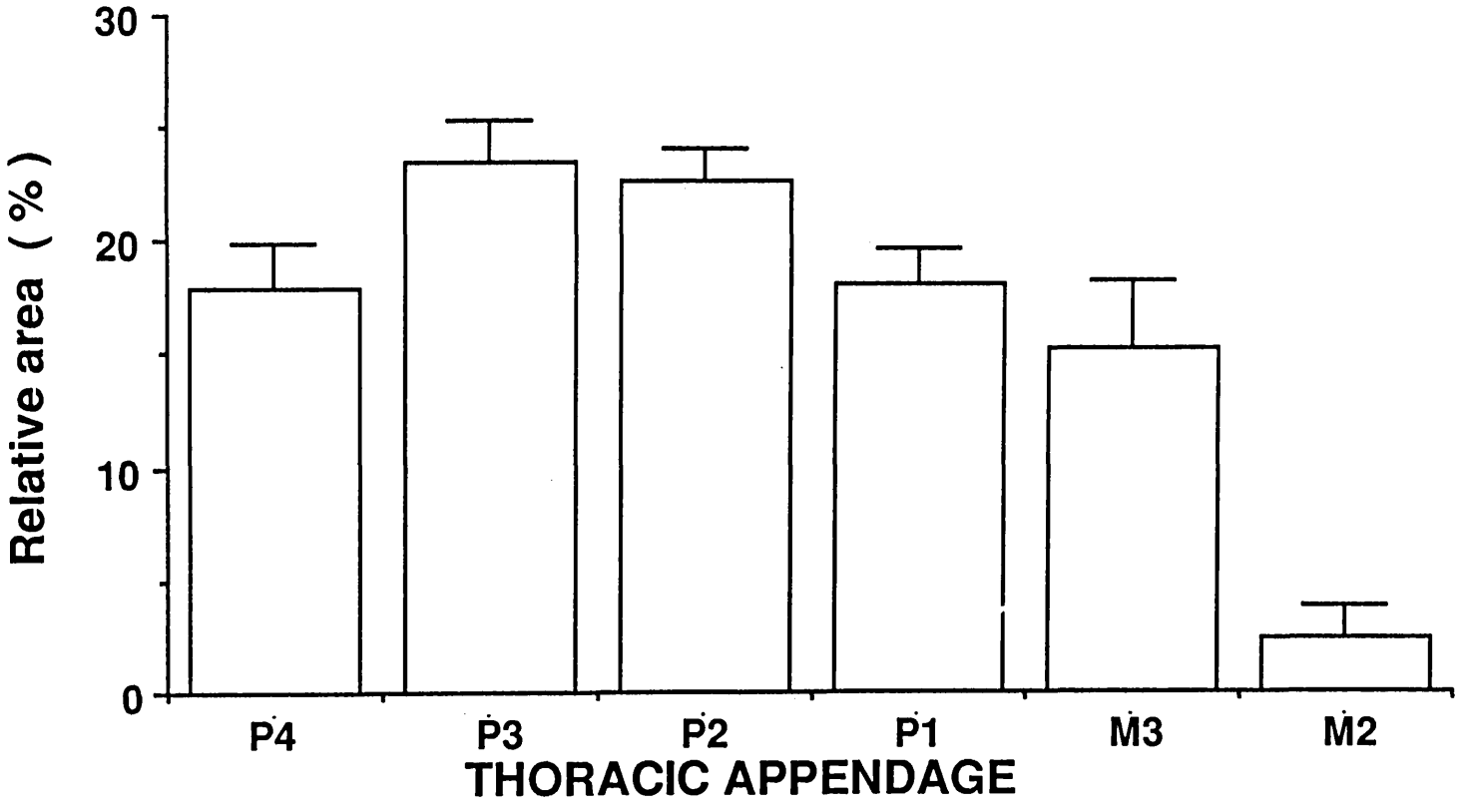

Maximum length of P3 filament (mm)
6
5
4
3
2
1
0
1
2
3
Fresh weight (g)

FIG. 3.13 Percentage contribution to the total gill area of gills (on the left side) from each thoracic appendage in ***Calocaris macandreae*** (means and standard deviations calculated from 14 individuals).


30
20
10
0
Relative area (%)
P4
P3
P2
P1
M3
M2
THORACIC APPENDAGE

blood vessels are lined with a membrane, varying in thickness from 0.7 to 2.7 μm. The external surfaces of both axis and filaments are bounded by an epithelial layer external to which was cuticle. The thickness of the filament cuticle varied from 1.2 -2.3 μm proximally to 0.8 - 1.2 μm at the distal end, as can be seen from longitudinal sections (Fig. 3.15c). The axis cuticle also decreased in thickness distally but was more substantial, varying from 2.3 - 5.2 μm. As with other crustaceans, the branchial cuticle is continuous with the general body cuticle and is probably chitinous. Several cells were usually present between the epithelial layer and the internal blood vessel of a filament, resulting in the wide range of total diffusion distances measured (44 to 74 μm).

The mastigobranchs in *Calocaris* comprise a lamellar structure. The external cuticle is relatively thick (1.7 - 2.7 μm). An epithelial cellular layer enclosed a thick (2.3 - 3.2 μm) basement membrane and a further cuticular layer of 1.4 - 3.0 μm. The centre space of the mastigobranch is continuous with the haemolymph vessels of the coxa.

The light micrographs of intact *Calocaris* gills (Fig. 3.14A) show gills that have been stained with AgCl as described by Holliday (1988). This stain is supposed to preferentially stain the Cl-transporting ATP'ases of the branchial surface. *Calocaris* gills which were tested invariably stained uniformly even after considerable experimentation with different concentrations and exposure times of AgCl and developer. Similar results have been obtained with other species (J.I. Spicer, pers. comm.) and the usefulness of this technique may be open to question. However, the densely stained gills were very opaque and were more visible for light microscopy and photomicrography.

FIG. 3.14 Trichobranchiate gills from *Calocaris macandreae*.

A) arthrobranchs stained with AgCl (see text for details). Scale bar 1 mm).

B) Scanning electron micrograph of part of an arthrobranch (Scale bar 1 mm). Note sediment trapped between filaments.

A

B

FIG. 3.15 Sections of trichobranchiate gills from ***Calocaris macandreae***.

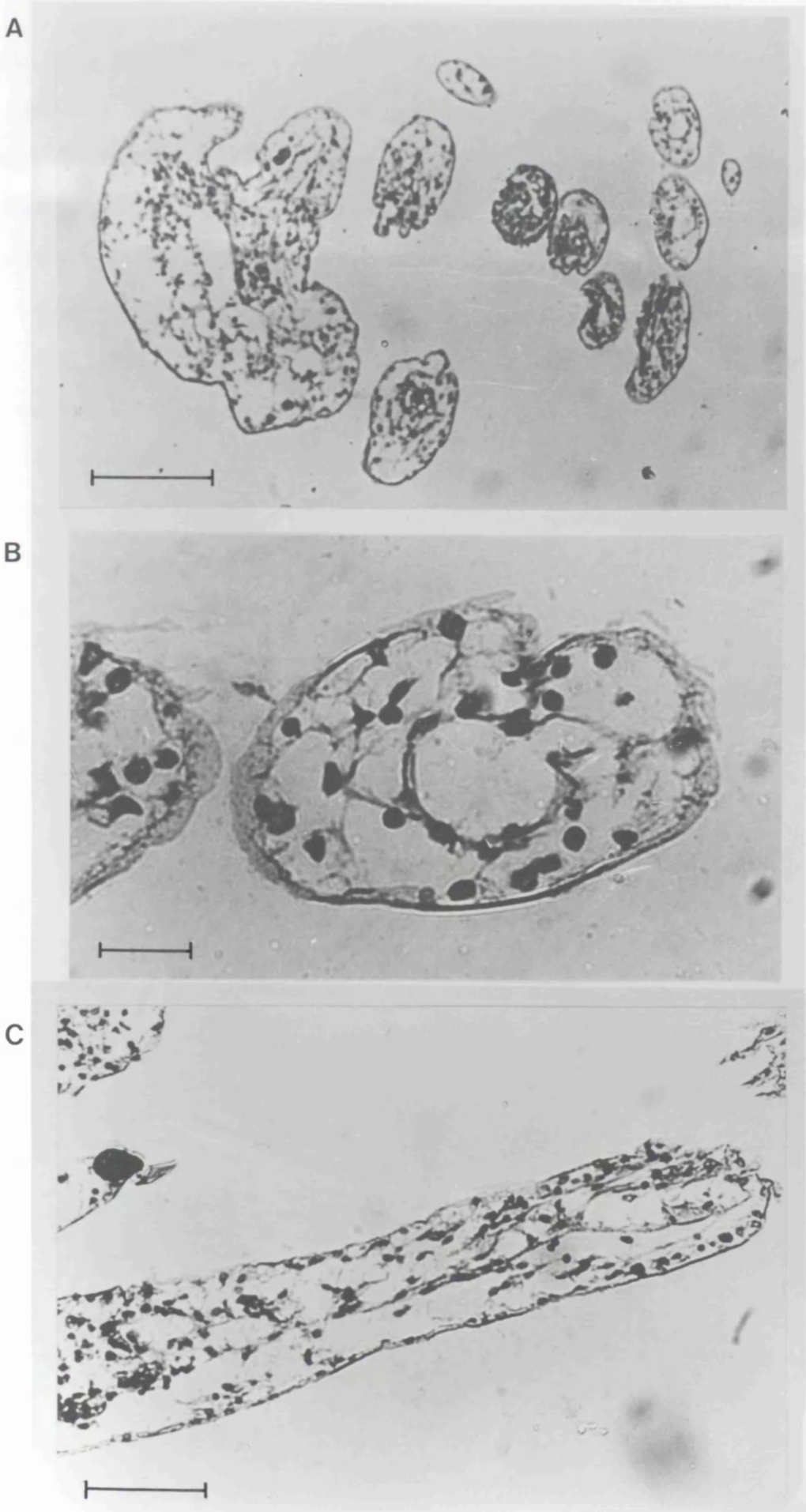
A) Transverse section of axis and several filaments. Note 2 blood channels in the axis, but single channel in filaments. Scale bar 100 um.

B) Transverse section of a single filament.

Scale bar 10 um.

C) Longitudinal section of a filament.

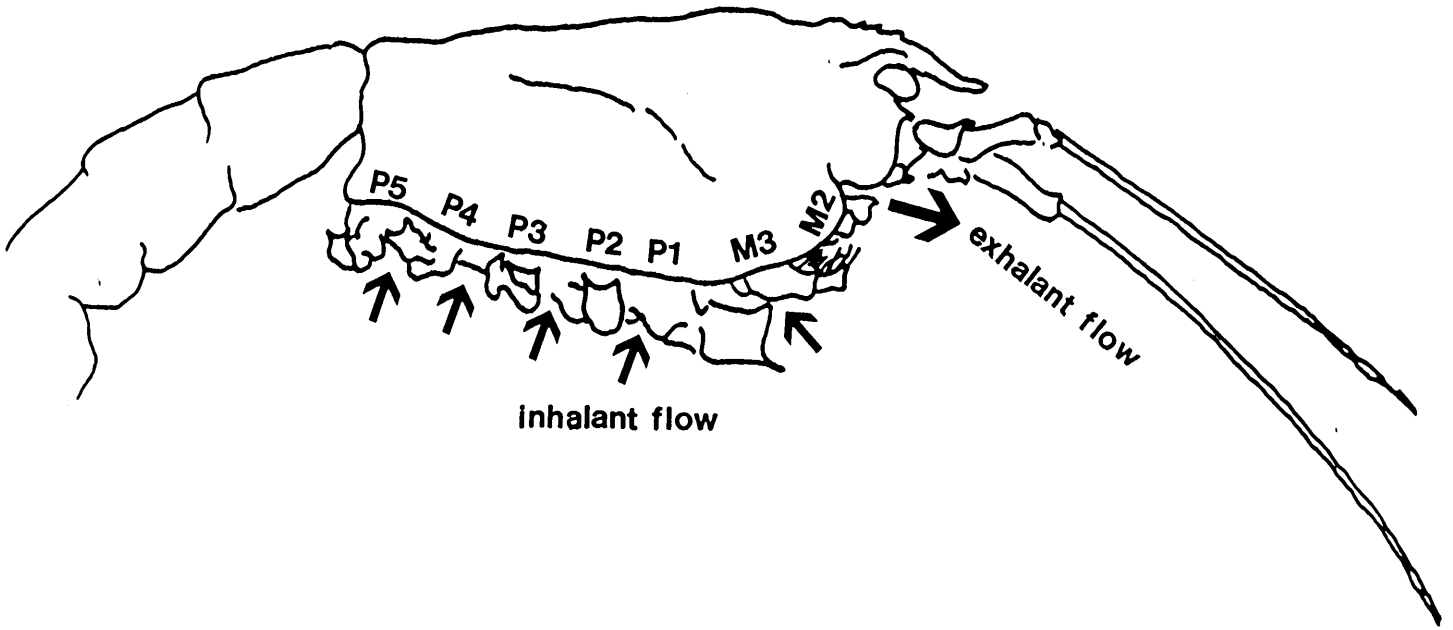
Scale bar 100 um.


A
B
C

3.3.4 Gill Ventilation.

The general pattern of water flow through the branchial chamber is presented in Fig. 3.16. The detailed flow pattern over the branchial surfaces could not be determined (see discussion below). Water flowed into the branchial chamber via several inhalant openings formed between the ventral edge of the branchiostegite, the ventro-lateral thoracic wall and the pereiopods. The ventilatory current then passed through the branchial chamber into the scaphognathite pumping chamber (the pre-branchial chamber) and was exhaled through an opening under the branchiostegite ventral to the antennae. The direction of ventilatory current flow was thus mainly in a postero-ventral to antero-dorsal direction.

FIG. 3.16 Directions of ventilatory water flow in *Calocaris macandreae*. Inhalent flow occurs equally into the openings formed between the branchiostegite and maxilliped 3 and pereiopods 1 - 5. Exhalent flow is directed dorsal to maxilliped 2.


P5
P4
P3
P2
P1
M3
M2
exhalant flow
inhalant flow

3.5 DISCUSSION

3.5.1 The branchial anatomy of the Thalassinidea.

The branchial anatomy of the Thalassinidea has not attracted much attention in recent years, although several earlier authors compared (and usually tabulated) information on the distribution, type and basic structure of the gills. Borradaile (1903), Calman (1909), Selbie (1914) and Gurney (1942) gave information regarding the gill formulae of a number of species. The gill formulae of the Laomediidae were discussed by Wear and Yaldwyn (1966) and Yaldwyn and Wear (1972). Biffar (1971) describes the branchial anatomy of ***Callianassa*** in his definition of the genus. The gill formulae of the U.K. thalassinid species have been tabulated (and inferred) from the references cited above and the present study in Table 3.2. Information concerning the branchial anatomy of the Axiidae is widely scattered in the taxonomic literature and is collated in Table 3.3.

There is close agreement between the branchial descriptions given by these authors, and those described in this study. With the exception of ***Calocaris macandreae***, the gills present on maxillipeds 1 and 2 were obscured by the maxillipeds and were not examined, as this would have necessitated further dissection. Also, the presence or absence of rudimentary gills on maxillipeds 1 and 2, and on pereiopod 5 is liable to vary between individuals (see above for ***Calocaris macandreae***). There is therefore some disagreement, for example, on the presence of a rudimentary pleurobranch on pereiopod 5 of ***Axius stirhynchus***, or the presence of a rudimentary arthrobranch on maxilliped 2 of ***Calocaris macandreae*** (this was observed on only 2 of the 25 specimens examined in this study; although Saint Laurent (1972) gives this character as present in ***Calocaris macandreae***). Disagreements concerning the gills present on maxilliped 1 and pereiopod 3 of the laomediid genera ***Jaxea***

TABLE 3.2 Branchial formulae taken from the literature, for the U.K. species of Thalassinidea. (M2 and M3: maxillipeds 2 and 3; P1 - P4: pereiopods 1 - 4).

Key:

M2 - 2nd maxilliped	Po - podobranch
M3 - 3rd maxilliped	A - arthrobranch
P1 - 1st pereiopod	Pl - pleurobranch
P2 - 2nd pereiopod	M - mastigobranch
P3 - 3rd pereiopod	r - rudimentary
P4 - 4th pereiopod	

Thoracic appendage

	M2			M3			P1			P2			P3			P4			P5		
	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl
Axius stirhynchus																					
Borradaile (1903)	r+r	-	0	-	-	-	1+M	-	-	1+M	-	1	1+M	-	1	1+M	-	1	-	-	r
Calman (1909)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	1	1+M	2	1	1+M	2	1	0	0	r
Selbie (1914)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	1	1+M	2	1	1+M	2	1	0	0	r
Gurney (1942)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	1	1+M	2	1	1+M	2	1	0	0	r
Calocaris macandreae																					
Borradaile (1903)	-	1	0	-	-	0	1+M	-	0	1+M	-	0	1+M	-	0	M	-	0	-	-	0
Selbie (1914)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	0	0	0
Gurney (1942)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Saint-Laurent (1972)	1+M	1	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Jaxea nocturna																					
Borradaile (1903)	M	-	-	1+M	-	-	1+M	-	-	1+M	-	-	1+M	-	-	M	-	-	-	-	-
Selbie (1914)	1+M	0	0	1+M	1	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Gurney (1942)	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Wear & Yaldwyn (1966)	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Upogebia sp.																					
Calman (1909)	0	0	0	0	2	0	0	2	0	0	2	0	0	2	0	0	2	0	0	0	0
Gurney (1942)	M	0	0	r+M	2	0	0	2	0	0	2	0	0	2	0	0	2	0	0	0	0
Calllanassa sp.																					
Gurney (1942)	r+M	0	0	0	2	0	0	2	0	0	2	0	0	2	0	0	2	0	0	0	0
Biffar (1971)	r	-	-	-	2	-	-	2	-	-	2	-	-	2	-	-	2	-	-	-	-

TABLE 3.3 Branchial formulae taken from the literature, for axiid species. (M2 and M3: maxillipeds 2 and 3; P1 - P4: pereiopods 1 - 4). Key as in Table 3.2.

Thoracic appendage

	M2			M3			P1			P2			P3			P4			P5		
	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl
Axius stirhynchus Borradaile (1903)	r+r	-	0	-	-	-	1+M	-	-	1+M	-	1	1+M	-	1	1+M	-	1	-	-	r
Calman (1909)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	1	1+M	2	1	1+M	2	1	0	0	r
Selbie (1914)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	1	1+M	2	1	1+M	2	1	0	0	r
Gurney (1942)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	1	1+M	2	1	1+M	2	1	0	0	r
A. (Elconaxius) sp. Gurney (1942)	M	0	0	1+M	0	0	1+M	2	0	1+M	2	1	1+M	2	1	1+M	2	0	0	0	0
A. (Iconaxiopsis) sp. Gurney (1942)	0+r	r	0	1+M	2	0	1+M	2	0	1+M	2	1	1+M	2	1	1+M	2	1	0	0	0
A. (Paraxius) sp. Gurney (1942)	1+r	2	0	1+r	2	0	1+r	2	0	1+r	2	0	0	2	0	0	2	0	0	0	0
Calocaris macandreae Borradaile (1903)	-	1	0	-	-	0	1+M	-	0	1+M	-	0	1+M	-	0	M	-	0	-	-	0
Selbie (1914)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	0	0	0
Gurney (1942)	1+M	r	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Saint-Laurent (1972)	1+M	1	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Calocaris (Calastacus) hirsutimana Boesch & Smalley (1972)	1+M	r	0	1+M	2	0	1+M	2	0 [CONFIRMED BY WILLIAMS (1974)]	1+M	2	0	M	2	0	1+M	2	0	0	0	0
C.(Calastacus) amakusana Miyake & Sakai (1967)	1+M	1	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
C. (Calastacus) laevis Saint Laurent (1972)	M	r	0	r+M	2	0	r+M	2	0	r+M	2	0	r+M	2	0	M	2	0	0	0	0

Thoracic appendage

	M2			M3			P1			P2			P3			P4			P5		
	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl	Po	A	Pl
Calocarides sp. Gurney (1942)	r+r	r	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	0	2	0	0	0	0
Axiopsis sp. Borradaile (1903)	M	1	0	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0
Axiopsis (Axiopsis) polyacantha Miyake & Sakai (1967)	M	0	0	1+M	1	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Axiopsis (Axiopsis) serratifrons Kensley (1980)	1+M	0	0	1+M	2	0	1+M	2	0	1+M	2	0	1+M	2	0	M	2	0	0	0	0
Coralaxius abelei Kensley & Gore (1980)	M	-	-	1+M	2	0	0	2	0	0	2	0	0	2	0	0	2	0	0	0	0
Axiorygma nethertoni Kensley & Simmons (1988)	r+M	0	0	r+M	2	0	r+M	2	0	r+M	2	0	r+M	2	0	M	2	0	0	0	0

and ***Naushonia*** are discussed by Wear and Yaldwyn (1966) and Yaldwyn and Wear (1972).

Several trends are evident on comparison of the branchial formulae/anatomy of the thalassinid species described above:

1. Of the species examined in this study, pleurobranchs were present only in ***Axius stirhynchus***. This is in accord with literature data for the axiids (Table 3.3), in which pleurobranchs are present only in the subgenera ***A. (Eiconaxius)***, ***A. (Iconaxiopsis)*** and ***A. (Paraxius)***, (as used by Gurney, 1942). These subgenera are now synonomous with ***A. (Axius)***. The subgenus ***A. (Neaxius)*** does not possess pleurobranchs (Borradaile, 1903; De Man, 1925). A review of the taxonomy of the axiids is given in Chapter 1.

Whether or not pleurobranchs should be regarded as a 'primitive' character (either within the Axiidae; or within the Decapoda) is unclear at present.

2. Mastigobranchs are present in ***Axius stirhynchus***, ***Calocaris macandreae***, and ***Jaxea nocturna***. Mastigobranchs were present in the axiids and laomediids (Table 3.2), and in ***Thalassina*** which Borradaile (1903) examined, but not in the callianassids. Mastigobranchs are present in a variety of astacid decapods, e.g. ***Homarus***, and crayfishes from the northern (but not the southern) hemisphere (Lochhead, 1950). Mastigobranchs are absent from pagurids (McLaughlin, 1980, 1983). In brachyuran crabs, the epipod structure and function is probably different to that of the mastigobranch (as defined by McLaughlin, 1983). The function of mastigobranchs is discussed below.

3. There is a pronounced trend towards simplification of the gill formula in more 'advanced' thalassinids (i.e. in ***Upogebia*** and ***Callianassa*** species.). Pleurobranchs are completely absent in upogebiid and callianassid species (Biffar, 1971; de Saint-Laurent, 1973; Rodrigues, 1978) and podobranchs are rudimentary, if present. The total number of trichobranchs in ***Upogebia*** and ***Callianassa*** spp was 10, compared to 15 in ***Jaxea*** and ***Calocaris***, and 18 in ***Axius***. Due to the differences in gill structure, however, the resultant gill area may be similar (see below). (NB the callianassid genus ***Callianidea*** is exceptional in possessing so-called branchial filaments on abdominal pleopods 2 - 5; e.g. Saint-Laurent, 1973.)

4. There is a trend towards a phyllobranchiate structure in the gills of ***Callianassa*** species and ***Jaxea nocturna***, with flattening occurring to varying extents (Borradaile, 1903; Selbie, 1914; Biffar, 1971; present study). The detailed anatomy of thalassinid 'tricho/phyllobranchiate' gills has not been studied. The only detailed descriptions of trichobranchiate thalassinid gills are those of Drach (1930) on ***Upogebia pusilla***, and the present study.

3.5.2 The gill areas of *Calocaris macandreae* and *Upogebia stellata*.

Two methods of gill area measurement were used in this study, direct measurement (calculation based on solid geometry: i.e. the gills are considered to be constructed from cones and cylinders), and pictorial measurement (calculation based on plane geometry: i.e. two dimensional gills). For trichobranchous gills there was a consistent difference between the methods, with the pictorial method giving estimates of approximately half the area as obtained using the direct method.

An estimate of the likely reasons for the discrepancy can be made. Using the pictorial method, with the gill filaments assumed to be approximate triangles, the estimated area (A) would be

A = 1/2 x length x width of the filament.
(= length x radius)

Using the direct method, and assuming the filaments to be cones, the estimated area is

A = pi x slope length x radius of the filament.

Since the length and slope length of the filaments are approximately equal (i.e. the filaments are much longer than their width), the difference between the methods should be a factor of pi. In fact, due to flattening of the filaments before drawing in the pictorial method, the observed discrepancy is smaller than this. Of the two methods, the direct measurement method is probably more accurate. Scammell and Hughes (1982) have also used this method for filamentous gills.

The specific gill areas measured by the direct method in this study for ***Calocaris macandreae*** were in the range 187 - 352 $mm^2.g^{-1}$; with gill areas for ***Upogebia stellata*** and ***U. deltaura*** approximately 344 and 122 $mm^2.g^{-1}$ respectively.

The first quantitative measurements of gill areas in decapods were those of Gray (1957), who examined the gills of sixteen species of brachyuran crabs from a variety of habitats. The gill areas varied from an average of 325 $mm^2.g^{-1}$ in the ocypodid ***Ocypode quadrata*** (as *O. albicans*: a semi-terrestrial ghost crab) to 1367 $mm^2.g^{-1}$ in the portunid ***Callinectes sapidus*** (the blue crab; an active, sublittoral crab capable of swimming considerable distances). Specific gill area was correlated with both habitat (decreasing with increasing

terrestrialisation) and metabolic activity (assessed on a subjective scale). The decrease in gill size in semi-terrestrial and terrestrial crabs is accompanied by the development of alternative gas exchange surfaces (e.g. Farrelly and Greenaway, 1987).

A similar correlation was noted by Scammell and Hughes (1982): "the surface area of the gills being greater in those species which are thought to have a more active mode of life". Values for specific gill areas, interpolated for animals of approximately average weight for each species, are given: ***Carcinus maenas***: 777 $mm^2.g^{-1}$; ***Cancer pagurus***: 425 $mm^2.g^{-1}$; ***Corystes cassivelaunus***: 460 $mm^2.g^{-1}$; ***Maia squinado***: 400 $mm^2.g^{-1}$ (all brachyurans); ***Pagurus bernhardus***: 450 $mm^2.g^{-1}$ (anomuran); ***Astacus fluviatilis***: 400 $mm^2.g^{-1}$; ***Homarus gammarus***: 170 $mm^2.g^{-1}$; ***Nephrops norvegicus***: 160 $mm^2.g^{-1}$ (all macrurans). Further examples of decapod gill areas are given by McMahon and Wilkens (1983): ***Cancer magister***: 440 $mm^2.g^{-1}$; ***Maia squinado***: 490 $mm^2.g^{-1}$. Bergmiller and Bielawski (1970) found a specific gill area of 550 $mm^2.g^{-1}$ in ***Astacus leptodactylus***. Although interspecific comparisons are limited due to the differences in size range (a large ***Homarus*** or ***Cancer*** being 1000-fold greater in weight than an average ***Calocaris macandreae***), it is evident that the range of weight-specific gill areas recorded in the thalassinids is comparatively low.

An additional observation is that those species with trichobranchiate gills (the macrurans above) tend to have smaller gill areas. It is most likely, however, that the low gill areas recorded in thalassinids relate to low rates of behavioural activity (Chapter 2) and hence to low oxygen uptake rates (see Chapter 4) and to the possession of a high affinity respiratory pigment (Chapter 5).

3.5.3 Functional morphology of the gills of *Calocaris macandreae.*

The gills of decapods have multiple functions. They are the major site of respiratory exchange of oxygen and carbon dioxide (with resultant interactions with the acid-base balance of the haemolymph). In addition, the gills are a major location of active and passive ionic exchange with the environment, an aspect which will not be considered further here.

There are two main levels at which the morphology of the gills might exhibit adaptive responses to environmental, behavioural or metabolic selective pressures. There are obvious differences in the gross morphology and fine anatomy of decapod gills at all taxonomic levels. These presumably result in functional differences, e.g. in characteristics of gas diffusion and blood perfusion. Secondly, the respiratory function of the gills will be dependant on the ventilatory flow patterns (in turn dependant on morphological characteristics of the branchial chamber, and on physiological responses of scaphognathite activity to both external and internal stimuli).

Although the functional anatomy/morphology of phyllobranchiate gills (usually of brachyuran crabs) has been well studied (e.g. Hughes *et al.*, 1969; Taylor and Butler, 1978; and in relation to air-breathing, Burggren and McMahon, 1988), there are few studies of the relationships between structure and function of trichobranchiate gills (Burggren *et al.*, 1974) and only one previous study of thalassinid trichobranchs (those of ***Upogebia pusilla***; Drach, 1930).

The functional anatomy of the trichobranchs of the crayfish ***Procambarus***

clarkii was studied by Burggren *et al.* (1974), building on the previous anatomical studies of Huxley (1880), Bock (1925 and Fisher (1972). The major anatomical factors which affect the efficiency of oxygen uptake at the gills are thought to be the blood-water diffusion distance, and the structure of the vascular system of the gills, controlling the perfusion pattern.

Burggren *et al.* (1974) found that although the podobranch filament epithelium thickness in ***Procambarus*** was 3.15 - 8.70 μm, this may be functionally reduced by the presence of plasma membrane folding in the epithelium, possibly resulting in a sinus system (this has been observed in ***Artemia salina***, in which the diffusion distance is only 1-2 um although the epithelial thickness is about 4 μm; Copeland, 1967). Additionally, the filament basement membrane may not represent a complete barrier to the movement of respiratory proteins (Fisher, 1972; Burggren *et al.*, 1974). The observed total epithelial thickness in ***Calocaris macandreae*** (44-74 μm) was much greater than in the above species or in ***Astacus pallipes*** (Fisher, 1972). Plasma membrane folding of the type described by Copeland (1967) and Burggren *et al.* (1974) was not observed at the magnifications used. It therefore appears that the diffusion distances in the trichobranchs of ***Calocaris macandreae*** may be comparatively large. However, further study of the fine structural characteristics may modify these conclusions.

The gill filaments of the crayfish ***Astacus pallipes*** (Fisher, 1972), ***Procambarus clarkii*** (Burggren *et al.*, 1974), and the thalassinid ***Upogebia pusilla***; (Drach, 1930) contain a relatively complex vascular system. In ***Procambarus***, modification of oxygen uptake efficiency at the gills may be produced by separation of oxygenated/deoxygenated blood, modification of perfusion by blood shunting, and a partial

countercurrent flow of blood and water (Burggren *et al.*, 1974). ***Calocaris macandreae*** appeared to possess a comparatively simple system, with a single blood channel and no septa, partitions etc. as described for other species. Clearly, the absence of these features may limit the efficiency of ***Calocaris*** gills compared to those of brachyuran crabs (Hughes *et al.*, 1969) and fish (Randall, 1970).

The pattern of ventilatory flow of water over the gills has been described for a number of macruran species, e.g. ***Astacus fluviatilis*** (Huxley, 1880); ***Penaeus setiferus*** (Young, 1959); ***Procambarus clarkii*** (Burggren *et al.*, 1974); ***Nephrops norvegicus*** (Scammell and Hughes, 1982). There is a general similarity in that water appears to enter the branchial chamber via openings between the pereiopods and exit anteriorly at the distal end of the scaphognathite. In more heavily calcified types (e.g. ***Homarus*** and ***Astacus***) there is a tendency for the inhalant flow to be primarily located at the posteroventral angle of the branchiostegite (Lochhead, 1950). In ***Calocaris macandreae***, however, inhalant flow appears to occur equally between all pereiopods.

The ventilatory flow patterns of ***Nephrops norvegicus*** and ***Procambarus clarkii*** have been described in detail (by Scammell and Hughes, 1982 and Burggren *et al.*, 1974 respectively), using the technique of replacing part of the branchiostegite with a transparent plastic mould. In ***Nephrops***, (Scammell and Hughes, 1982) the mastigobranchs divide each inhalant flow into two components: the main one "traverses the gill bases, passes across and through the proximal gill filaments, then distally as it meets the epipodite [= mastigobranch] of the next anterior group of gills. The subsidiary stream is directed distally immediately after entering the inhalant opening and passes through the

podobranch filaments". In ***Procambarus***, (Burggren *et al.*, 1974) a similar effect is produced by folds in the distal quarter of each podobranch. Although the ventilatory flow pattern in ***Calocaris*** was not observed (due to the small size), it is reasonable to suppose that the mastigobranchs have a similar function.

Despite the several studies of functional morphology of phyllobranchiate gills in decapods which demonstrate that the morphology of these gills is comparable with that of fish, there are insufficient data in the literature to allow a functional comparison of the gill types found in the decapods (and particularly in the thalassinids). Assessments of gill efficiency, measured as % oxygen extraction, show that efficiency is similar at approximately 20 - 50% in a variety of brachyuran (phyllobranchiate) and macruran (trichobranchiate) decapods during quiescent behaviour (McMahon and Wilkens, 1983). Extraction efficiency may be as much as 79% under stress (McMahon *et al.*, 1974). If the practical difficulties related to their small size could be overcome, a comparison of gill efficiency in a range of thalassinid species including trichobranchiate axiids and (almost) phyllobranchiate callianassids would be instructive.

CHAPTER 4. MECHANISMS OF OXYGEN UPTAKE IN *CALOCARIS MACANDREAE*.

4.1 INTRODUCTION.

In the first part of this thesis, the respiratory conditions present in the burrows of ***Calocaris macandreae*** and other thalassinid species were described. It was found that oxygen availability varied greatly in different regions of the burrow, and that permanent, severe hypoxia (< 30 Torr) occurred in the deeper areas.

There are several approaches to assessing the efficiency of the respiratory physiology of animals. Metabolic rate under different environmental conditions may be measured using calorimetric techniques, or assuming metabolism to be entirely aerobic, by measurement of rates of oxygen uptake. This chapter investigates the ability of ***Calocaris macandreae*** to maintain oxygen uptake in hypoxic environments. Rates of oxygen uptake were measured using two methods: 'open respirometry', in which the animal was exposed to a constant flow of water of varying oxygen tension; and 'closed respirometry', in which the animal was enclosed in a limited volume of water and oxygen depletion results from the metabolism of the animal itself.

Aerobic respiration in decapod crustaceans, and other animals, may be considered as the sum of several unit processes (McMahon and Wilkens, 1983). In terms of oxygen uptake these include:

ventilation, the transport of oxygen from the environment to the respiratory exchange area;

oxygen uptake, from the environment across the respiratory exchange area;

perfusion, oxygen removal from the respiratory exchange area;

transport, to the tissues by the haemolymph;

metabolism, oxygen utilisation by the tissues.

The functional anatomy and morphology of the gills in ***Calocaris macandreae*** were investigated in the previous chapter. The physiological mechanisms of ventilation and perfusion in ***Calocaris*** are investigated in this chapter. The physiology of oxygen transport will be considered in Chapter 5. Finally, metabolic responses to failure of the respiratory system to maintain a sufficient supply of oxygen to the tissues during environmental anoxia, will be described in Chapter 6.

4.2 MATERIALS AND METHODS.

4.2.1 Rates of oxygen uptake in normoxia and hypoxia.

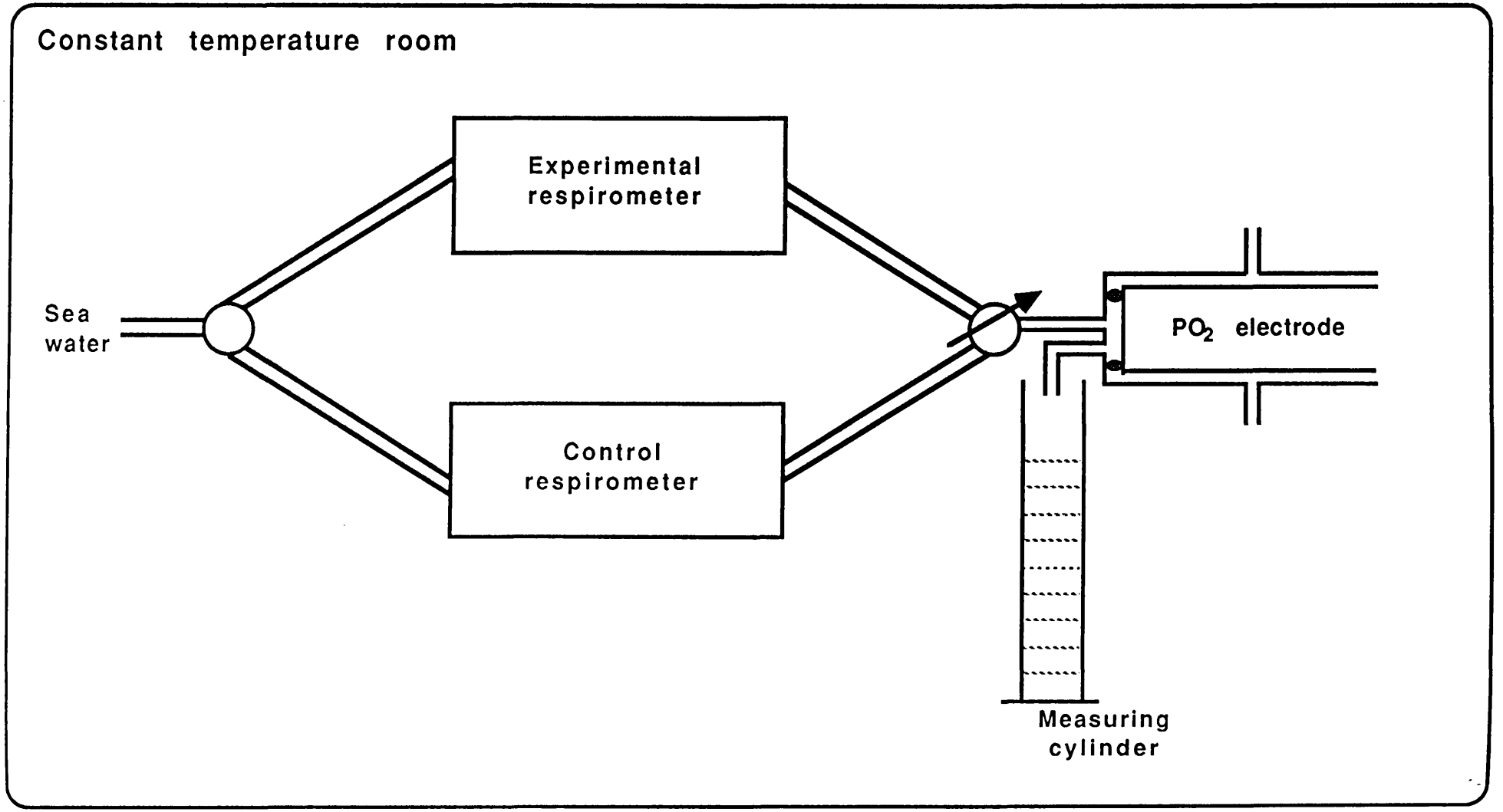
4.2.1.1 Open respirometry.

The rate of oxygen consumption by *Calocaris macandreae* in normoxic conditions was measured by open respirometry (animal size range approximately 0.2 to 2.5 g fresh weight). The experimental apparatus consisted of a constant level header tank, which supplied aerated natural sea water (salinity = 32 $^{o}/oo$) at a constant rate to the respirometer chamber (Fig. 4.1). Initial experiments suggested that the optimum shape for the chamber was cylindrical, resulting in a consistent oxygen gradient within the chamber and to some extent resembling in shape the burrow habitat of the species. The respirometer chamber volume (i.e. the diameter and length of the perspex tube) was varied from 15 to 35 ml according to the size of the animal. Two identical chambers were set up, in order that control PO_2 values could be obtained.

The sea water outflows from both the respirometer and control chambers were led via narrow cannular tubing (of as short a length as possible) and a three-way valve, to an oxygen electrode (Radiometer E5046). The electrode was connected to a Strathkelvin Instruments PO_2 meter, the output of which was recorded on a chart recorder. The complete apparatus was situated in a constant-temperature room at $10^{o}C$.

Each experimental run consisted of an electrode calibration with aerated seawater (as described in Chapter 2), followed by 30 minutes of control measurement. Control values were always within 1 Torr of aerated sea water values (155 - 160 Torr). The three-way valve was then switched so that water from the respirometer chamber flowed past

FIG. 4.1 Diagram of the open respirometer. See text for explanation.


Constant temperature room
Experimental respirometer
Sea water
Control respirometer
PO_2 electrode
Measuring cylinder

the electrode at a rate between 0.8 and 1.5 ml. min^{-1}. The flow rate was measured by diverting the outflow from the electrode into a measuring cylinder for a measured time. The apparatus was adjusted so that control and respirometer sea water flow rates were similar. The experiment was then run for a period of 12 - 16 hours (usually overnight) followed by another period of control PO_2 measurement.

Rates of oxygen uptake were then calculated from the formula:

$$\dot{M}O_2 = \frac{PO_2 \times \text{alpha} \times \text{Flow rate (ml.min}^{-1})}{\text{animal fresh weight (g)} \times 60} \quad \mu\text{mol } O_2.g^{-1}.\ h^{-1}.$$

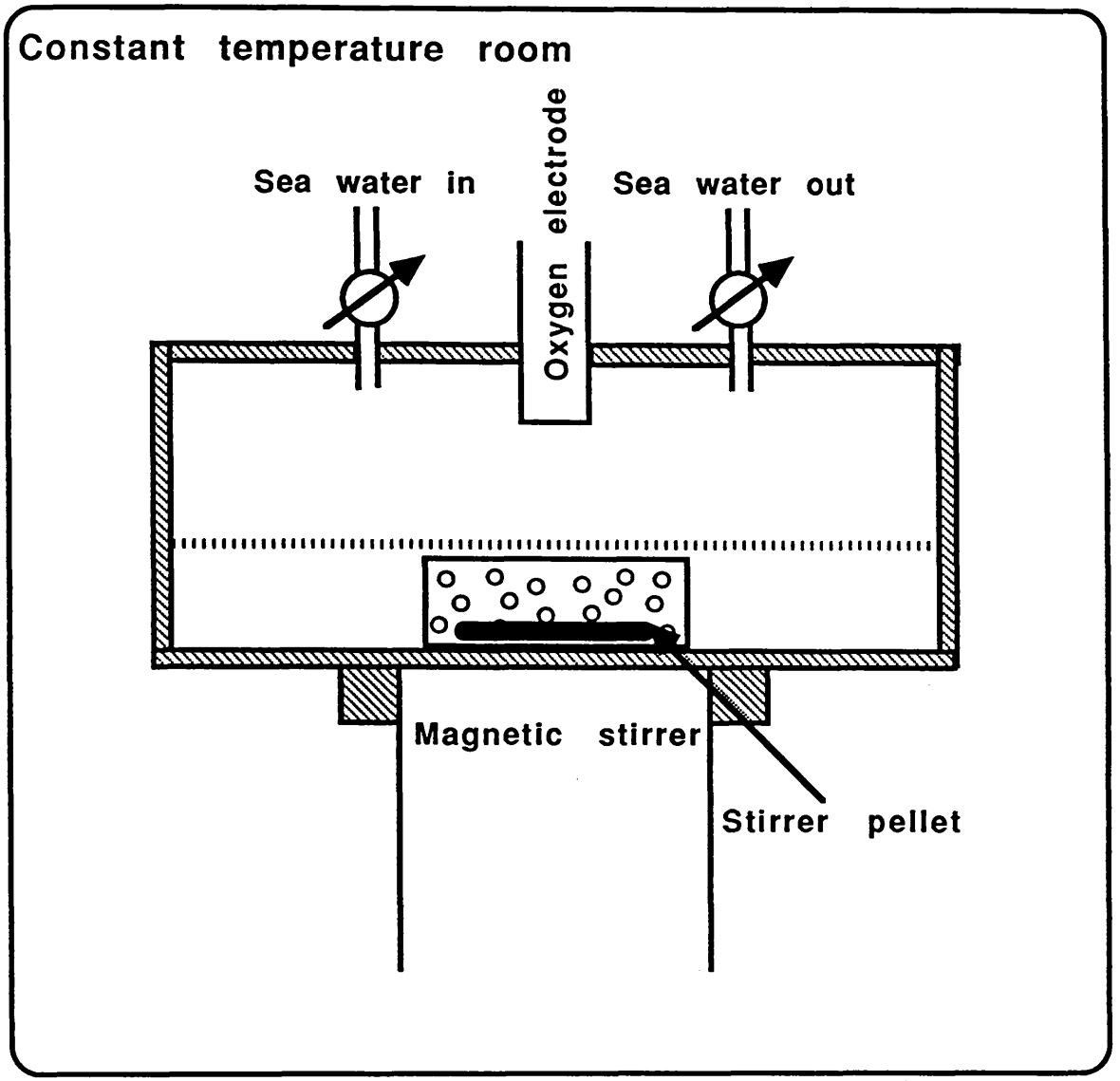
where PO_2 = respirometer PO_2 - control PO_2 (Torr);

alpha = solubility coefficient of oxygen in sea water at 10°C
= 1.39 μmol O_2. ml^{-1}. $Torr^{-1}$.

4.2.1.2 Closed respirometry.

A closed respirometer was used in order to investigate the rates of oxygen consumption of *Calocaris* under conditions of declining oxygen tension. The closed respirometer apparatus (shown in Fig. 4.2) consisted of a perspex respirometer chamber containing an enclosed stirring bar, into which projected an oxygen electrode (Radiometer E5046) connected to a PO_2 meter (Strathkelvin Instruments) and a chart recorder. After initial experiments, a respirometer chamber volume of 16 ml was found to allow experiments over a reasonable timescale (complete depletion of oxygen in approximately 12 hours). During initial experiments in which the respirometer chamber was immersed in a water bath, difficulties were experienced due to electrical interference from the water bath electronics. More satisfactory results were obtained by placing the apparatus in a temperature-controlled room maintained at 10 °C. Artificial sea water ('Tropic Marin' made up in fresh deionised water) at a salinity of 32 °/oo was

FIG. 4.2 Diagram of the closed respirometer. See text for explanation.


Constant temperature room
Oxygen electrode
Sea water in
Sea water out
Magnetic stirrer
Stirrer pellet

used for these experiments, in order to reduce background oxygen consumption by the microflora present in natural sea water (see later).

After electrode calibration with aerated water, each experimental run consisted of placing an animal in the respirometer and allowing a short settling time of approximately 30 minutes (settling times are discussed below, 4.4.1). The inflow and outflow taps were then closed and the animal allowed to deplete the oxygen within the chamber. The oxygen partial pressure declined to 0 Torr over a period of 6 - 12 hours, and a period of 2 - 6 hours of anoxia was usually measured. The rate of oxygen depletion (expressed as Torr. h^{-1}) was then measured from the chart recording at various PO_2's. (Where $\dot{M}O_2$ varied rapidly, the instantaneous rate of oxygen depletion was measured by drawing tangents to the trace.) After each run the electrode calibration was checked to ensure that no electrode drift had occurred during the experiment.

Several control experiments were run in which background respiration rates were measured. These rates were always low (<10% of the experimental rates) and were subtracted from the results obtained during experiments with animals. The cause of the background respiration is unclear since the respirometers were sterilised (by rinsing with 'Milton's fluid') between experiments and freshly-made artificial sea water was always used. The background respiration was distinct from the effects of electrode drift (which was very low) and a consistently high initial oxygen uptake rate when starting a run, which appeared to be an experimental artifact related to water flow within the respirometer chamber.

The rate of oxygen consumption was calculated from:

$$\dot{M}O_2 = \frac{\text{alpha} \times \Delta PO_2 \times V}{\text{time}} \qquad \mu\text{mol } O_2 . g^{-1} . h^{-1}$$

where: ΔPO_2 = change in oxygen partial pressure (Torr)

V = chamber volume (ml)

The true rate of oxygen consumption of the animal (MO_2) was then obtained by subtraction of the background rate.

Initial experiments were carried out in which longer settling times (up to 24 hours) were allowed. Several reports in the literature describe protracted settling periods during which oxygen uptake rates decline to a so-called "resting level" (see Discussion below). This response was never observed in ***Calocaris***. Results from both open and closed respirometry suggest that the rate of oxygen uptake in ***Calocaris*** contained in respirometers is constant over a time period of <30 minutes - >24 hours. Animals held in closed respirometers for longer than 24 hours suffered increased mortality, possibly as a result of the accumulation of waste products. In addition, ***Calocaris*** always showed some degree of activity during experiments, usually in the form of continual walking and turning movements. Thus the results obtained from these experiments should be considered as oxygen uptake rates during 'normal' rather than 'resting' levels of activity. This subject will be discussed in more detail below (see Discussion, 4.4.1).

4.2.2 Heart and scaphognathite rate measurements.

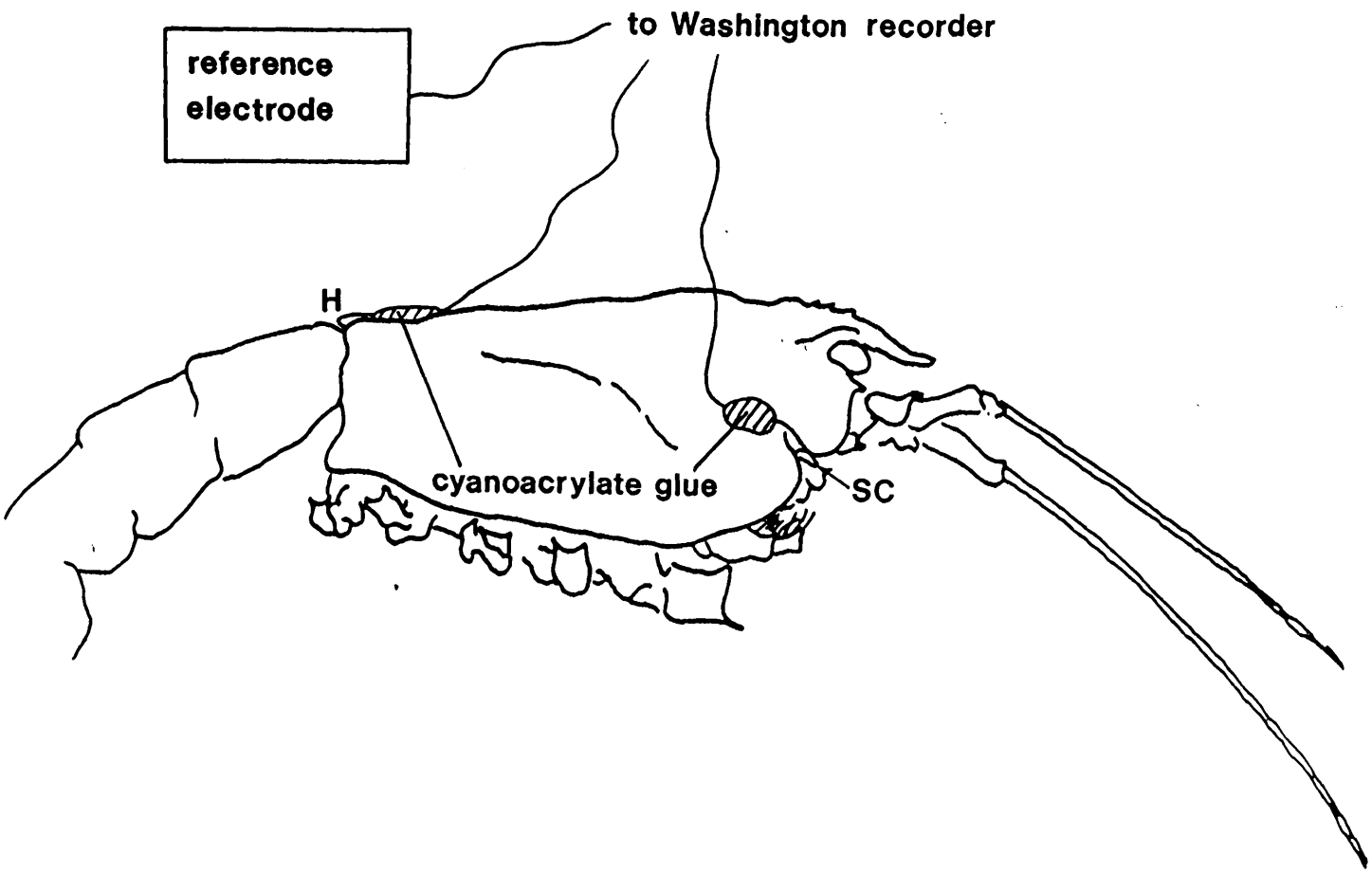
The heart and scaphognathite rates of ***Calocaris macandreae*** were recorded using an impedance technique similar to that used originally by Hoggarth and Trueman (1967) and modified by Spaargaren (1973) and Dyer and Uglow (1973). A small oscillating current (2μA; 25kHz) is induced between a fine wire electrode fixed to the animal and a larger

reference electrode situated in the water of the holding tank. Any impedance change between the electrodes (due to heart or scaphognathite movements) produces a proportional voltage change which is amplified and recorded on a chart recorder.

The electrodes used to record scaphognathite movements were made from fine (S.W.G. 44) shellac-coated copper wire, with the shellac removed from the last millimetre. The wire used was as fine as possible in order to allow the animal freedom of movement; this resulted in many breakages of the electrode wires. The wire was bent to form a hook which was placed under the anterior margin of the branchiostegite so that the bared end of the electrode was positioned close to the scaphognathite (Fig. 4.3). The heart electrode was similar (although with a slightly shorter hook) and was placed over the dorsal posterior edge of the cephalothorax to lie over the pericardium (Fig 4.3). The electrodes were fixed in place using cyano-acrylate adhesive (Permabond) with the setting time accelerated to a few seconds using methyl methacrylate. The animals were quickly dried with a tissue before the electrodes were attached, and the total time of aerial exposure was as short as possible (under 1 minute). There were few mortalities associated with the operation; some animals remained alive while connected to an impedance recorder for up to 10 days. A recovery time of at least 24 hours was allowed before any recordings were made.

The reference electrode consisted of either an aluminium sheet (10 cm x 5 cm) or a piece of aluminium foil placed in the tank. Both electrodes were connected to a George Washington impedance pneumograph and pen recorder (Palmer Bioscience). Four channels were available for recording but no more than two electrodes were connected to any one

Fig. 4.3 Diagram of the placement of impedance electrodes to record scaphognathite and heart rates. The scaphognathite electrode (SC) is hooked around the anterior edge of the branchiostegite. The heart electrode (H) is hooked over the dorsal posterior edge of the carapace. The reference electrode is placed in the sea water medium. All electrodes are connected to a George Washington impedance pneumograph. The electrodes are fixed in position with cyanoacrylate adhesive. See text for further details.


reference
electrode
to Washington recorder
H
cyanoacrylate glue
SC

animal. Recordings were made of single scaphognathite rates; both scaphognathites; heart rate only and heart and one scaphognathite. It was necessary to use animals weighing in excess of 1 g (fresh weight) since smaller animals were unduly restrained by the wires. Although some signal noise was generated by locomotory movement of the animals, in general the animals remained stationary over relatively long periods of time so that satisfactory recordings could be made. In the case of animals exposed to severe hypoxia and anoxia, complete cessation of locomotory behaviour was always observed.

After the electrodes had been attached, the animals were placed in small (2-5 l capacity) aquaria for the duration of experiments. Several attempts were made to record heart and scaphognathite rates from animals in burrows. Animals were, however, reluctant to enter burrows while connected to the recorder and in most cases the wires broke. Due to the difficulty of removing animals from the burrows subsequently (without destroying the burrow) and a shortage of empty burrows in the laboratory, these attempts were abandoned. Some results were obtained from animals positioned inside lengths of clear perspex tubing, within which the animals appeared to exhibit normal behaviour patterns such as preening and ventilatory pleopod movements.

Scaphognathite and heart rates were recorded from animals held under a variety of conditions. The chart recordings were analysed by counting the beats recorded over short time intervals (usually 1 minute) although continuous recordings were made over a period of several hours. In experiments in which both scaphognathites, or one scaphognathite and the heart rate were monitored, the traces were examined for evidence of unilateral scaphognathite pumping; phase linking of the scaphognathites; and correlation between heart and

scaphognathite rates. In addition, some high-speed recordings were made to investigate the form of the pump actions of the heart and scaphognathites.

Scaphognathite and heart rates were measured in animals exposed to a variety of respiratory conditions. Control experiments were conducted in which animals were held in normoxic conditions (in aerated sea water). Scaphognathite and heart responses were then investigated in conditions of gradually declining oxygen tension, and in conditions of extended anoxia (up to 12 hours). During these experiments, hypoxic conditions with controlled pH and PCO_2 were produced by bubbling appropriate nitrogen/carbon dioxide mixtures through the water. pH and PCO_2 were occasionally measured using the instruments described in Chapter 5. All experiments were carried out at $10^{o}C$.

4.3 RESULTS.

4.3.1 Oxygen consumption in normoxia.

The rates of weight-specific oxygen consumption in 19 specimens of ***Calocaris macandreae*** were determined using open respirometry. No mortalities were recorded during the open respirometry experiments. Oxygen consumption ($\dot{M}O_2$) varied from a minimum of 0.28 $\mu mol.g^{-1}.h^{-1}$ in an animal which had been stationary for a considerable time (probably > 12 hours) to 1.67 $\mu mol.g^{-1}.h^{-1}$ in another animal immediately after being placed in the respirometer. $\dot{M}O_2$ did not change significantly in any animal, over the duration of an experiment (correlation analysis).

The relationship between log $\dot{M}O_2$ and log fresh body weight (Fig. 4.4) was non-significant, although an inverse trend was observed. The lack of significance may be attributed to the relatively small size range of ***Calocaris*** which was studied. Many other studies of oxygen uptake in crustaceans (and other animals) which considered a sufficiently wide size range have found that $\dot{M}O_2$ is proportional to a constant power function of body weight, according to the general equation:

$$\dot{M}O_2 = a.wt^{b-1}.$$

Values of the 'a' and 'b' parameters derived from the present study for ***Calocaris macandreae*** are:

$$\begin{aligned} a &= 0.74 \\ b-1 &= -0.33 \\ b &= 0.67. \end{aligned}$$

4.3.2 Oxygen consumption in hypoxia.

The rates of weight-specific oxygen consumption of five *Calocaris* under conditions of declining oxygen partial pressure, as measured by closed respirometry, are shown in Fig. 4.5. The elevated rates at PO_2's of 130 - 155 Torr are likely to be due to disturbance of the animal. These elevated rates were similar both in settled animals at the initiation of the experimental run (i.e. on stopping the water flow), and in animals which were placed in respirometers immediately prior to commencing the run. After 2 - 4 hours the chamber PO_2 had decreased to 130 Torr, and oxygen consumption rates recorded over the range 130 - 20 Torr were in close agreement with rates recorded in normoxia by open respirometry. The mean rate of weight-specific oxygen consumption recorded in quiescent animals, using both methods (in the PO_2 range 155 - 20 Torr) was approximately 0.74 $\mu mol.g.h^{-1}$. (Note: this weight-specific rate applies over the entire size range measured).

Oxygen consumption was strongly regulated over the environmental PO_2 range 15 - 155 Torr in *Calocaris macandreae*. Fig. 4.5 shows no significant decrease in $\dot{M}O_2$ from a rate of 0.74 $\mu mol.g^{-1}.h^{-1}$ until a 'critical' PO_2 ('Pc') of 10 Torr was reached (t-tests, $P>0.05$). An examination of individual $\dot{M}O_2$ curves, however, showed that individual Pc's varied within the range 10 - 20 Torr. Below a PO_2 of 10 Torr, oxygen consumption decreased. At a PO_2 of 5 Torr, $\dot{M}O_2$ was not significantly greater than zero (t-test, $P>0.10$).

FIG. 4.4 The relationship between rate of oxygen consumption ($\dot{M}O_2$; $\mu mol.g^{-1}.h^{-1}$) and fresh body weight (g) in ***Calocaris macandreae***. The equation of the regression line is:

$$\log y = -0.33 \log x - 0.13$$

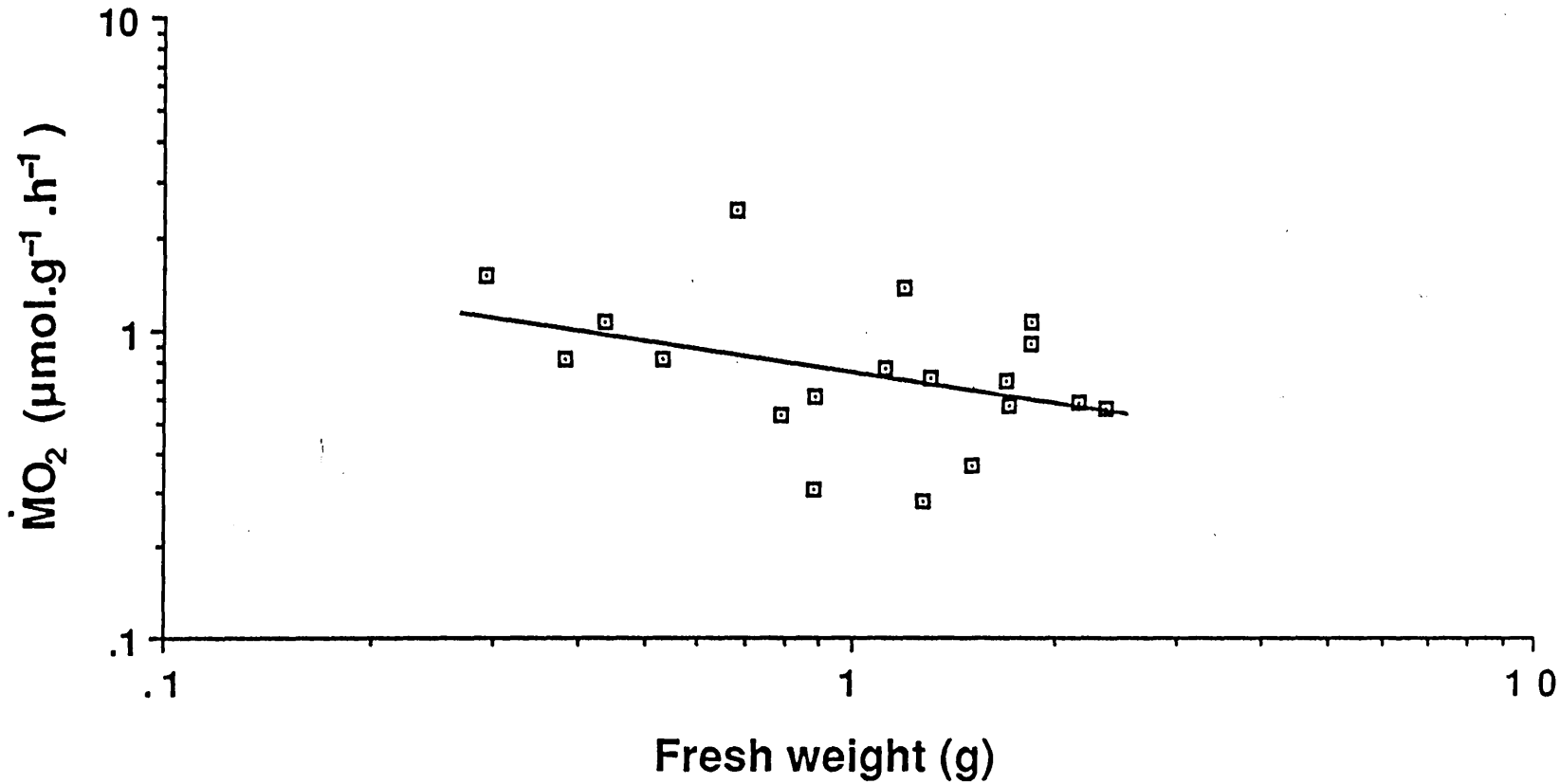
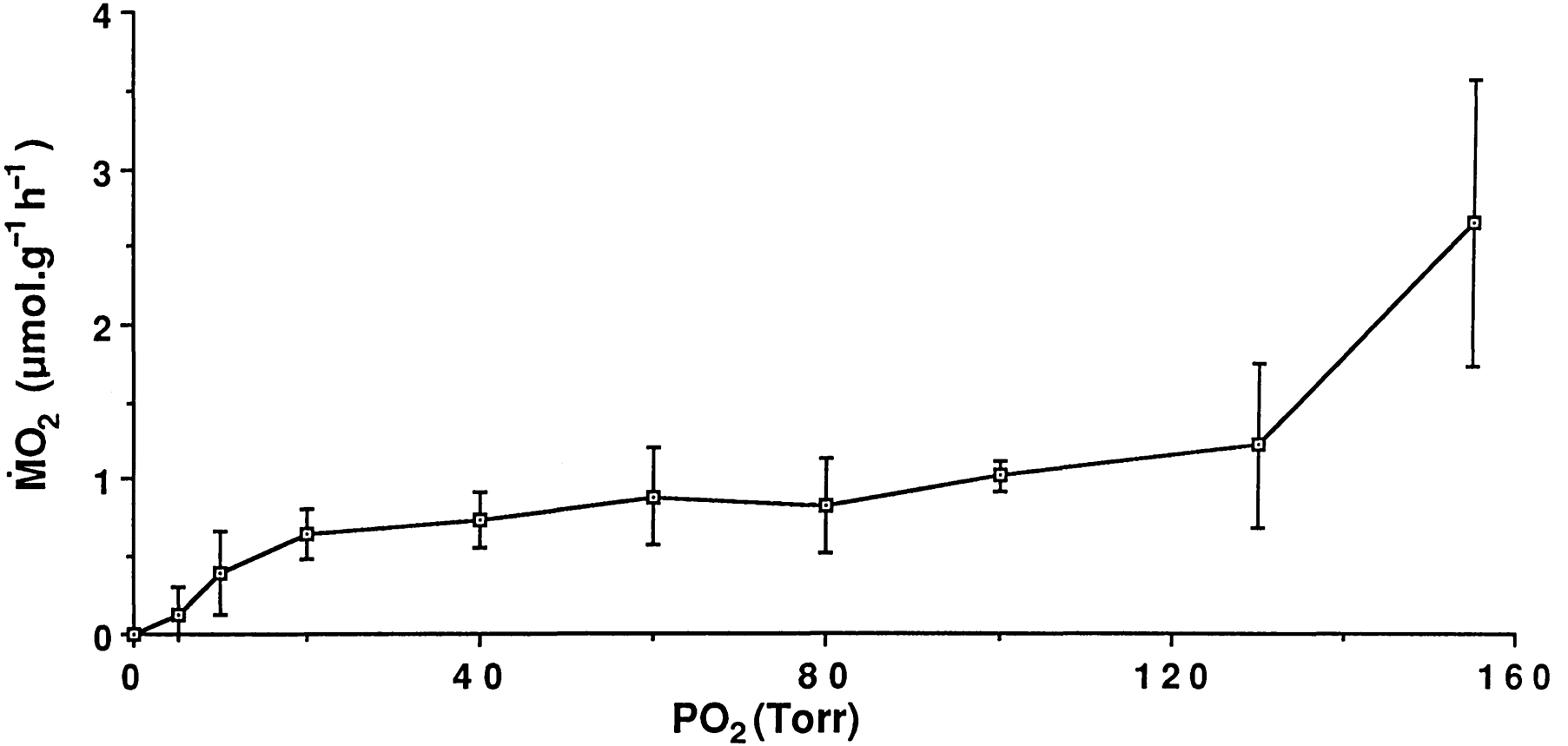

10
1
.1
.1
1
10
$\dot{M}O_2$ (µmol.g^{-1}.h^{-1})
Fresh weight (g)

FIG. 4.5 The effect of declining oxygen tension (PO_2; Torr) on the rate of oxygen consumption in ***Calocaris macandreae***. Means and standard deviations from measurements on 5 individuals are presented.


4
3
2
1
0
$\dot{M}O_2$ (µmol.g^{-1} h^{-1})
0
40
80
120
160
PO_2(Torr)

4.3.3 Branchial ventilation and heart rate in normoxia.

There was considerable variation in the pattern of branchial ventilation shown by individual *Calocaris* maintained in normoxic conditions (e.g. Figs. 4.6, 4.7, 4.10A). This apparent variation was due in part to differences in the position of the electrode and in the amplitude of the recorded beat. In most cases, periods of electrical noise were also recorded, which were due mainly to movement by the animal relative to the reference electrode. However, the continuing scaphognathite beat pattern could usually be observed superimposed on the larger fluctuations produced by movement of the animal (e.g. Figs. 4.6, 4.7).

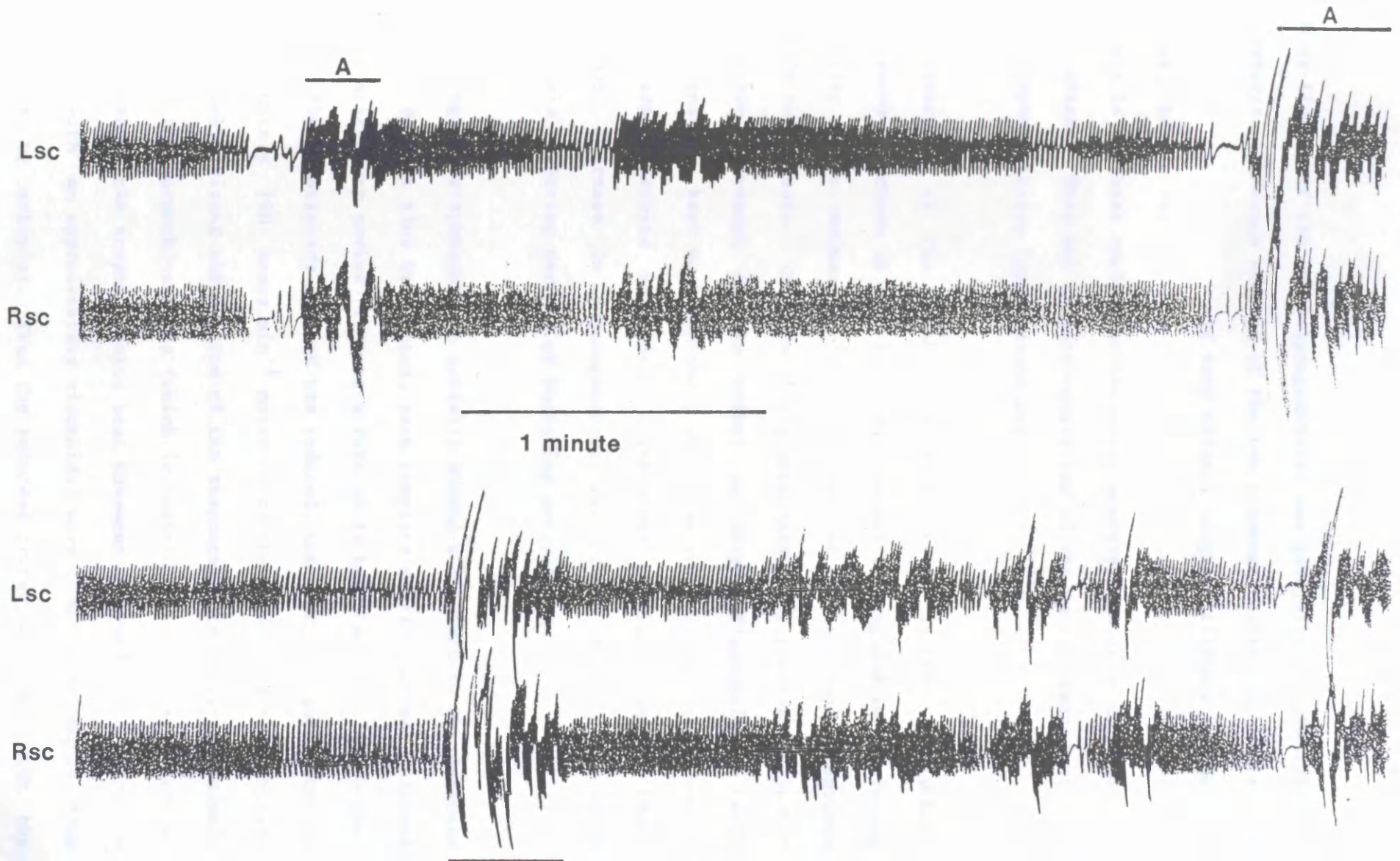
In all cases in which the rate of beating of both scaphognathites was measured, tight coupling of the two rates was observed (i.e. there was a constant ratio (usually = 1) between the beat rates). Fig. 4.6 shows a recording of right and left scaphognathites from an animal in normoxic conditions within a perspex tube 'burrow', 16 hours after electrode attachment. The animal showed sporadic locomotory activity (reflected as periods of noise on the recording) and occasional brief decreases and pauses in scaphognathite activity. This animal had probably not completely recovered from the operation (since the ventilation rate is relatively high compared with 'fully recovered' animals). However, Fig. 4.6 clearly shows that the rates of beating of both scaphognathites were nearly identical. In addition, decreases in rate and pausing occurred simultaneously in both scaphognathites. This was the case in all animals studied and very few observations of unilateral beating or pausing were made. (The stroke volume cannot be deduced from impedance recordings; the recorder amplification had been adjusted in Figs. 4.6 and 4.7 to give equal amplitudes and it is possible that the respiratory current flow is unequal.)

Fig. 4.6 Recordings of scaphognathite activity from an individual *Calocaris macandreae* in a perspex tube 'burrow' under normoxic conditions.

Lsc) Left scaphognathite.

Rsc) Right scaphognathite.

A) periods of locomotory activity, recorded as large oscillations on the trace.


A
A
Lsc
Rsc
1 minute
Lsc
Rsc

Phase-linking of the two scaphognathites was generally not observed in *Calocaris*. Although beating of the two scaphognathites was coupled (as described above), there was very often a slight difference in the two rates. As a result, the two scaphognathites were usually at different points in the beat cycle, and there was usually slow drift between the two phases. This may suggest separation of the neural control of the two scaphognathites (see Discussion).

A recording of the scaphognathite rates of another individual *Calocaris* is shown in Fig. 4.7. This animal was engaged in burrowing activity on the sediment surface, interspersed with stationary periods of 30s to 1 minute. The upper (left-scaphognathite) trace was adjusted to show movement of the animal as large fluctuations (with scaphognathite beat superimposed), while the lower (right) trace shows only scaphognathite movements. There was a significant (t-test, $P<0.05$) increase in scaphognathite rate (from 8-10 to 40-60 beats.min^{-1}) during periods of burrowing activity.

Recordings of scaphognathite activity showed a distinctive beat-pattern (Fig. 4.8). At slow beat rates, each complete oscillation was followed by a stationary period (2.5s at a rate of 12 beats.min^{-1}). At higher rates the stationary period was reduced, until at a beat rate of approximately 100 beats.min^{-1} movement of the scaphognathite became continuous. Direct observation of the scaphognathite in living animals through the branchiostegite (which is translucent in most animals) showed that the scaphognathite beat movement occurred in a vertical plane, with an approximately sinusoidal wave-form which moved from posterior to anterior. Thus the recorded traces conformed with the expected pattern of movement of the anterior end of the scaphognathite

FIG. 4.7 Recordings of scaphognathite activity from an individual ***Calocaris macandreae*** engaged in burrowing activity on the sediment surface.

Lsc) Left scaphognathite. The trace has been adjusted to show burrowing activity as large oscillations.

Rsc) Right scaphognathite.

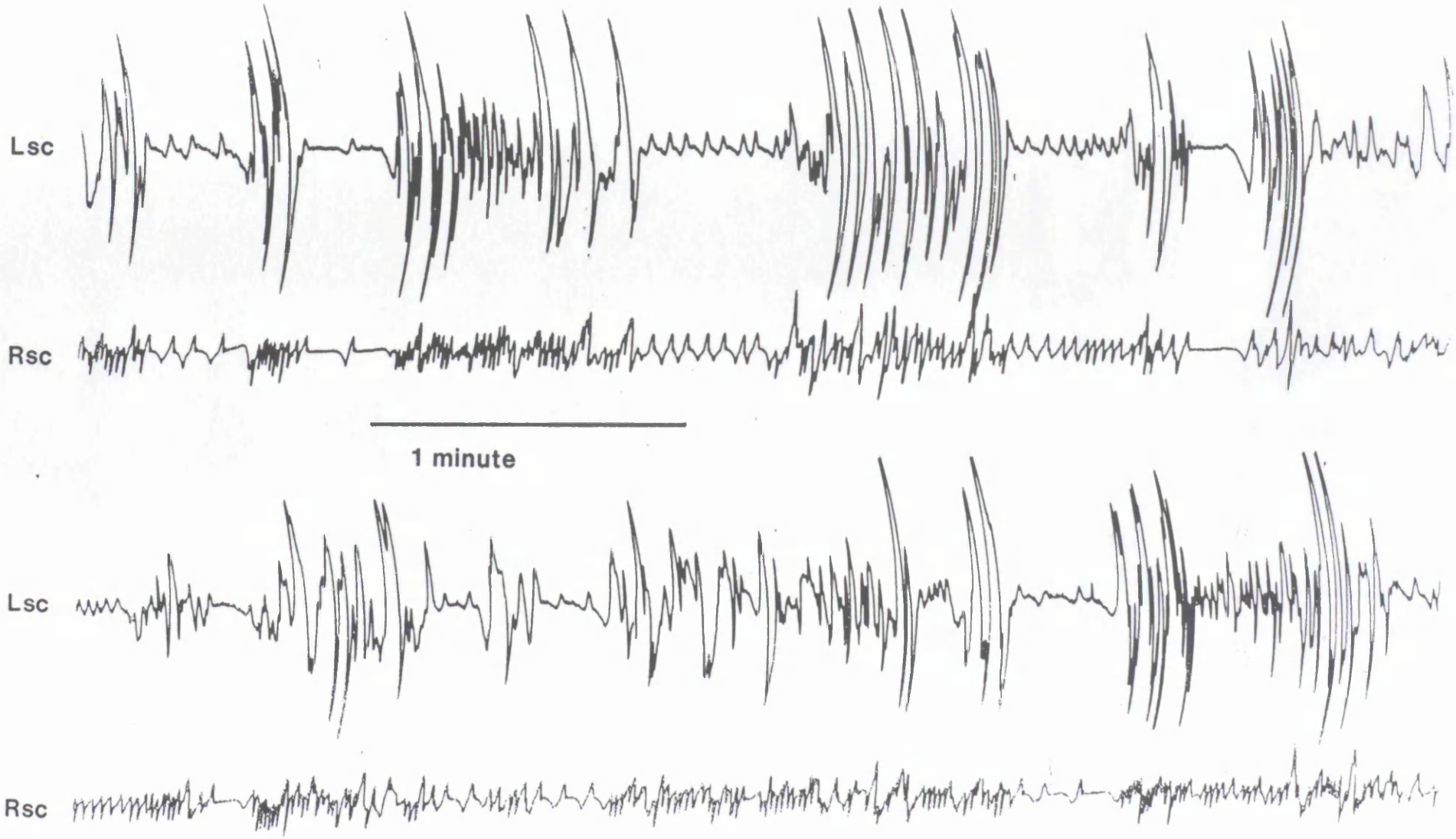
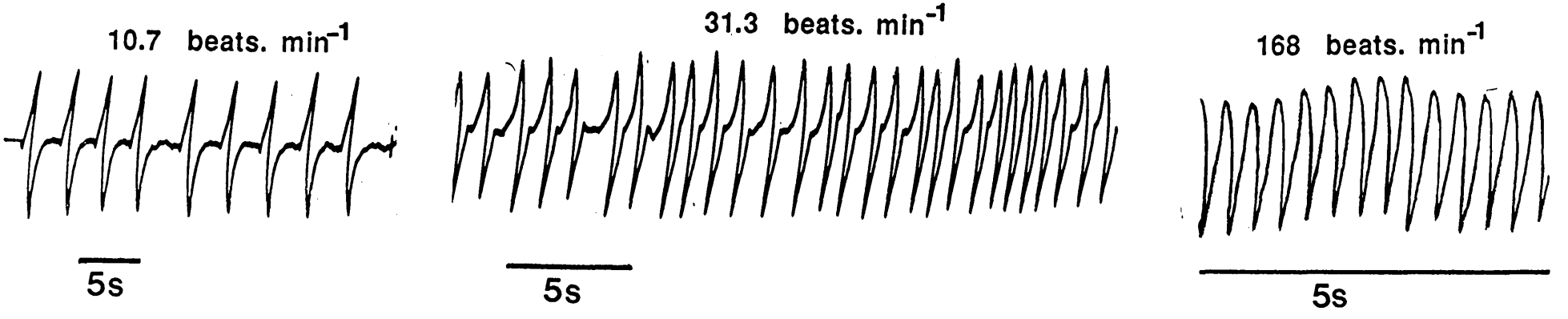

Lsc
Rsc
1 minute
Lsc
Rsc

FIG. 4.8 Variations in the detailed beat pattern of the scaphognathite of ***Calocaris macandreae***, at different scaphognathite beat rates. The shape of the beat trace changes as the beat becomes continuous (for more explanation, see text).


10.7 beats. min-1
5s
31.3 beats. min-1
5s
168 beats. min-1
5s

(which is closest to the position of the electrode; Fig. 4.3).

Recordings made of the heart rate of ***Calocaris*** under normoxic conditions showed a rate which varied from 30 to 70 beats.min^{-1}. The beat-pattern was complex, and was not tightly coupled to scaphognathite rates (Figs. 4.9, 4.10A).

Many individual ***Calocaris*** which were apparently in a settled condition in normoxia, showed periods of cardiac and ventilatory arrest (apnoea). These periods were generally of short duration, 10 - 30 seconds (e.g. Fig. 4.10A), but in some individuals periods of bilateral ventilatory pausing of up to 2 minutes were recorded. Periods of cardiac arrest were usually shorter (Figs. 4.9 and 4.10A). In general, individual animals showed either consistent coupling (e.g. Fig. 4.10A) or uncoupling (e.g. Fig 4.9) between periods of ventilatory pausing and cardiac arrest. No observations were made of periods of obvious reversal of the scaphognathite beat (similar to those which occur in many brachyuran crabs). These are most easily detected by pressure transducer recordings from the branchial chamber; it is possible that reversals in ***Calocaris*** were not detected by the impedance recordings used in this study, although reversals in crabs are usually easily detected on impedance recordings (A.C. Taylor, pers. comm.).

4.3.4 Branchial ventilation and heart rate in hypoxia.

The beat-patterns of the right scaphognathite and heart of an individual ***Calocaris***, exposed to progressive hypoxia are shown in Fig. 4.10. The mean beat rates of five individuals in similar conditions are shown in Figs. 4.11 and 4.12. The scaphognathite rate increased from a mean value of 27 beats.min^{-1} at 155 Torr to a maximum rate of

Fig. 4.9 Recordings of heart (H) and scaphognathite (SC) activity in an individual ***Calocaris macandreae*** under normoxic conditions. Note that scaphognathite and heart rates were not tightly coupled in this individual.

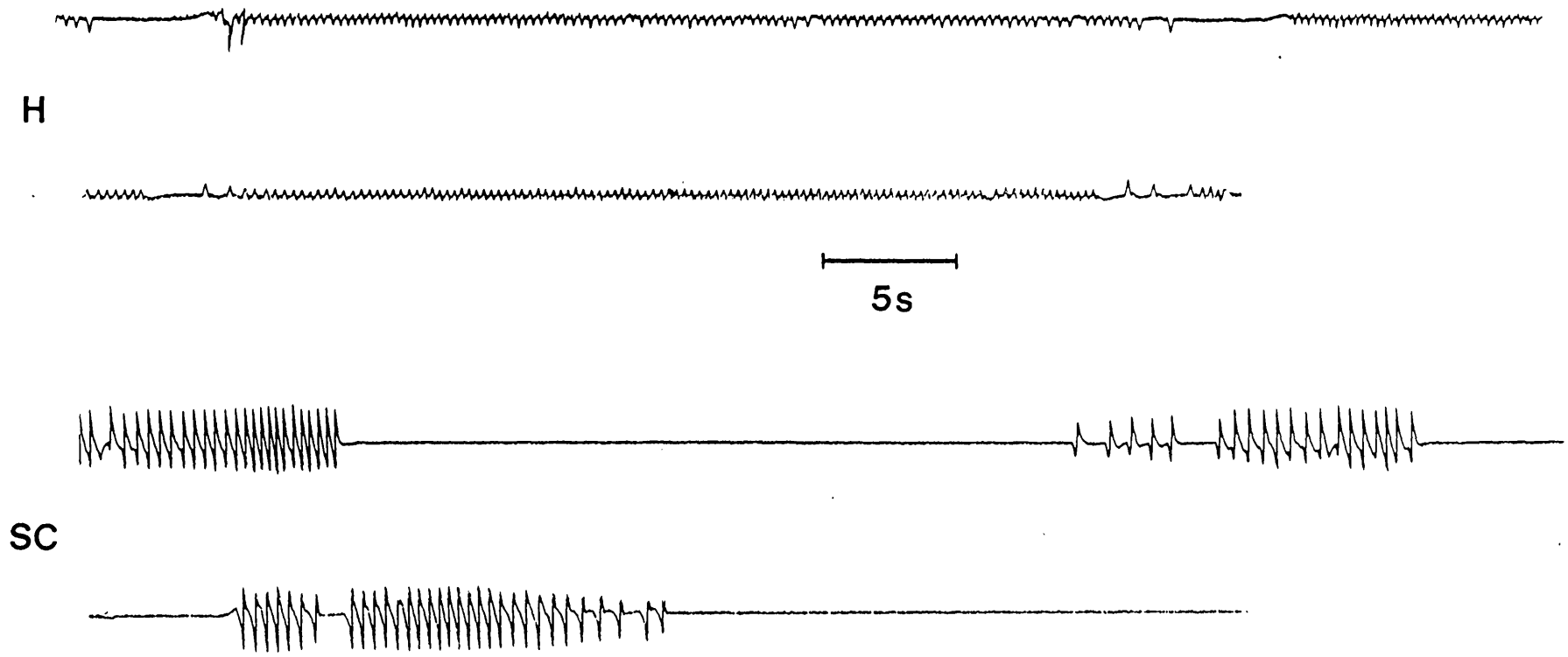
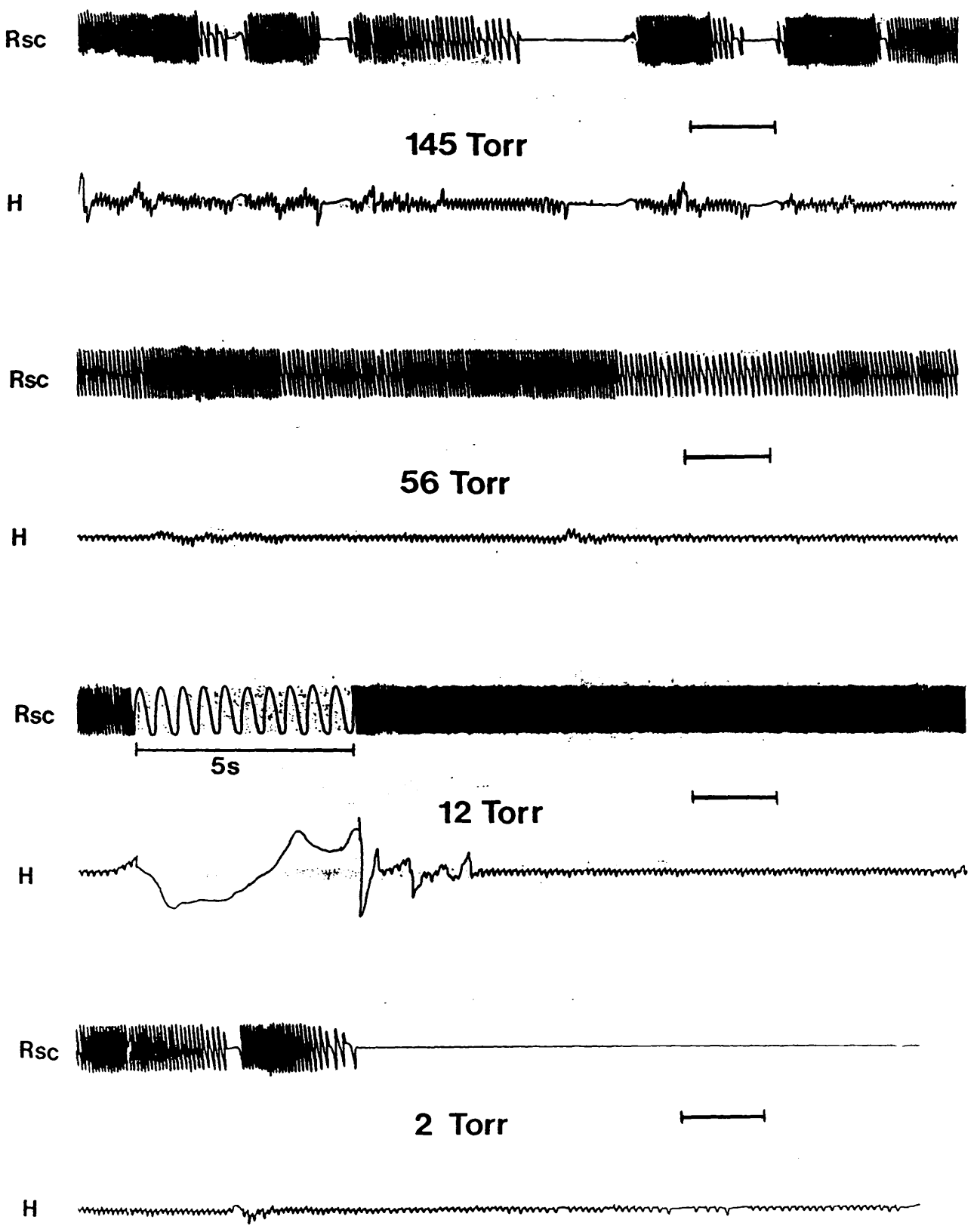

H
5s
SC

FIG. 4.10 Recordings of heart (H) and right scaphognathite (Rsc) in an individual ***Calocaris macandreae*** in conditions of declining oxygen tension. Hyperventilation is evident at PO_2's of 56 and 12 Torr (relative to scaphognathite rates at 145 Torr), however, there is no significant change in heart rate. Apnoea occurs at a PO_2 of 2 Torr. Scale bar: 10 seconds (A 5 second high-speed recording is included in the 12 Torr trace).


Rsc
145 Torr
H
Rsc
56 Torr
H
Rsc
5s
12 Torr
H
Rsc
2 Torr
H

110-160 beats.min^{-1} at approximately 15 Torr. Below this 'critical' PO_2 (Pc) the scaphognathite rate decreased until nearly all animals showed ventilatory arrest when conditions became anoxic. The increased scaphognathite rate resulted initially from a decrease in the frequency of pausing to produce continuous beating, followed by a large increase in the beat rate. The response shown in Fig. 4.10 had an unusually abrupt ventilatory arrest at 2 Torr; most individuals showed a more complicated response with longer periods of arrest interspersed with rapid beating as the PO_2 decreased from a Pc of 10 - 20 Torr to anoxia. Occasionally, animals maintained a ventilatory flow even at very low PO_2's (< 1 Torr). All individuals studied, however, showed complete ventilatory arrest after 30 minutes of anoxia.

In contrast to the pronounced change in scaphognathite rates, no significant change in heart rate was recorded in any individual *Calocaris* exposed to hypoxia. Similarly, brief exposures to anoxia had no effect (Fig. 4.10; Fig. 4.12). During exposure to varying degrees of oxygen availability, heart rate was maintained at a mean rate of 52 beats.min^{-1}. In several experiments with different animals exposed to long periods of anoxia (12 - 24 hours), the heart rate was observed to be maintained (although occasionally at a slow rate, approximately 10 beats.min^{-1}).

FIG. 4.11 The effect of declining oxygen tension (PO_2; Torr) on scaphognathite rate (F_{sc}) in ***Calocaris macandreae***. Different symbols represent 5 individuals. Time-course of oxygen depletion approximately 12 hours.

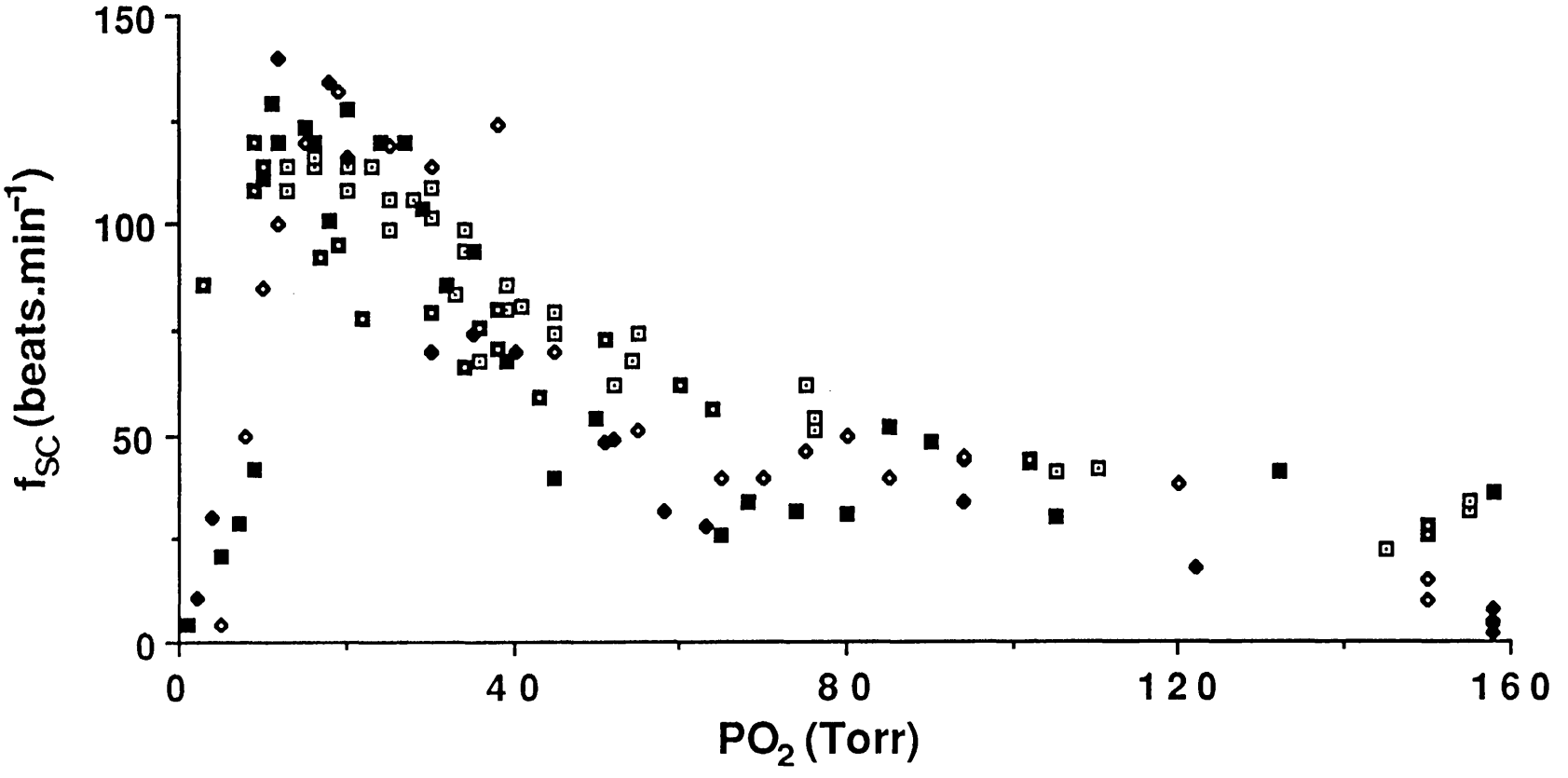
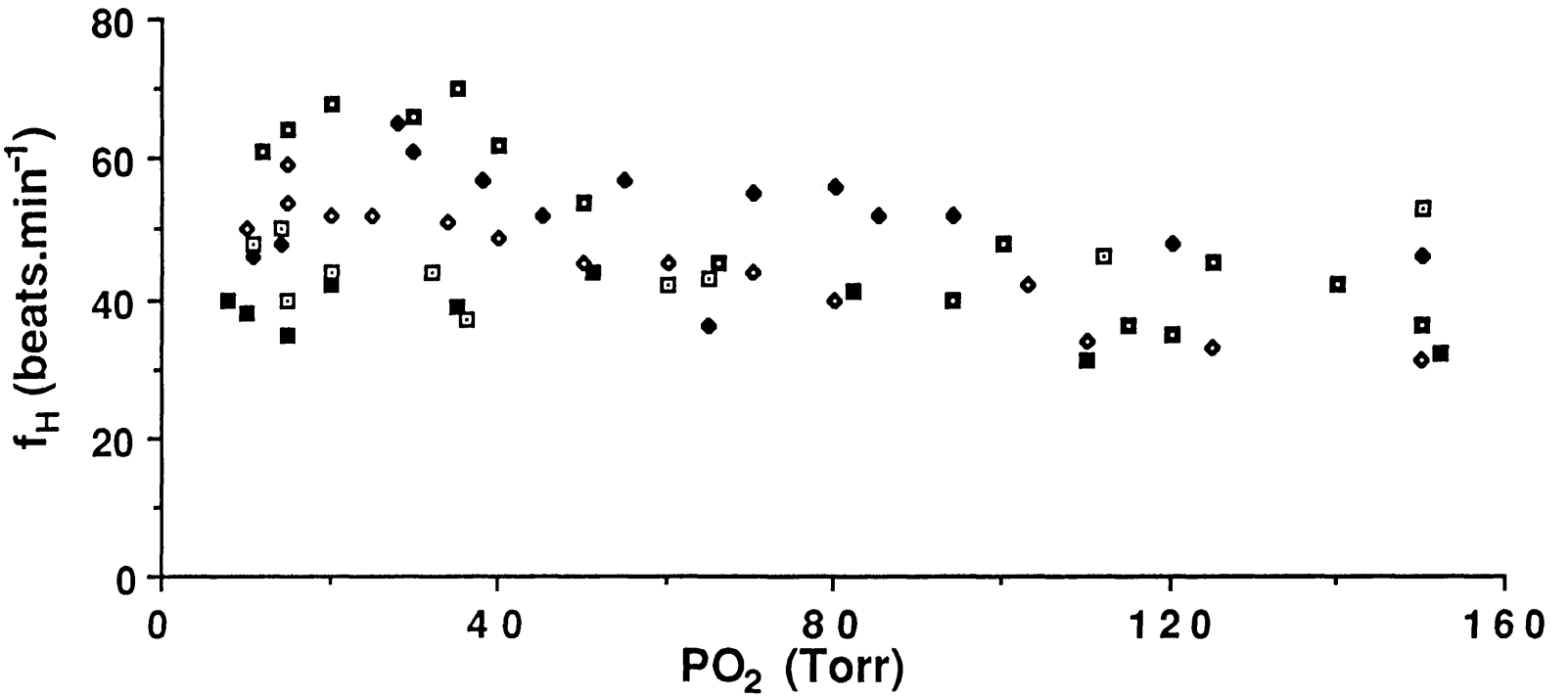

150
100
50
0
f_{SC} (beats.min^{-1})
0
40
80
120
160
PO_2 (Torr)

FIG. 4.12 The effect of declining oxygen tension (PO_2; Torr) on heart rate (f_H) in ***Calocaris macandreae***. Different symbols represent 5 individuals. Time-course of oxygen depletion approximately 12 hours.


80
60
40
20
0
f_H (beats.min^{-1})
0
40
80
120
160
PO_2 (Torr)

4.4 DISCUSSION

4.4.1 Comparative aspects of oxygen uptake by *Calocaris macandreae*.

In this study, rates of oxygen consumption ($\dot{M}O_2$) of ***Calocaris macandreae*** were measured using both open and closed respirometry. $\dot{M}O_2$ was measured for animals thought to be in a 'normal' state of disturbance (see below), in normoxic conditions and in conditions of progressive hypoxia.

McMahon and Wilkens (1983) state that "oxygen uptake (consumption) is the most frequently used (and perhaps misused) parameter in crustacean respiratory physiology". There is a large literature concerning oxygen uptake rates by crustaceans (see review of Wolvekamp and Waterman, 1960), characterised by inconsistency in methods and large variability even in reports for the same species under apparently similar conditions. McMahon and Wilkens (1983) suggest that the best approach to understanding oxygen consumption is to define the rate limits : the basal rate, "that level of O_2 uptake necessary for bodily maintenance under a particular set of environmental conditions that approximate the seasonal norm"; and the active rate "resulting from sustained maximal activity". The range of oxygen uptake rates thus defined is termed the "aerobic metabolic scope" (Bennet, 1978).

Probably the majority of studies of oxygen uptake in crustaceans (including this one) have used some form of closed respirometry, although the method has been repeatedly criticised. Tang (1933) and Kamler (1969) led the criticism of the simplest form of closed respirometry (the "Winkler bottle"). More recently, von Oertzen (1984) listed 4 categories of problems:

1. Disturbance of the animal during transfer to the bottle (this can be extended in more complex experimental systems, to include disturbance due to a change in water flow when starting a run).
2. Uncontrolled pH variation due to CO_2 excretion during an experiment.
3. Uncontrolled variation in the effects of metabolic waste-product accumulation, due to variation in the biomass/volume ratio.
4. The absence of mixing leading to sub-optimal supply of oxygen to the animal.

Although criticism (4) is usually overcome in closed respirometers such as that used in this study (by including an enclosed stirring bar in the respirometer), the remaining criticisms are inherent in the design of closed respirometers. Open respirometers avoid these problems, although there are practical problems in detecting the small PO_2 differences necessary, and in ensuring adequate control conditions. In addition, the rate of data acquisition using open respirometry is usually slow in comparison with that made possible by using large numbers of closed bottle respirometers. In the present study, very similar rates of oxygen consumption were recorded using both methods of respirometry, suggesting that the methodological criticisms outlined above were not limiting (in addition, pH did not vary throughout the course of the closed respirometer experiments).

The *Calocaris* used in this study continued to show sporadic locomotory activity throughout the experiments (or until low PO_2's were reached in closed systems), and furthermore did not show a significant decrease in $\dot{M}O_2$ during 12 - 16 hours following disturbance. The physiological state of these animals therefore does not conform to either 'basal' or 'active' according to the terminology of McMahon and Wilkens (1983) and

has been defined here as 'normal'. Definitions of 'resting' or 'standard' metabolism (e.g. Prosser, 1973) involve quantification of activity levels in animals, followed by extrapolation to 'zero activity'; while other studies have defined a physiological state according to a fixed recovery time from handling disturbance (e.g. 'routine': 12h (Innes, 1985)).

Several studies have found that oxygen uptake of apparently inactive animals declines significantly for 24 - 48 hours following handling stress (e.g. McMahon *et al.*, 1974; McMahon and Wilkens, 1975, 1977; Butler *et al.*, 1978; Taylor and Butler 1978). The relevance of long-running studies such as these to conditions in the natural habitat is probably limited; in addition the effects of starvation and confinement will become significant and species-specific.

In conclusion, lack of comparability in methods complicates the use of oxygen uptake data for inter-specific or inter-study comparison. The use of such comparisons in the present discussion will be confined to studies in which experimental conditions were similar to those used in this study, and were chosen to represent typical environmental conditions for the species in question. Also, only oxygen uptake rates measured under comparable activity states (e.g. "quiescent, bilateral ventilation"; "routine") will be compared. These comparisons must nevertheless be regarded with caution.

Values for rates of oxygen consumption by thalassinid species, taken from the literature, are tabulated below:

TABLE 4.1 Literature data for rates of oxygen consumption in the Thalassinidea.

SPECIES	$\dot{M}O_2$ ($\mu mol.g^{-1}.h^{-1}$)	Temp (°C)	Reference
Calocaris macandreae	0.50**	5	Dries, 1975
	0.75	10	present study
Callianassa californiensis	0.80-1.16*	10	Thompson and Pritchard, 1969
	1.43*	10	Miller *et al.*, 1976
Callianassa jamaicense	2.9*	25	Felder, 1979
Upogebia pugettensis	2.7*	10	Thompson and Pritchard, 1969
Upogebia africana	4.5*	20	Hill, 1981
Upogebia stellata	0.90	10	present study

* re-calculated from authors' units
** re-calculated from author's units and data from Calderon-Perez (1981)

The lower oxygen uptake rates recorded in *Calocaris macandreae* by Dries (1975), compared to the present study, are probably due to the different temperatures used. Rates of oxygen consumption by *Calocaris macandreae* are comparatively similar to those of *Callianassa* species (recorded in studies conducted at 10°C); in the approximate range 0.5 - 1.5 $\mu mol.g^{-1}.h^{-1}$. Rates of oxygen consumption in *Upogebia* species are slightly higher, in the range 0.9 - 4.5 $\mu mol.g^{-1}.h^{-1}$. This may relate to the more oxygenated habitat of *Upogebia*, and the more active behavioural patterns shown (see previous chapters).

A representative sample of oxygen uptake rates reported in the literature for a range of brachyuran and macruran decapods, was tabulated by McMahon and Wilkens (1983). In summarised form (with units converted to $\mu mol.g^{-1}.h^{-1}$), these reports include:

TABLE 4.2 Literature data for rates of oxygen consumption in brachyuran and macruran decapods.

BRACHYURA	$\dot{M}O_2$ ($\mu mol.g^{-1}.h^{-1}$)	Reference
Cancer magister	2.04-3.66	McMahon *et al.* (1979)
Cancer productus	1.62	McMahon and Wilkens (1977)
Carcinus maenas	1.20-1.32	Taylor (1976)
Callinectes sapidus	2.82	Mangum and Weiland (1975)
Libinia emarginata	3.00	Burnett (1979)
MACRURA		
Homarus americanus	1.32	McMahon and Wilkens (1977)
Homarus gammarus	1.02	Butler *et al.* (1978)
Pacifastacus leniusculus	1.92-7.62	Rutledge (1981)
Orconectes rusticus	0.90	Wilkes and McMahon (1982)

Thus oxygen uptake rates measured in a variety of non-thalassinid decapod crustaceans are in the range 0.9 - 3.0 $\mu mol.g^{-1}.h^{-1}$. Oxygen consumption rates in the thalassinids ***Calocaris macandreae*** and ***Callianassa*** species are comparatively lower; this may be of potential functional significance (see below). In contrast, oxygen uptake rates measured in ***Upogebia*** species are within the range documented for non-burrowing, 'typical' decapods. The greater metabolic rates of ***Upogebia*** species are possibly correlated with the less hypoxic burrows and more active lifestyle (i.e. a filter-feeding strategy: see Chapter 2).

The ability of decapod crustaceans (and other invertebrates) to maintain oxygen consumption at a constant rate, over a wide range of oxygen partial pressures, has been the subject of much research (reviewed by e.g. Wolvekamp and Waterman, 1960; Mangum and van Winkle, 1973; Herreid, 1980). Initial reports suggested that regulatory abilities in crustaceans were limited (Wolvekamp and Waterman, 1960), for reasons largely related to gill and blood performance. More recent reviewers, however, have concluded that the majority of malacostracan crustaceans have considerable ability to maintain routine or quiescent

levels of oxygen consumption, in the face of environmental oxygen depletion (Taylor and Butler, 1979; McMahon and Wilkens, 1983).

Historically, animals have been classified into two categories based on MO_2 regulatory ability: 'oxyregulators' vs. 'oxyconformers'. This approach has since been repeatedly criticised as an oversimplification (e.g. Mangum and van Winkle, 1973: "an inherently futile attempt to force a continuously varying quantitative phenomonen into a simple verbal dichotomy"). In addition, regulatory ability is greatly influenced by many variables (Herreid, 1980; McMahon and Wilkens, 1983). Most recent authors have described regulatory ability simply in terms of the 'critical point' (Pc; defined above), usually quoted as a range measured on a number of individual animals.

Values for the Pc reported for burrowing decapods have been listed by Atkinson and Taylor (1988): 10-25 Torr in the thalassinids ***Callianassa californiensis*** (Thompson and Pritchard, 1969; Miller *et al.*, 1976; Torres *et al.*, 1977) and ***Callianassa jamaicense*** (Felder, 1979); and 45-50 Torr in ***Upogebia pugettensis*** (Thompson and Pritchard, 1969). Pc's of 20-40 Torr have been recorded for the burrowing crab ***Goneplax rhomboides*** (A.C. Taylor, unpubl. obs.); and 40 Torr in ***Nephrops norvegicus*** (Hagerman and Uglow, 1985). These data compare with Pc values measured in ***Calocaris macandreae***, which varied individually between 10 - 20 Torr. In general, Pc values in burrowing decapods, and particularly in the thalassinids ***Calocaris macandreae*** and ***Callianassa*** species are lower than in decapods from 'normal' marine environments (e.g. 130 Torr in ***Ebalia tuberosa***: Schembri, 1979; 60-80 Torr in ***Cancer pagurus***: Bradford and Taylor, 1981).

4.4.2 Physiological mechanisms of oxyregulation in *Calocaris macandreae*.

Between 1970 and 1982, a great deal of research was conducted, aimed towards elucidation of the respiratory features and mechanisms of decapod crustaceans. This allowed McMahon and Wilkens (1983) to review the interactions of ventilation, perfusion and oxygen uptake in large decapods, in some detail. Since this review, relatively little new information has been reported, although recent research is beginning to consider neural and hormonal control of ventilation and perfusion in more detail.

The mechanisms of regulation of oxygen uptake in decapods are similar whether the stress results from activity or hypoxia (McMahon and Wilkens, 1983). Regulatory processes may include ventilation (oxygen supply to the respiratory surface); perfusion (maintenance of a favourable oxygen diffusion gradient); oxygen transport in the haemolymph (considered in the next chapter); and oxygen utilisation rates at the tissues.

In decapods, hyperventilation (increased frequency of scaphognathite pumping and resultant increased ventilatory flow) is a frequent response to environmental hypoxia, presumably related to regulation of oxygen uptake. Hyperventilation has been recorded in ***Homarus gammarus*** (Butler *et al.*, 1978; McMahon *et al.*, 1978); ***Homarus americanus*** (McMahon and Wilkens, 1975); ***Cancer productus*** (McMahon and Wilkens, 1977); ***Callinectes sapidus*** (Batterton and Cameron, 1978); ***Libinia emarginata*** and ***Ocypode quadrata*** (Burnett, 1979); ***Cancer pagurus*** (Bradford and Taylor, 1981; Burnett and Bridges, 1981); ***Austropotamobius pallipes*** (Wheatly and Taylor, 1981); ***Palaemon elegans*** (Morris and Taylor, 1985); ***Carcinus maenas*** (Arudpragasam and Naylor,

1964; Taylor *et al.*, 1973; A.C. Taylor, 1976) and the thalassinids ***Callianassa californiensis*** and ***C. affinis*** (Farley and Case, 1968). The hyperventilatory pattern observed in ***Calocaris macandreae*** was unremarkable, except for the low critical PO_2 at which hyperventilation ceased in declining oxygen tensions.

The reasons for cessation of the hyperventilatory response when environmental PO_2 decreases below the Pc, are thought to be related to the high energy demand of increased ventilatory pumping (Hughes and Shelton, 1962; Bradford and Taylor, 1981). The mechanical work and energetic costs of scaphognathite pumping have been investigated in the shore crab, ***Carcinus maenas*** (Wilkens *et al.*, 1984), showing that the cost of ventilation for resting crabs is equivalent to 30% of total MO_2.

Coupling between the Pc's observed in oxygen uptake and ventilation rate was oberved in ***Calocaris***, as in most other species studied. The maximum rates of scaphognathite pumping achieved, may be limited by mechanical factors in addition to energetic costs (McMahon and Wilkens, 1983). An additional interaction which is frequently observed is that between the hyperventilation response and acid-base balance in the haemolymph: hypoxia-induced hyperventilation often results in a pronounced alkalosis (e.g. Truchot, 1976; McMahon *et al.*, 1978; Sinha and Dejours, 1980; see Chapter 5).

The neural control of scaphognathite function has been reviewed by McMahon and Wilkens (1983). Several crustaceans show a similar pattern of bilateral coordination between scaphognathites, in which periods of phase constancy alternate with periods of drift in relative frequency. This pattern is thought to demonstrate independence of the pattern

generators (Wilkens and Young, 1975; Young and Coyer, 1979). The synchronising mechanism is not well understood, although communication may occur at the motor neuron level (Wilkens and Young, 1975; Pilkington and MacFarlane, 1978).

The cardiovascular response to environmental hypoxia in decapods is not so consistent as that of the ventilatory system. Many species which are not strong oxyregulators (e.g. ***Austropotamobius pallipes***: Wheatly and Taylor, 1981; ***Homarus gammarus***: McMahon, Butler and Taylor, 1978) show a progressive bradycardia in declining oxygen tensions. Other species which exhibit more ability to regulate oxygen consumption (e.g. ***Palaemon elegans***: Morris and Taylor, 1985; ***Calocaris macandreae***: present study) show little if any response in heart rate. The cardiovascular system is more complex than the respiratory pumps, however, since changes in heart stroke volume may occur (McMahon and Wilkens, 1977) which are not measured using impedance techniques (Depledge, 1979). Measurements of perfusion rates based on the Fick principle (e.g. Taylor, 1976; Bradford and Taylor, 1981) suggest that cardiac output remains independant of PO_2 above the Pc.

In normoxic conditions, some individual ***Calocaris macandreae*** exhibit pausing in both respiratory ventilation and blood circulation (usually tightly coupled). Similar pausing patterns have been described in many decapods, although pausing in brachyuran crabs generally occurs less frequently but for longer periods (5 - 20 minutes: Bridges, 1976; McMahon and Wilkens, 1977; Bradford and Taylor, 1981; Taylor, 1984). The adaptive advantages of respiratory pausing are probably related to energetic costs: during bilateral ventilation (and normal perfusion) in normoxic conditions, the respiratory pigment remains almost fully

saturated on return to the gills, and acts as a venous reserve during pauses. The full capabilities of the respiratory/cardiovascular systems are reserved for periods of activity or hypoxic stress (e.g. Burnett and Bridges, 1981).

Calocaris macandreae exhibited a somewhat unusual physiological response in that the heart rate (and presumably the haemolymph circulation) was maintained even after several hours of anoxia, during which time the venous reserve is almost certainly depleted and the blood ceases to deliver oxygen to the tissues. It is possible that this response (or non-response) is related to the accumulation of metabolic end-products (e.g. L-lactate) in the haemolymph, since maximum lactate concentrations appeared to be in equilibrium in tissue and blood during anoxia (see Chapter 6). In the absence of blood circulation, localised lactate accumulation in the tissues and haemolymph might limit the anaerobic capability of the animal. A circulated, mixed haemolymph might represent a larger 'sink' for metabolic end-products (and may also minimise localised acid-base disturbances). An alternative explanation for the maintenance of blood flow may be that the neural PO_2 detector in decapods appears to be located in the vascular system. A maintained circulation might allow a rapid response to improved environmental conditions during recovery from hypoxia (A.C. Taylor, pers. comm.)

4.4.3 Respiratory physiology in *Calocaris macandreae*, in relation to the burrow environment.

As discussed above, there appears to be a correlation between both low oxygen consumption rates and low Pc (i.e. well-developed regulatory ability), and burrowing behaviour in decapod crustaceans. Both these characteristics of the respiratory physiology are usually interpreted

as being of obvious benefit to burrowing animals, in view of the conditions of restricted oxygen availability often prevalent within the burrow (see previous chapter). An alternative reason for a low oxygen consumption rate (low metabolic rate) might be a low level of behavioural activity, as observed in ***Calocaris***. Other factors such as longevity and moult cycles are almost certainly involved, and should be accounted for when interspecific comparisons are made.

The aerobic metabolic scope (= 'active' $\dot{M}O_2$ rates / 'quiescent' $\dot{M}O_2$) has been measured in only a few decapod species (McMahon and Wilkens, 1983), but appears to be generally in the range 4 - 5 fold at 8-10°C. Results obtained in this study suggest that the aerobic scope in ***Calocaris macandreae*** may be approximately 2 - 3 fold (maximal rates recorded were 1.4 - 2.8 $\mu mol.g^{-1}.h^{-1}$). It may be, therefore, that the low activity rates observed in ***Calocaris*** are imposed by respiratory/metabolic limitations. Meaningful comparisons are especially difficult due to the paucity of comparative, quantitative data concerning activity patterns of decapods in natural conditions (most authors being content to label species as "sluggish", "active" etc.).

Although the oxygen consumption rates observed in ***Calocaris macandreae*** were comparatively low, it is evident that regulation of oxygen consumption during periods of hypoxia was highly efficient. The main regulatory mechanism appears to be modification of the respiratory ventilatory rate (coupled with a highly efficient blood pigment; see Chapter 5). In terms of the unit processes outlined in the Introduction; ventilation -> uptake -> perfusion -> transport -> metabolism; the evidence is that physiological oxyregulation occurs

mainly by modulation of the ventilation and transport processes. *Calocaris* demonstrates a respiratory system which is well adapted to maintaining a low rate of oxygen consumption in the variable, hypoxic conditions of the burrow; in contrast to most other decapods which are adapted to long periods of inactivity interspersed with short periods of activity during which there is a large metabolic oxygen demand.

In conclusion, it appears likely that characteristics of the respiratory physiology of *Calocaris macandreae* and of other thalassinids may reflect an adaptation to:

1. The respiratory conditions within the burrow, i.e. the severe spatial gradient of oxygen availability.
2. The activity regime possible within the burrow: a consistent, low level of activity, made possible by the high degree of protection from predation and self-sufficiency with regard to food supply etc., afforded by the burrow. There is little requirement for a high aerobic scope, as found in 'active' decapods.

These interactions between environment, ecology, behaviour and physiology will be considered further in later chapters.

CHAPTER 5. RESPIRATORY PROPERTIES OF THE HAEMOLYMPH OF *CALOCARIS MACANDREAE*

5.1 INTRODUCTION.

The fourth physiological process in the unit process scheme of respiration outlined by McMahon and Wilkens (1983; see also introductions to previous chapters), was respiratory gas transport between the gills and tissues. This chapter investigates the oxygen and carbon dioxide transporting properties of the haemolymph of ***Calocaris macandreae***, both *in vitro* and *in vivo*.

The physiological characteristics of respiratory gas transport in decapod crustaceans are strongly influenced by the possession of a respiratory pigment, haemocyanin. The structure of the haemocyanin molecule is known to vary between species, mainly as a result of variation of the aggregation state (i.e. the number of polypeptide sub-units contained in each native molecule; reviewed by Mangum, 1983a). The haemocyanins of thalassinids have been recognised for their exceptionally high molecular weight since the work of Svedberg (1933), who examined haemocyanins from a large number of species from different phyla (including ***Calocaris macandreae***), using ultracentrifugation techniques. More recent work (e.g. Miller and van Holde, 1981a, b) has shown that large native haemocyanin molecules (eikositetramers, composed of 24 monomer subunits) are also present in other thalassinid species. The structure of ***Calocaris*** haemocyanin was investigated using gel electrophoresis to estimate monomer molecular weight, and gel filtration to determine the size of the native molecule.

The oxygen transporting properties of crustacean haemocyanins have been the subject of recent intensive research, in particular following the

introduction of diffusion chamber techniques (used to construct *in vitro* dissociation curves). It is now evident that the oxygen affinity of crustacean haemocyanins is affected (modulated) by a number of physical factors (e.g. calcium concentration, temperature) and organic 'modulators'(e.g. L-lactate, urate), and that 'fine-tuning' of oxygen transporting properties of the haemolymph may be a response to changing external and internal conditions. However, the relations between characteristics measured *in vitro* and the *in vivo* function of the vascular oxygen transport system are not completely understood. This problem is particularly evident in studies of small animals, since it is usually not possible to measure haemolymph PO_2 *in vivo*. The characteristics of ***Calocaris*** haemocyanin were studied *in vitro* and related to *in vivo* function, and ecological factors, as far as was possible within the constraints of animal size and practical techniques.

The ionic composition of the haemolymph was determined, since many of the techniques used required physiological saline solutions (as a dialysis medium, gel filtration buffer etc.). In addition, the magnesium concentration of the haemolymph has been linked to the activity patterns of different species (Robertson, 1949, 1960).

Finally, a brief study was made of the carbon dioxide transporting properties and acid-base status of the haemolymph. Knowledge of these areas of crustacean physiology is rapidly increasing, and it is becoming apparent that both CO_2 transport/excretion and acid-base regulation may have important interactions with respiratory processes.

5.2 MATERIALS AND METHODS.

5.2.1 Ionic composition of the haemolymph.

Haemolymph samples from *Calocaris macandreae* were taken from the pericardium of freshly caught animals. Approximately 100 ul was obtained from each animal (mean fresh weight approximately 1 g). Samples were usually centrifuged (10000g; 5 minutes) to remove cellular debris and frozen at -20°C. Pooled samples were thoroughly mixed prior to centrifugation and frozen at -20°C in aliquots of 0.5 or 1.0 ml.

Sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}) and chloride (Cl^-) concentrations were measured in several pooled haemolymph samples, sampled on a number of dates.

Initially, all ionic concentrations were measured by atomic absorption spectrophotometry (AAS) using a Pye SP90 spectrophotometer. Later, only Ca^{2+} and Mg^{2+} were measured by AAS (using a Pye Unicam PU9200). Samples were diluted using Millipore quality distilled water (dilution factors were 121 and 363 for Ca^{2+} and Mg^{2+} respectively). Diluted samples for Ca^{2+} determination included 1% LaCl in order to reduce interference (Whiteside and Milner, 1984).

Na^+ and K^+ determinations were carried out by flame spectrophotometry (Corning Flame Photometer 410) using dilution factors of 6171 (Na^+) and 121 (K^+). Cl^- concentrations were measured in undiluted haemolymph using 20 ul samples and a chloride titrator (Jenway PCLM3).

5.2.2 Oxygen carrying capacity of the haemolymph.

The total oxygen carrying capacity (cO_2) of haemolymph containing a respiratory pigment is equal to the oxygen bound to the pigment ($c_{HCY}O_2$) plus the physically dissolved oxygen fraction. Three different methods were used to estimate the oxygen carrying capacities of ***Calocaris*** haemolymph and haemocyanin. Since haemocyanins bind one oxygen molecule for every two copper atoms, it is possible to estimate $c_{HCY}O_2$ by determination of the Cu concentration of the haemolymph. $c_{HCY}O_2$ can also be estimated from the protein concentration of the haemolymph, with the assumptions that 1.) all protein = haemocyanin

2.) $E^{1\%}$ of protein = 24.3 (this study, 5.3.2)

3.) the functional sub-unit molecular weight = 75 000k (Mangum, 1983a).

In addition cO_2 can be determined directly using the method of Tucker (1967): this uses KCN to displace bound O_2 from the pigment (see below). The amount of physically dissolved oxygen can be calculated from the solubility coefficient of oxygen in sea water at the appropriate temperature, or can be measured directly (in sea water) using Tucker's method.

Copper concentrations of the haemolymph were determined by Atomic Absorption Spectrophotometry (AAS), using a Pye Unicam PU9200. At the dilution level necessary for Cu determination in haemolymph (100-fold), there was a large 'flare' due to sodium. Standard Cu solutions (Spectrosol $CuNO_3$; BDH) were therefore diluted using both Millipore quality distilled water and Ringer solutions with appropriate NaCl, KCl, $MgCl_2$ and $CaCl_2$ concentrations. There was little difference between standard curves constructed with distilled water and Ringer-diluted standards. The absorbances of 12 haemolymph samples at 335 nm

were measured using a Pye Unicam 8700 spectrophotometer, after 100-fold dilution in deionised water. The extinction coefficient ($E^{1\%}_{335}$) of the haemolymph copper was then calculated from the regression equation.

The protein concentration of haemolymph samples from 15 individuals was measured by the Coomassie blue method (Read and Northcote, 1981). The protein extinction coefficient ($E^{1\%}_{280}$) was then calculated from absorbances of undiluted haemolymph at 280 nm.

Tucker's (1967) method gives a direct measurement of the total oxygen content of a haemolymph sample. The method used was that given by Bridges (1979). A 1% solution of KCN was degassed and placed in a small (approx. 350 µl) thermostatted chamber at 30°C (an elevated temperature was used to produce a faster electrode response). The KCN displaced bound oxygen from the haemocyanin, as dissolved oxygen. An oxygen electrode (Radiometer E5046), connected to an oxygen meter (Radiometer PHM73) and chart recorder, measured PO_2 within the cell. The contents of the chamber were mixed by a small magnetic stirrer. The increase in PO_2 was measured following the injection of haemolymph samples (10 - 20 µl). The cO_2 of the original sample can be calculated as:

$$cO_2 = \frac{PO_2 \cdot \alpha_{CN} \cdot \text{chamber volume} \cdot 100}{\text{sample volume} \cdot 760}$$

where,

α_{CN} = solubility coefficient for oxygen in KCN
(= 0.0261 vol % at 30°C);

and,

$$PO_2 = PCO_2 \text{ (final)} - PO_2 \text{ (initial)} \cdot \frac{\text{chamber volume} - \text{sample volume}}{\text{chamber volume}}$$

cO_2 and $c_{HCY}O_2$ values are usually reported in the literature using units of vol % (= ml. 100 ml^{-1}) or $mmol.l^{-1}$. The latter units are used here.

5.2.3 The subunit composition of the haemocyanin.

The molecular weights of the monomer sub-units and native haemocyanin of ***Calocaris macandreae*** were estimated using two different methods.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to estimate the molecular weight of the haemocyanin monomers under reducing conditions. For comparative purposes, haemolymph samples from ***Calocaris macandreae***, the galatheid squat lobster ***Munida rugosa***, the lobster ***Nephrops norvegicus***, and the brachyuran crabs ***Carcinus maenas*** and ***Liocarcinus puber*** were resolved on a 5 - 25% (w/v) gradient acrylamide gel (Pharmacia GE 2/4 LS), with a constant current (35 mA), variable voltage (100V initial - 300V maximum) supply for approximately 4 hours. Gels were fixed using 10% acetic acid, 28% methanol and 1% glycerol, stained with Coomassie blue and vacuum dried.

The use of gradient gels (as above) allows rapid determination of unknown molecular weights, since good separation is achieved over a wide molecular weight range. However, non-gradient gels provide better separation of proteins of similar weight, if an appropriate gel is chosen. Therefore, more accurate results were obtained for the haemocyanin of ***Calocaris macandreae*** using a 7.5 % (w/v) gel as follows: The sample was diluted to produce a range of dilutions with a sample buffer containing 0.0625 mol.l^{-1} Tris-HCl (pH 6.8), 2% SDS, 59% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue. The diluted sample was then heated to 90^0 C for 10 minutes. Samples were then loaded onto a 3.75 % stacking gel, and were resolved in a 7.5 % gel (1.5 x 160 x 120 mm) at 8 mA. gel^{-1} overnight. The gels were stained with Coomassie blue. The following molecular weight markers were run in the outside tracks (bovine albumin: Mr = 66k; glyceraldehyde 3-phosphate dehydrogenase: 36k; carbonic anhydrase: 29k; trypsinogen:

24k; trypsin inhibitor: 20.1k; alphalactalbumin: 14.2k.). N.B. all molecular weights are given in 'Daltons', however, the term 'Daltons' is not consistently used in the literature and does not strictly apply to values of Mr).

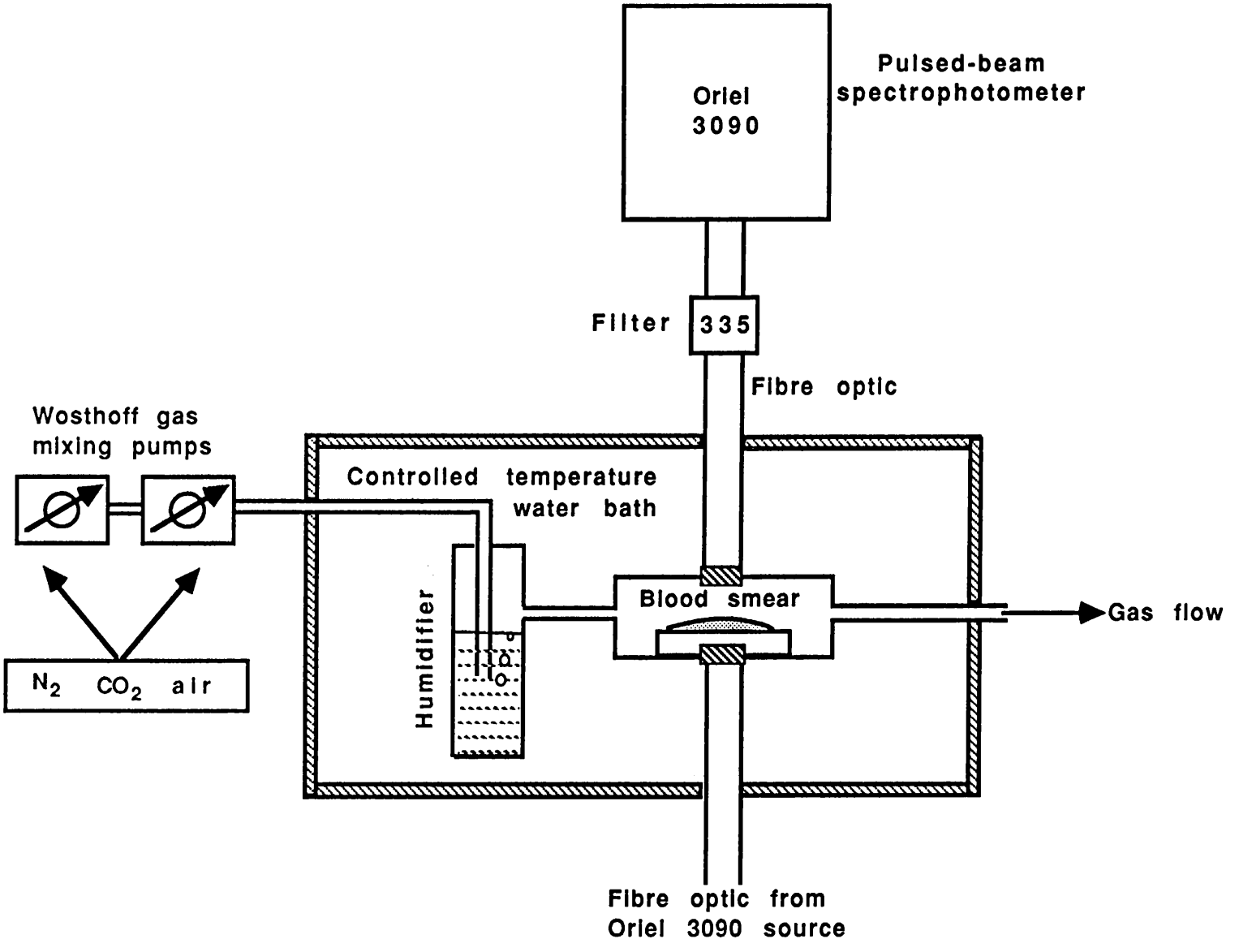
The molecular weight of the native molecule was estimated using Sephacryl S400 high resolution gel filtration. Haemolymph samples (1ml) were applied to a sephacryl (Pharmacia) column and eluted with an appropriate physiological saline, at a flow rate of 1 $ml.min^{-1}$. 1 ml fractions were collected using a fraction collector. The Cu concentration of each fraction was measured using AAS, in order to positively identify the haemocyanin-containing fractions. The column was calibrated using blue dextran (Mr = approximately 2000 kdaltons), alpha macroglobulin (725k) and apoferritin (443k).

5.2.4 Oxygen affinity of the haemocyanin.

Oxygen dissociation curves were constructed for ***Calocaris macandreae*** haemocyanin using a modification of the diffusion chamber originally described by Sick and Gersonde (1967). The system used is shown in Fig. 5.1. Blood samples from several animals were pooled, and the carrying capacity and ionic composition determined as described above. The L-lactate concentrations of these samples were determined using the method outlined in Chapter 6 and detailed in Appendix 4, since lactate is known to be a modulator of haemocyanin oxygen affinity in some species (Truchot, 1980; Mangum, 1983a; Bridges *et al.*, 1984).

The technique used measured the relative oxygen saturation of a haemolymph smear (2 - 10 µl) placed on a microscope slide within the diffusion chamber. The basis of the method is that changes in the

FIG. 5.1 Diagram of the Diffusion Chamber. The haemolymph smear is supported on a glass slide. Gas mixtures of known composition are produced by the Wosthoff pumps, humidified and passed over the haemolymph smear. The absorption of the haemolymph smear at 335 nm is measured by an Oriel 3090 pulsed-beam spectrophotometer. Temperature is accurately controlled by a water bath.


Oriel
3090
Pulsed-beam
spectrophotometer
Filter
335
Fibre optic
Wosthoff gas
mixing pumps
Controlled temperature
water bath
Humidifier
Blood smear
Gas flow
N_2 CO_2 air
Fibre optic from
Oriel 3090 source

absorbance of oxygenated haemocyanin (at 335 nm) are measured while the haemolymph sample is successively equilibrated to several oxygen tensions. A precision gas mixing system (H. Wösthoff, two type 2M301 in serial) supplied a gas mixture of known PO_2 and PCO_2 which was bubbled through a humidifier tube and passed over the haemolymph smear. The absorbance of the smear at 335nm was measured by a pulsed beam spectrophotometer (Oriel Scentific 3090). The spectrophotometer beam was directed through the haemolymph sample by two fibre optics. The temperature of the diffusion chamber was controlled by a circulating water bath.

For each curve, the relative saturation (S) of the haemolymph was measured at 5 - 8 different PO_2's; while pH was controlled by the PCO_2 of the gas mixture. Another sample was tonometered in a Radiometer BMS II Mk2 haemolymph micro system with the same gas mixture. At approximately 50% saturation, the pH of this sample was measured, using the microcapillary pH electrode of the BMS 2, connected to a Corning ion analyser 255.

Oxygen dissociation curves were constructed by plotting PO_2 against S. The resulting sigmoid dissociation curves indicate the relative oxygen affinity (since a high affinity haemolymph is saturated at a lower PO_2). The conventional quantitative parameter of oxygen affinity is the P50 (i.e. PO_2 for 50% saturation) which can be estimated from the x-intercept of the Hill plot (log PO_2 against log S/1-S). The slope of the Hill plot (N50) is a measure of the pigment cooperativity, resulting from interaction between the haemocyanin subunits.

The dependence of haemocyanin oxygen affinity on pH (the Bohr effect) is quantified as the 'b' value of the regression equation of log P50

plotted against pH. In this study, pH was controlled by PCO_2 alone, since most previous studies of crustacean haemocyanins have found that the specific effect of CO_2 is negligible. The alternative method is to use an exogenous buffer system (e.g. Tris maleate or Tris HCl); it was felt that the addition of exogenous buffers might introduce variations in the haemocyanin environment, and should be avoided where possible.

The effects of temperature on oxygen affinity of ***Calocaris*** haemocyanin were investigated by constructing oxygen dissociation curves in the pH range 7.2 - 8.2 at temperatures of 5, 10, 15 and 20°C. The change in enthalpy (ΔH) during oxygenation at different temperatures was calculated from the Van't Hoff equation in the form:

$$H = -2.303 \times R \times \frac{\log (\Delta P50)}{1/(T_1 - T_2)}$$

where R = gas constant;
$T_{1,2}$ = absolute temperature.

Modulation by L-lactate was investigated by varying the L-lactate concentration of the haemolymph sample. This was done using two methods:

1. Addition of a small volume (10ul in 500µl) of concentrated L-lactate (in buffered physiological saline); to give final concentrations of 5, 10, 20 and 40 $mmol.l^{-1}$;

2. Dialysis of haemolymph samples (0.5 ml; pH 8.25; 4°C; 16 hours) against buffered physiological salines containing several concentrations of L-lactate to give final concentrations (in the haemolymph) of 5 and 10 $mmol.l^{-1}$. The exogenous buffer used was Tris-HCl (10 $mmol.l^{-1}$).

Oxygen dissociation curves were constructed in the pH range 7.6 - 8.2 for each treatment. Control samples were prepared by addition of 0 $mmol.l^{-1}$ lactate, and dialysis against a saline without L-lactate.

5.2.5 Carbon dioxide transport in the haemolymph.

A brief investigation of the CO_2 transporting properties of ***Calocaris*** haemolymph was made. *In vitro* carbon dioxide dissociation curves were constructed using a Radiometer BMS II blood micro system to tonometer haemolymph samples against precision gas mixtures of varying PCO_2. The Haldane effect was quantified by comparing 0 and 10% air-equilibrated samples (corresponding to 0 and 100% oxygen saturation of the haemocyanin). The Haldane effect is defined as the difference in total CO_2 concentration between oxygenated and deoxygenated haemolymph at a specific PCO_2 (usually chosen to give a pH similar to *in vivo* values), and is quantified in functional terms as $\Delta cCO_2/cO_2$ (Truchot, 1976b).

The total CO_2 concentrations ($cCO_2 = CO_2 + HCO_3^- + CO_3$) of the tonometered samples were measured using the method of Cameron (1971); described below. In addition, the pH of each tonometered sample was measured. All measurements were made at 10^oC.

The method of Cameron (1971) for the determination of cCO_2 was used as follows. A PCO_2 electrode (Radiometer E5037) connected to a Radiometer PHM73 meter) measured PCO_2 in a thermostatted, stirred cell (volume 365 ul), filled with 0.01 M HCl. The equilibrium of the carbonate/ bicarbonate/carbon dioxide system is dependant on pH, such that the bicarbonate/carbonate contained in an injected haemolymph sample (volume 15 µl) was displaced as CO_2. The resultant increase in PCO_2 was detected by the electrode and recorded on a chart recorder. The system was calibrated using 12 $mmol.l^{-1}$ $NaHCO_3$.

CO_2 dissocation curves (similar to O_2 curves except that the curves are hyperbolic rather than sigmoid) were plotted as cCO_2 against the PCO_2 of the equlibration gas mixture. The capacitance coefficient, β, is defined as the ratio of the increment of CO_2 concentration to the increment of CO_2 partial pressure ($\beta = cCO_2/\Delta PCO_2$; Piiper *et al.*, 1971; Truchot, 1976b). β therefore measures the steepness of the CO_2 dissociation curve within the physiological PCO_2 range (0 - 4 Torr). PCO_2 is difficult to measure directly in aquatic animals, since low partial pressures are usually present (as a result of the high solubility and large diffusion coefficient of CO_2 in water). Direct measurement is made more difficult still, by the small sample volumes which can be obtained from ***Calocaris***. Therefore no direct measurements were made; instead *in vivo* PCO_2 was calculated from *in vivo* pH and cCO_2 as follows:

Measurement of pH and cCO_2 of haemolymph samples equilibrated to known PCO_2 *in vitro* (as above) allowed calculation of pK' from the modified Henderson-Hasselbalch equation:

$$pK' = pH - \log\left(\frac{cCO_2 - \alpha.PCO_2}{PCO_2}\right)$$

where α = solubility coefficient of CO_2, taken from the nomogram of Truchot (1976a) at appropriate temperature and 32 $^{o}/_{oo}$; (= 0.058 $mmol.l^{-1}$ $Torr^{-1}$ at 10°C).

In vivo PCO_2 may then be calculated by substitution of appropriate *in vitro* pK' values, with measured *in vivo* values for cCO_2 and pH in the same equation.

$[HCO_3]$ can be estimated from the approximation:

$$[HCO_3] = cCO_2 - \alpha.PCO_2$$

since $[CO_3]$ (and probably carbamates) are usually negligible at physiological pH values (Truchot, 1983). A diagram of $[HCO_3]$ vs. pH is a useful graphical representation of acid-base status within a system. A true Davenport diagram also includes PCO_2 isopleths calculated from an *in vitro* Astrup titration (i.e. measurement of pH and $[HCO_3]$ in samples tonometered at several known PCO_2's). These have been omitted in the present study, since insufficient data were collected for their calculation. However, a non-bicarbonate buffer line (i.e. buffering probably due to ionisable protein groups) can be plotted from *in vitro* data. Acid-base perturbations due to respiratory effects (i.e. changes in PCO_2) are represented as movement along the non-bicarbonate buffer line; metabolic effects result in deviations from the line (ideally along PCO_2 isopleths).

5.2.6 *In vivo* measurements.

The following measurements were made of *in vivo* haemolymph parameters relevent to O_2 and CO_2 transport in ***Calocaris macandreae***: pH, L-lactate concentration and cCO_2. cCO_2 and pH determinations were made as described above, using post-branchial haemolymph samples (10 - 20 µl) taken anaerobically from the pericardium using a Hamilton syringe. The assay used for L-lactate determination is described in Chapter 6 and Appendix 4.

Some attempts were made to determine *in vivo* PO_2 values for post-branchial haemolymph. However, it proved impossible to obtain samples of sufficient volume for injection into a PO_2 cell (Radiometer) without introducing air bubbles. The *in vivo* function of the haemocyanin is difficult to interpret if *in vivo* PO_2 is unknown, since the relative contributions of dissolved and pigment-bound oxygen cannot be

calculated.

A preliminary examination of oxygen transport in hypoxic conditions was made, using only cO_2 measurements. The cO_2's of post-branchial haemolymph samples (10 µl), taken from animals exposed to varying degrees of environmental hypoxia, were measured (as above). Further samples from the same animals were then tonometered against air, and maximum oxygen carrying capacity ($c_{max}O_2$) measured. The total haemolymph saturation (S_{total}), including both bound and dissolved fractions, was then calculated as:

$$S_{total} = cO_2 \; / \; c_{max}O_2.$$

Further explanation of the rationale of this experiment is given in the Discussion.

5.3 RESULTS.

5.3.1 Ionic composition of the haemolymph.

The concentrations of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and Cl^{-} measured in pooled haemolymph samples from animals taken on four dates (from the Loch Striven population) are given in Table 5.1:

TABLE 5.1: Blood ionic concentrations in *Calocaris macandreae* (mmol.l^{-1}).

date	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Cl^{-}
29/1/87	9.2	45.4	402	11.4	479
18/9 and 8/10/87 (pooled)	7.6	48.8	415	14.1	433
8/12/87	8.4	52.9	480	8.0	-
10/1/89	12.1	47.2	414	8.5	481

Replicate determinations indicated that the approximate accuracy of the measurements are: Ca^{2+}, Mg^{2+}, K^{+}: ± 0.1; Na^{+}, Cl^{-}: ± 5 mmol.l^{-1}. There is evidence, therefore, of some variation in pooled haemolymph sample ionic concentrations. Variation between individuals was not measured, due to the limited volume of haemolymph that could be obtained from each *Calocaris*.

5.3.2 Oxygen carrying capacity of the haemolymph.

The absorbance coefficients of copper (at approximately 335 nm) and protein (at approximately 280 nm) were calculated from data for absorbance of diluted haemolymph samples, and the concentrations of copper (measured by AAS) and protein (measured by the Coomassie blue method). Since absorption was measured by scanning between 250 - 450 nm, the maximum absorbance in each peak was used (the peak wavelength differed by approximately 1 - 2 nm between individuals). Fig. 5.2A, B shows individual variation in the measured parameters. Values for the absorbance coefficients (calculated for 1% solutions, 1cm path length) were: $E_{335} = 1702$

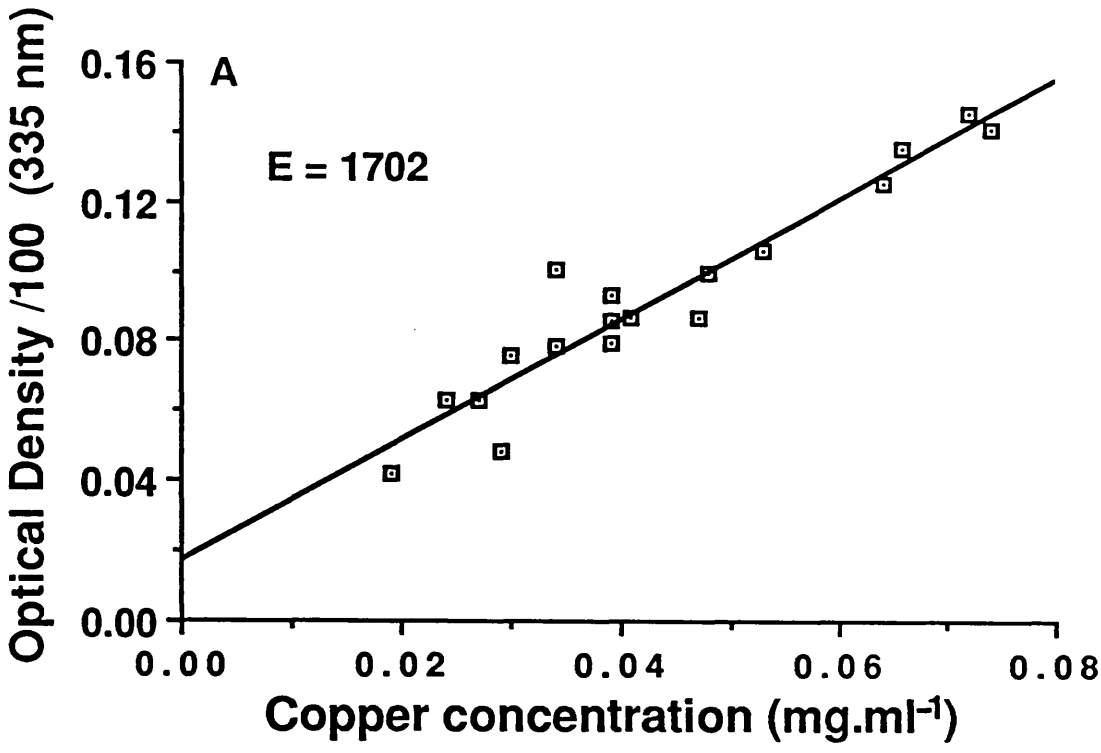
$E_{280} = 24.03$

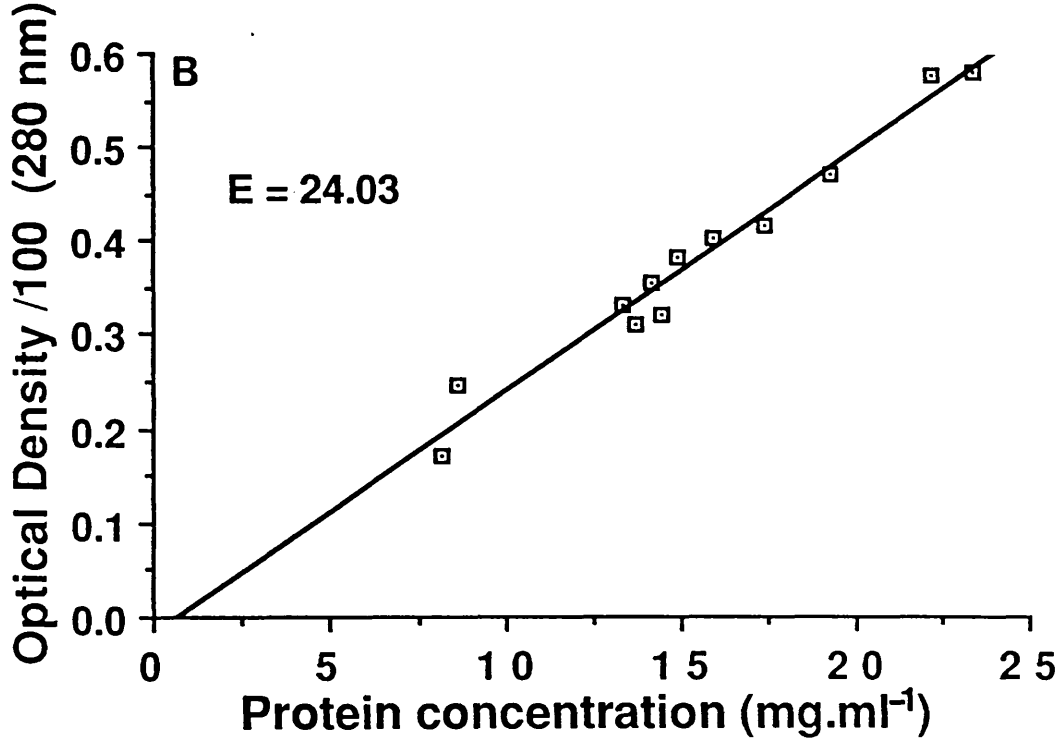
FIG. 5.2 A) The relationship between optical density at 335 nm and copper concentration (measured by Atomic Absorption Spectrophotometry) in haemolymph samples (diluted 100-fold) from individual ***Calocaris macandreae***. The equation of the regression line is:

$$y = 0.0177 + 1.72x \quad (r = 0.95;\ P<0.001)$$

B) The relationship between optical density at 280 nm and protein concentration (measured by the Coomassie Blue method) in haemolymph samples (diluted 100-fold) from individual ***Calocaris macandreae***. The equation of the regression line is:

$$y = -0.0177 + 0.0258x \quad (r = 0.98;\ P<0.001)$$


A
E = 1702
Optical Density /100 (335 nm)
0.16
0.12
0.08
0.04
0.00
0.00
0.02
0.04
0.06
0.08
Copper concentration (mg.ml-1)


B
E = 24.03
Optical Density /100 (280 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
Protein concentration (mg.ml-1)

The cO_2 and calculated $c_{HCY}O_2$ of the haemolymph of 19 individual *Calocaris* measured by the Tucker method are shown in Table 5.2. Values for $c_{HCY}O_2$ calculated from Cu (measured by AAS) and protein concentrations (measured by UVS) for the same samples are also shown. Copper concentrations of the haemolymph varied from 0.02 to 0.075 $mg.ml^{-1}$; total protein content was 7 - 24 $mg.ml^{-1}$.

TABLE 5.2: Oxygen carrying capacities of haemolymph and haemocyanin of *Calocaris macandreae*, measured by the Tucker cell and from copper and protein concentrations. cO_2 and $c_{HCY}O_2$ units are $mmol.l^{-1}$.

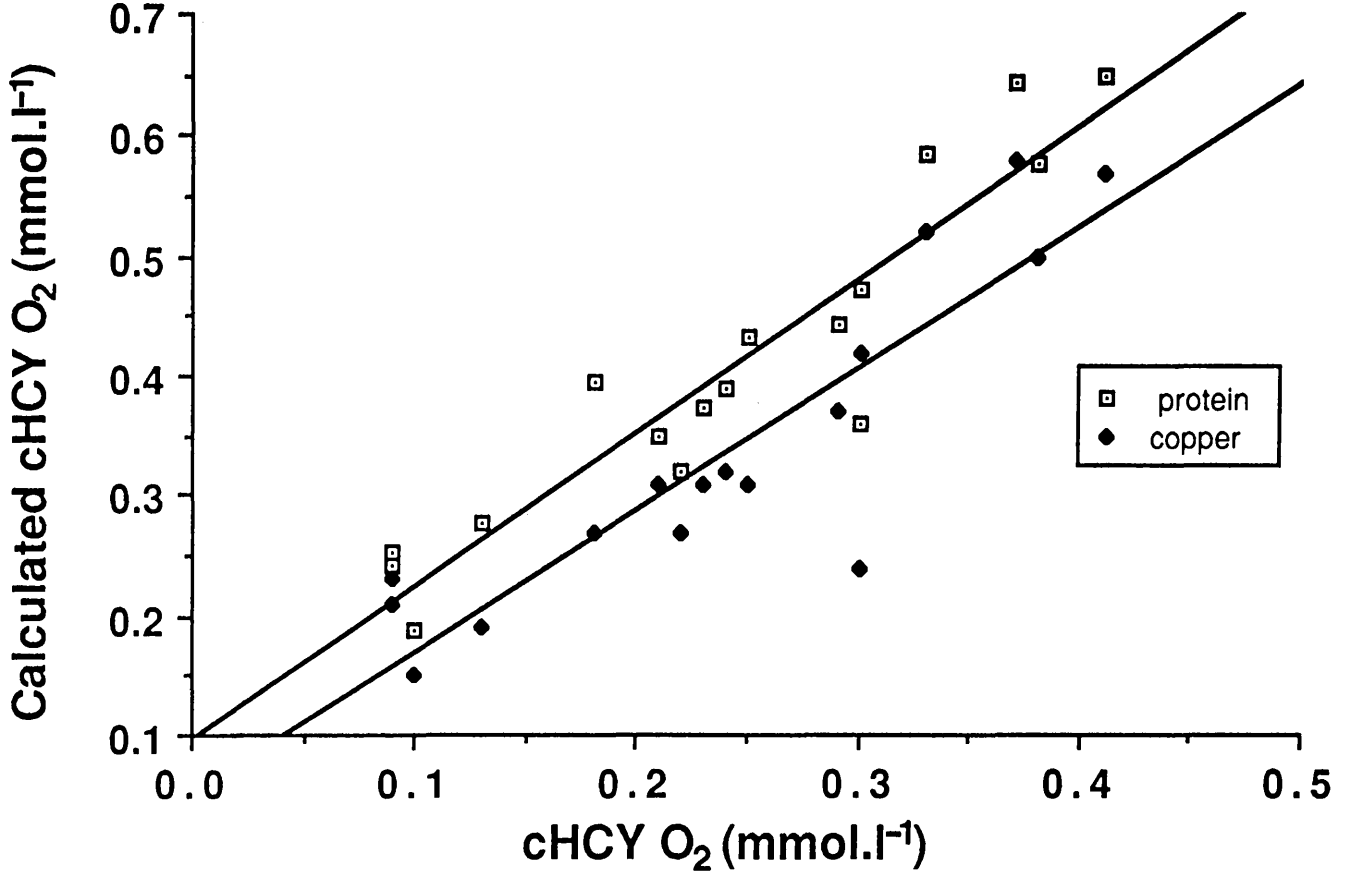
animal	cO_2	calculated $c_{HCY}O_2$ (Tucker)	calculated $c_{HCY}O_2$ (Protein)	calculated $c_{HCY}O_2$ (Copper)
1	0.50	0.22	0.46	-
2	0.57	0.29	0.44	0.37
3	0.38	0.10	0.19	0.15
4	0.52	0.24	0.39	0.32
5	0.58	0.30	0.36	0.24
6	0.62	0.34	-	-
7	0.66	0.38	0.58	0.50
8	0.51	0.23	0.37	0.31
9	0.53	0.25	0.43	0.31
10	0.41	0.13	0.28	0.19
11	0.46	0.18	0.40	0.27
12	0.49	0.21	0.35	0.31
13	0.69	0.41	0.65	0.57
14	0.65	0.37	0.64	0.58
15	0.58	0.30	0.47	0.42
16	0.61	0.33	0.59	0.52
17	0.50	0.22	0.32	0.27
18	0.37	0.09	0.25	0.21
19	0.37	0.09	0.24	0.23

The relationships between $c_{HCY}O_2$ (calculated from Tucker cell measurements), and the concentrations of Cu (measured by AAS) and protein (measured by UVS), are shown in Fig. 5.3. There was a significant positive correlation between $c_{HCY}O_2$ and both Cu concentration ($r = 0.90$; $P<0.001$) and protein concentration ($r = 0.94$; $P<0.001$). There was no significant difference between the two regression lines (covariance analysis). In addition neither regression

FIG. 5.3 The relationships between oxygen carrying capacity (cHCY O_2, $mmol.l^{-1}$) calculated from concentrations of copper and protein in the haemolymph, and measured directly using the method of Tucker (1969). The equations of the regression lines are:

protein: $y = 0.097 + 1.28x$ ($r = 0.94$; $P<0.001$)

copper: $y = 0.053 + 1.18x$ ($r = 0.90$; $P<0.001$)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.1
0.2
0.3
0.4
0.5
Calculated cHCY O2 (mmol.l-1)
cHCY O2 (mmol.l-1)
protein
copper

slope was significantly different from 1 (t-test, P>0.1).

The mean haemolymph cO_2 of animals sampled on this date (12/4/89) was 0.52 ± 0.10 (standard deviation) $mmol.l^{-1}$. Mean cO_2's of samples from at least 10 animals collected on three separate occasions were: 0.53 $mmol.l^{-1}$ (29/1/87, n=12); 0.77 $mmol.l^{-1}$ (18/9/87, n=10); 0.49 $mmol.l^{-1}$ (4/12/87, n=15). There was little evidence of seasonal variation in the oxygen carrying capacity of ***Calocaris macandreae*** haemolymph.

5.3.3 Sub-unit structure of the haemocyanin. A photocopy of the vacuum-dried SDS-PAGE gel used for the comparative study is shown in Fig. 5.4A. The banding patterns of the resolved proteins from each species were similar, with 4 major bands present, (approximate molecular weights of between 37k and 47k).

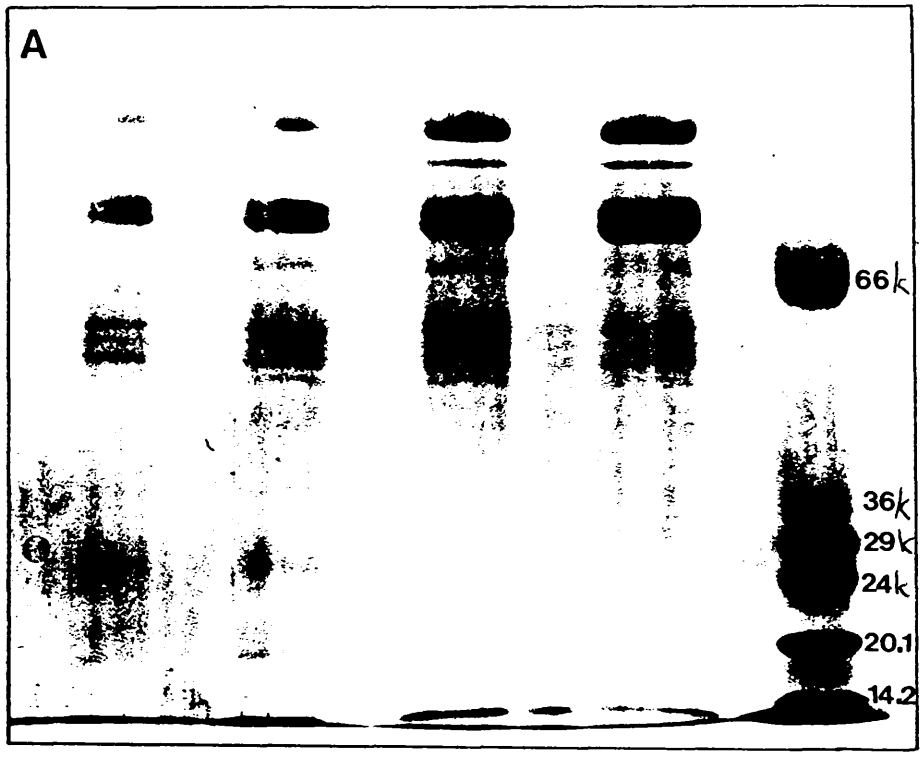
The 7.5% SDS-PAGE gel used for the more accurate determination of the monomer molecular weights of ***Calocaris*** haemocyanin, is shown in Fig. 5.4B. Four major protein bands were present, with molecular weights estimated from the calibration presented in Fig. 5.5 of between 100 and 240k.

The concentrations of copper in the fractions collected from sephacryl gel filtration of the native haemocyanin, are shown in Fig. 5.6A. When plotted on the regressed calibration line of the column (Fig. 5.6B), the calculated molecular weight (Mr) of the molecule was approximately 1650k.

FIG. 5.4 SDS-PAGE gels of haemocyanin.

A) Serial 10-fold dilutions of haemolymph samples from ***Calocaris macandreae*** run on a 7.5% homogenous gel. Standards (with molecular weights shown) were run in the right hand track.

B) Haemolymph samples from ***Calocaris macandreae*** (Cm), ***Nephrops norvegicus*** (Nn), ***Munida rugosa*** (Mr), ***Carcinus maenas*** (Cm') and ***Liocarcinus puber*** (Lp), run on a 5 - 25% gradient gel. Standards (with molecular weights shown) were run in the left and right hand tracks.


A
66k
36k
29k
24k
20.1k
14.2k

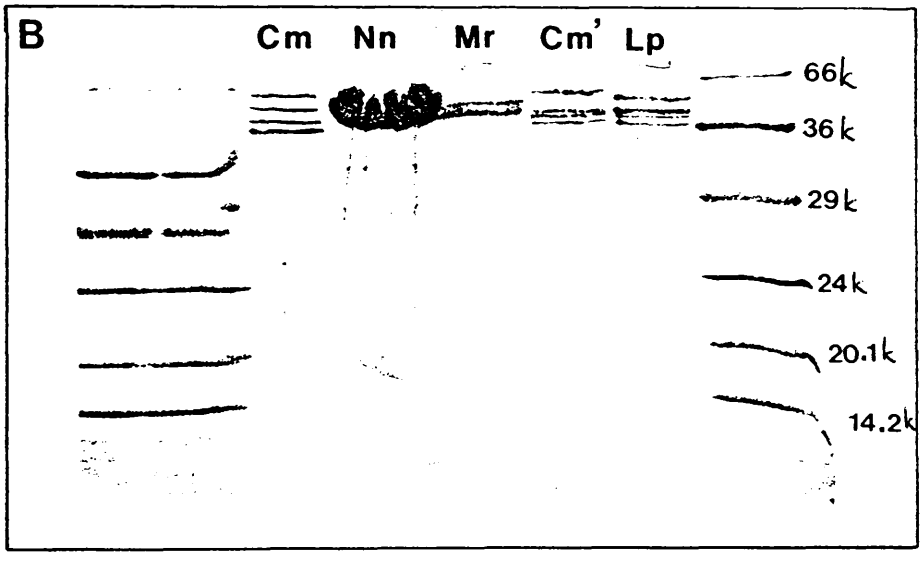
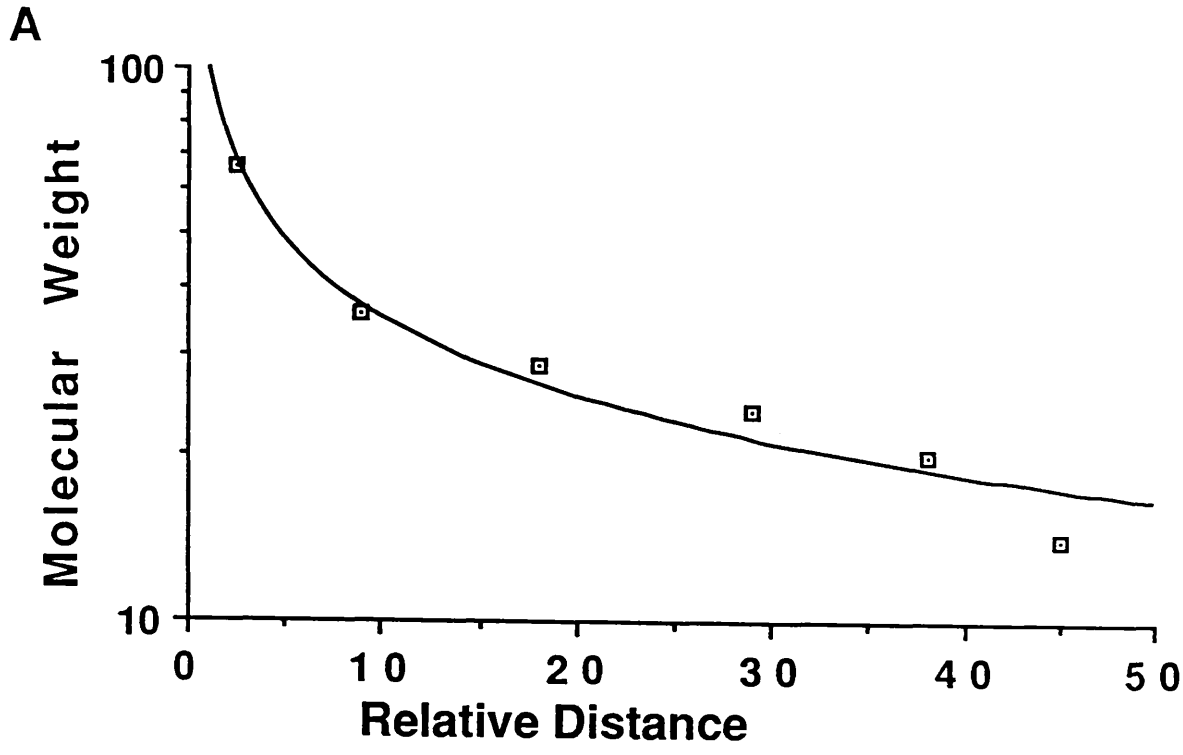

B
Cm
Nn
Mr
Cm'
Lp
66k
36k
29k
24k
20.1k
14.2k

FIG. 5.5 Calibration graphs for the SDS-PAGE gels presented in Fig. 5.4. Relative distance is measured from an arbitrary origin.


A
100
10
Molecular Weight
0
10
20
30
40
50
Relative Distance

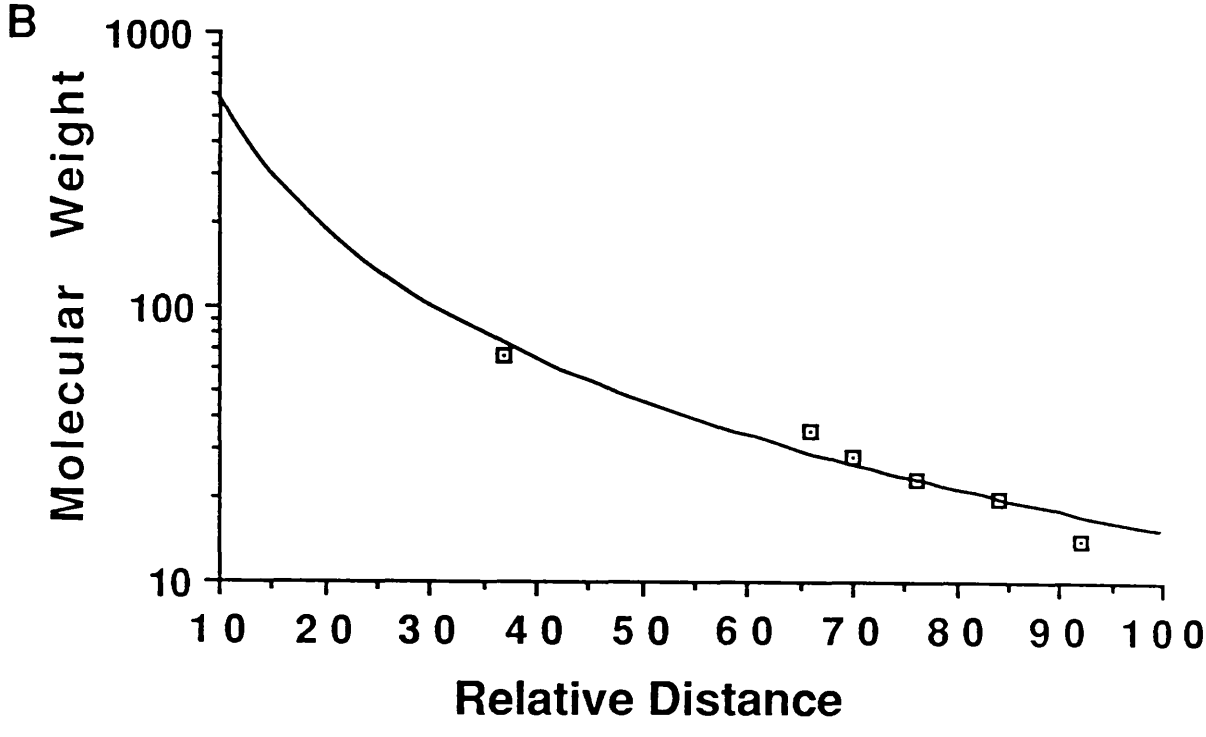
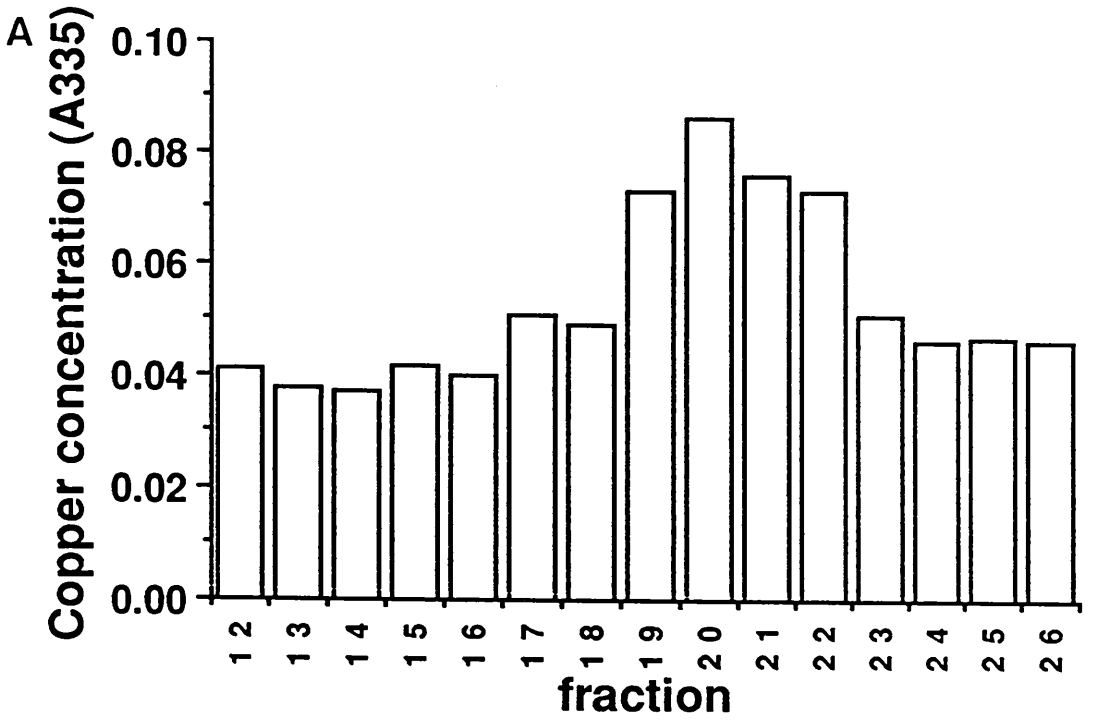
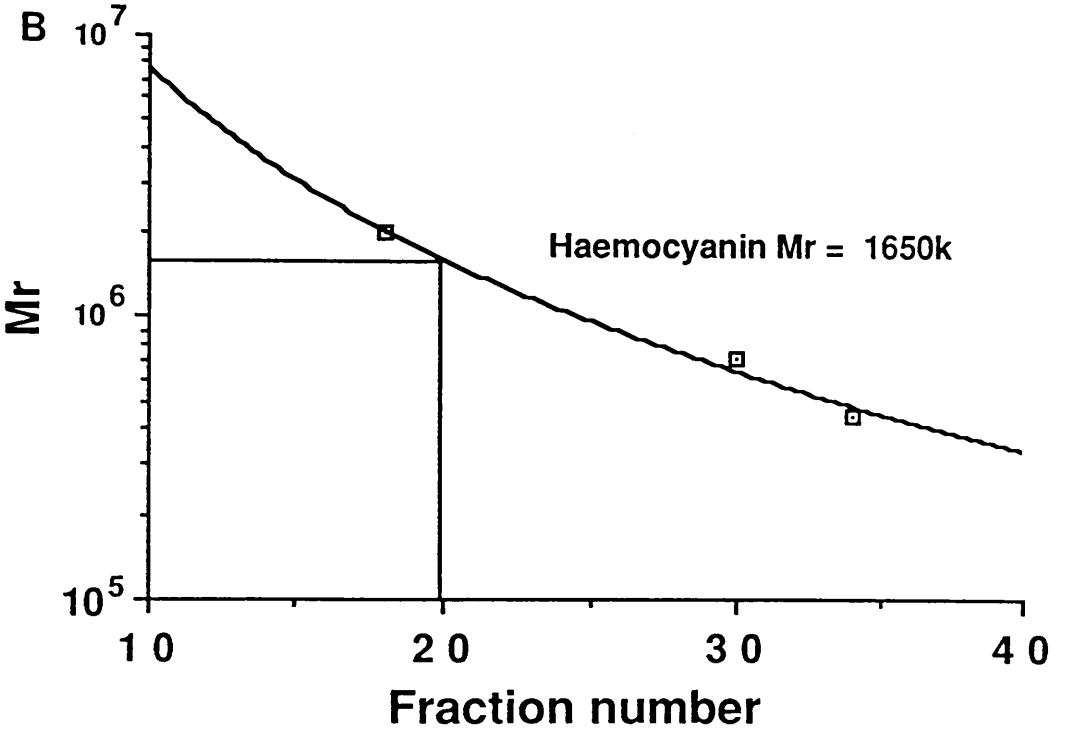

B
1000
100
10
Molecular Weight
10
20
30
40
50
60
70
80
90
100
Relative Distance

FIG. 5.6 Results from high resolution gel filtration (on a Sephacryl S400 column) of ***Calocaris macandreae*** haemocyanin.

A) Copper concentrations measured by absorption at 335 nm of fractions collected at 1 minute intervals. Absorption was measured relative to a distilled water blank. Haemocyanin was present in fractions 19 - 23 (this was confirmed by protein absorbance measured at 280 nm).

B) Calibration of the column, using blue dextran (Mr = 2000k), alpha macroglobulin (Mr = 725k) and apoferritin (Mr = 443k). The calculated haemocyanin Mr was 1650k.


A
Copper concentration (A335)
0.10
0.08
0.06
0.04
0.02
0.00
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
fraction


B
10^7
Mr
10^6
10^5
Haemocyanin Mr = 1650k
10
20
30
40
Fraction number

5.3.4 Oxygen affinity and modulation of the haemocyanin.

A family of oxygen dissociation curves, measured on neat (i.e. untreated, undialysed) *Calocaris* haemolymph at 10°C is shown in Fig. 5.7. The L-lactate concentration in this haemolymph sample was 3.1 $mmol.l^{-1}$. Oxygen dissociation curves represent relative affinity by position along the x-axis. Fig. 5.7 shows a family of curves, measured at a variety of pH's, as controlled by the PCO_2 of the gas mixture. It is evident that an increase in pH resulted in an increase in oxygen affinity, i.e. a positive Bohr effect was present.

Some of the data are shown in the form of a Hill plot, with regression lines fitted by the method of least-squares to data between 25 and 75% saturation (Fig. 5.8; only 4 curves are shown for clarity). This type of plot allows a more accurate calculation of oxygen affinity (log P50 = x-intercept) and cooperativity (n50 = slope) for each curve. A Bohr plot of the complete data set (Fig. 5.9) represents oxygen affinity as position relative to the y-axis; and quantifies the Bohr factor as the 'b'value of the regression equation of log P50 plotted against pH.

The oxygen affinity of *Calocaris* haemocyanin under physiological conditions is high (e.g. P50 = 1.7 Torr at pH 7.69 and 10°C; Figs. 5.7, 5.8). Values of haemocyanin cooperativity (n50) were in the range 2.38 to 3.65, but did not vary with pH (Fig. 5.10). There was a small but significant Bohr effect ($\varphi = \Delta \log P50 / \Delta pH$; = -0.58) under these conditions.

The effects of temperature (in the range 5 - 20°C) on the oxygen affinity of *Calocaris* haemocyanin are shown in Fig. 5.11. Significant modulation of both oxygen affinity and the Bohr effect

FIG. 5.7 Oxygen dissociation curves for untreated haemolymph of ***Calocaris macandreae***. The curves were constructed at 10^0C and the pH of the haemolymph was altered by adjusting the proportion of CO_2 in the gas mixtures with which the haemolymph was equilibrated.

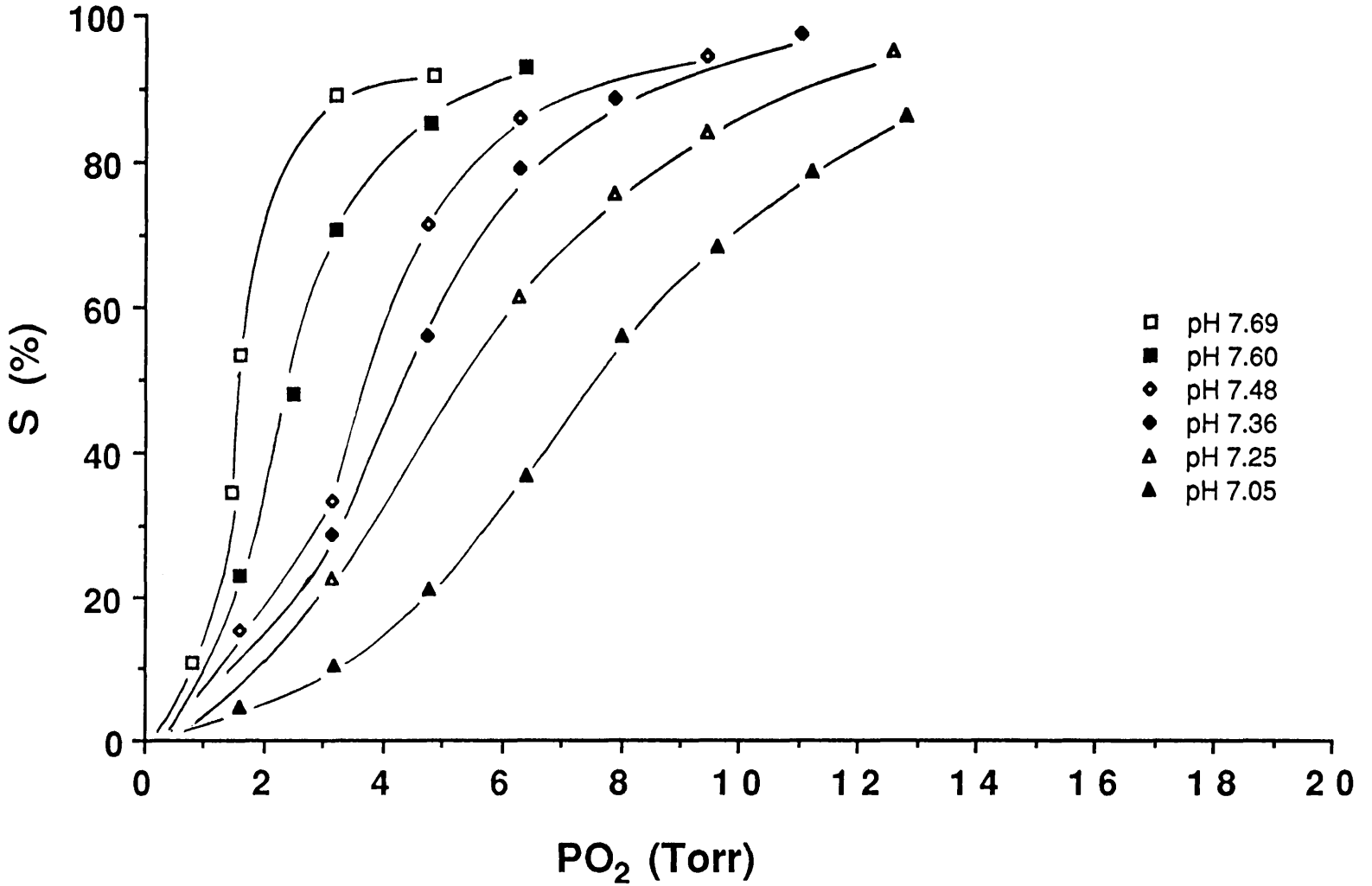

100
80
60
40
20
0
S (%)
0
2
4
6
8
10
12
14
16
18
20
PO2 (Torr)
pH 7.69
pH 7.60
pH 7.48
pH 7.36
pH 7.25
pH 7.05

FIG. 5.8 A Hill plot of four oxygen dissociation curves of the haemolymph of ***Calocaris macandreae***. The curves were constructed at 10^0C and the pH of the haemolymph was altered by adjusting the proportion of CO_2 in the gas mixtures with which the haemolymph was equilibrated.

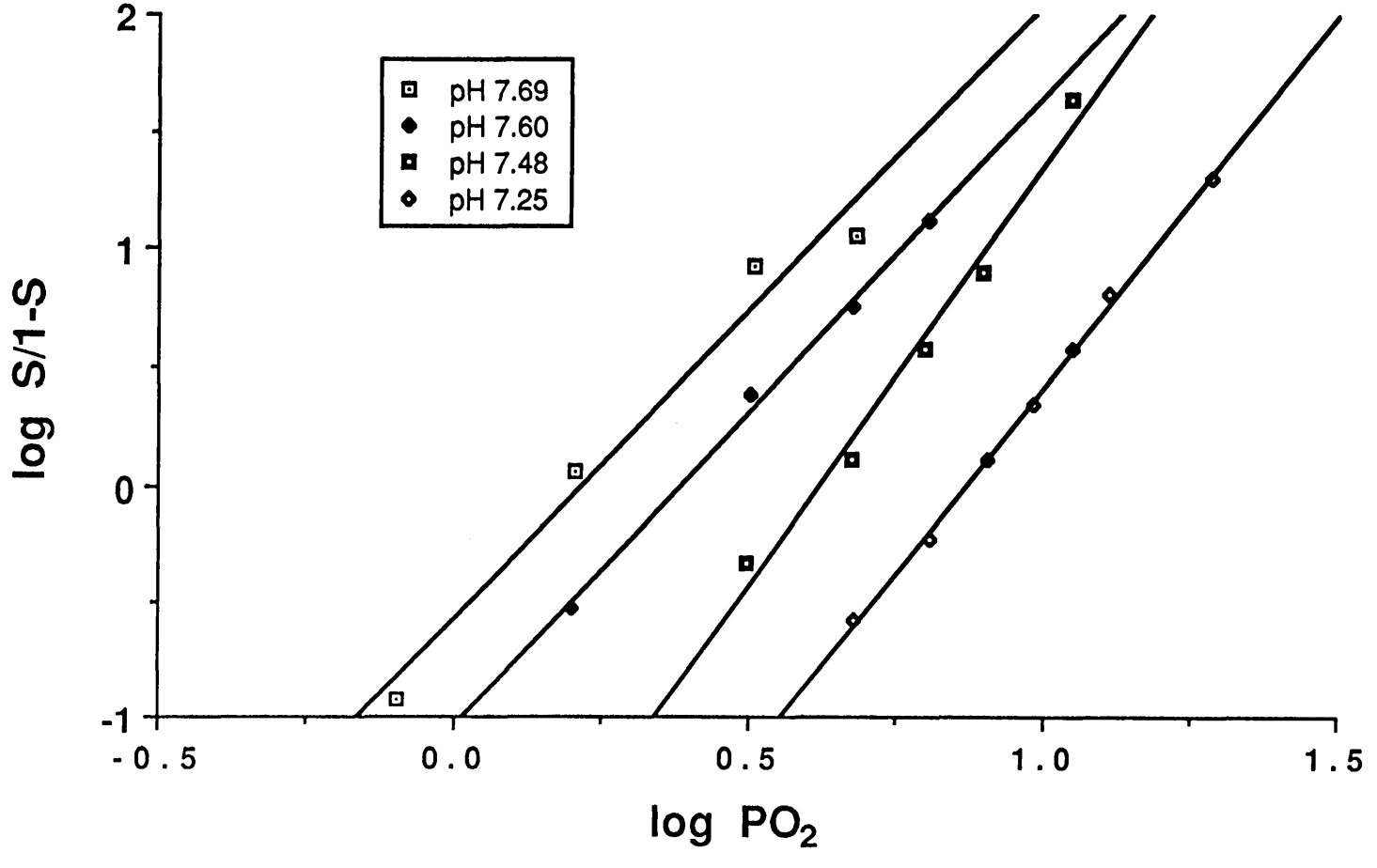

pH 7.69
pH 7.60
pH 7.48
pH 7.25
log S/1-S
log PO2
2
1
0
-1
-0.5
0.0
0.5
1.0
1.5

FIG. 5.9 The relationship between oxygen affinity (P50) and pH in the haemolymph of *Calocaris macandreae*. P50 was calculated from the Hill plot (measured at 10^0C) and the pH of the haemolymph was altered by adjusting the proportion of CO_2 in the gas mixtures with which the haemolymph was equilibrated.

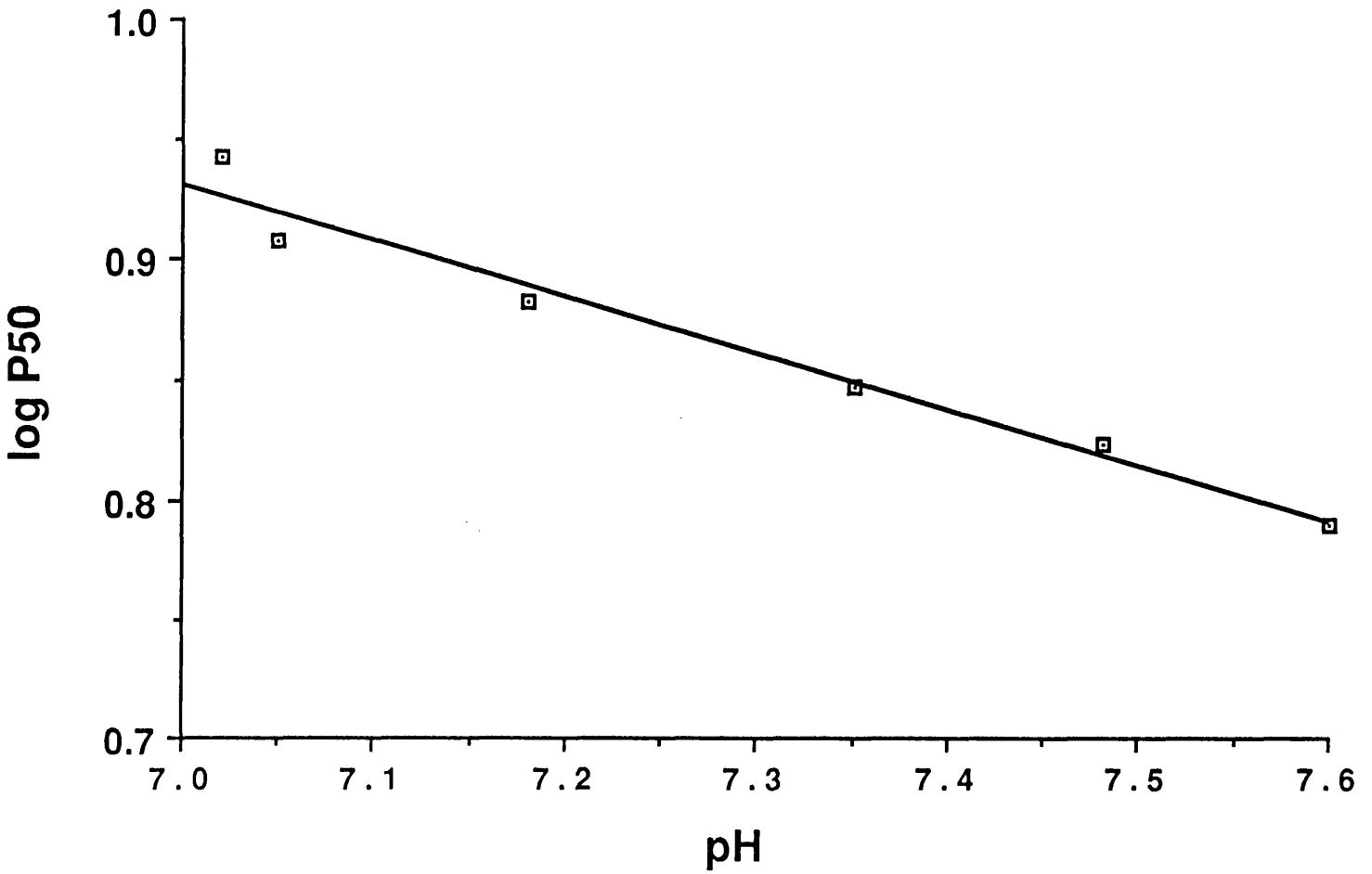
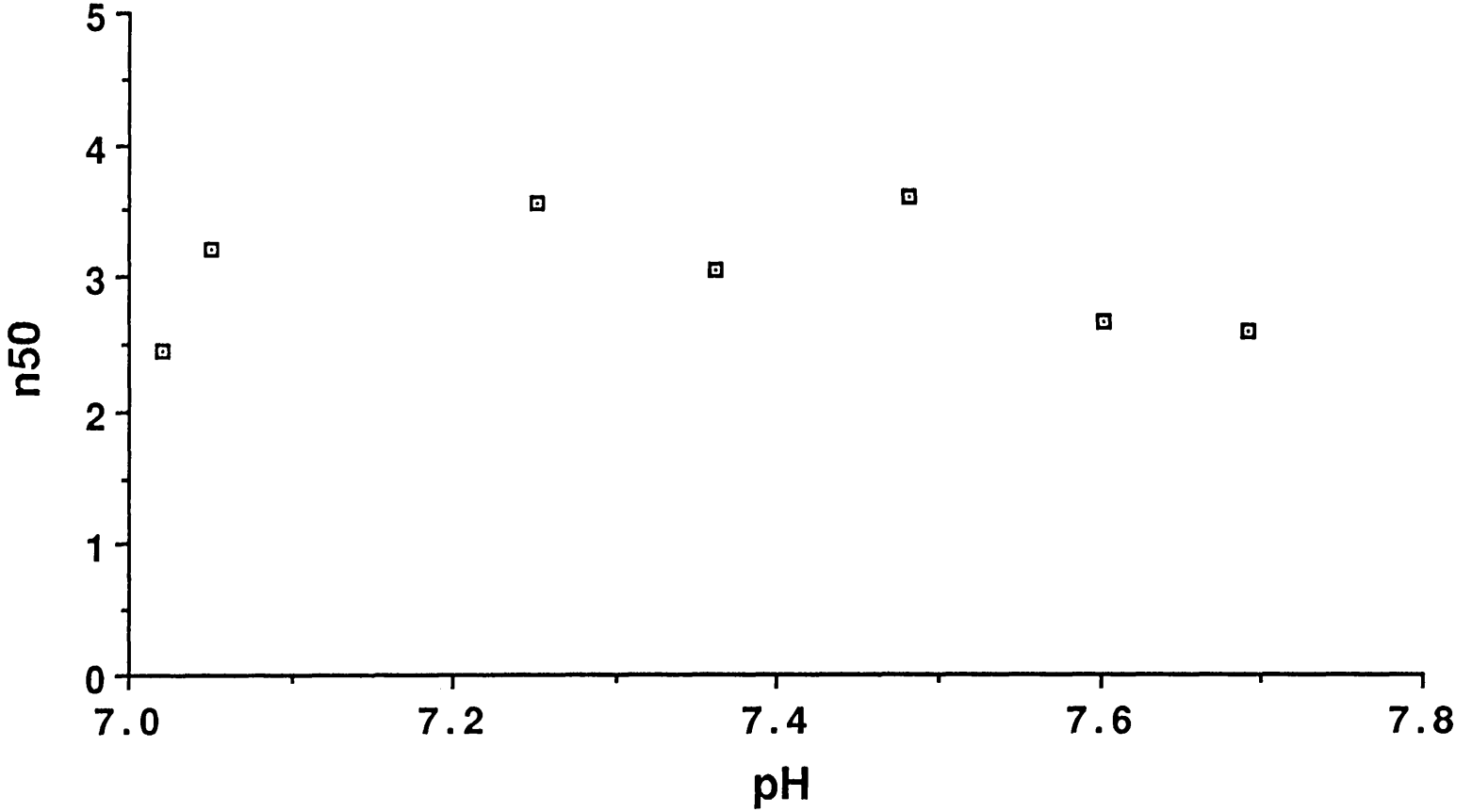

1.0
0.9
0.8
0.7
log P50
7.0
7.1
7.2
7.3
7.4
7.5
7.6
pH

FIG. 5.10 The relationship between cooperativity (n50) and pH in the haemolymph of ***Calocaris macandreae***. n50 was calculated from the Hill plot (measured at 10^0C) and the pH of the haemolymph was altered by adjusting the proportion of CO_2 in the gas mixtures with which the haemolymph was equilibrated.


5
4
3
2
1
0
n50
7.0
7.2
7.4
7.6
7.8
pH

shown by the haemocyanin was measured.

There was a relatively large decrease in oxygen affinity with increased temperature; P50 decreased from 1.38 Torr at 5°C to 6.17 Torr at 20°C (both at pH 7.6). Covariance analysis also showed a significant effect of temperature on the Bohr shift, which decreased from -0.71 ± 0.05 (S.D.) at 5°C, to -0.23± 0.03 at 20°C. There was a particularly large effect of temperature, on both oxygen affinity and the Bohr effect, between 10 and 15°C. The change in enthalpy, ΔH, (resulting from oxygenation) was -75.6 kJ mol^{-1} between 5°C and 15°C at pH 7.6.

Modulation of oxygen transport by ***Calocaris*** haemocyanin due to L-lactate could not be demonstrated *in vitro* (Fig. 5.12). Two methods of varying the lactate concentration of the sample were used (addition of a concentrated lactate solution, and dialysis against a lactate-containing Ringer) as described above. Neither experimental method resulted in significant modulation of oxygen affinity or Bohr effect, within physiological concentrations of lactate (up to 20 $mmol.l^{-1}$; covariance analysis). This finding was unlikely to be due to methodological problems, as was demonstrated by a preliminary experiment using haemolymph from ***Carcinus maenas***, which produced a lactate effect of significant magnitude using both methods. Also, the lack of effect of dialysis suggests that no dialysable 'unidentified factors' were present.

There was no evidence of modulation of haemocyanin cooperativity by any of the factors measured (pH, temperature, lactate or urate concentration).

FIG. 5.11 The effects of temperature and pH on oxygen affinity (P50) of the haemolymph of ***Calocaris macandreae***. The pH of the haemolymph was altered by adjusting the proportion of CO_2 in the gas mixtures with which the haemolymph was equilibrated. There are significant effects of temperature on both oxygen affinity (P50) and Bohr shift (slope).

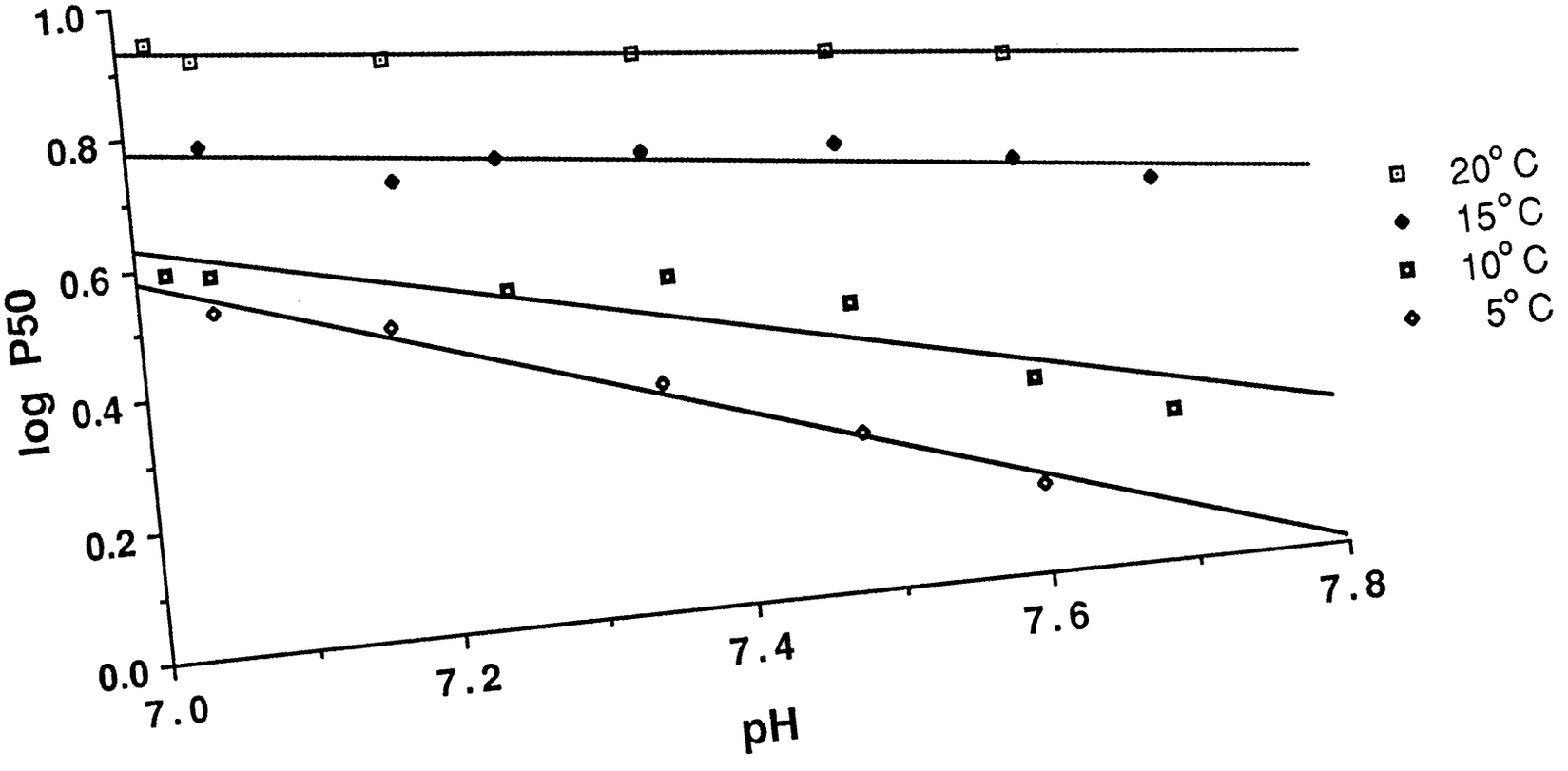
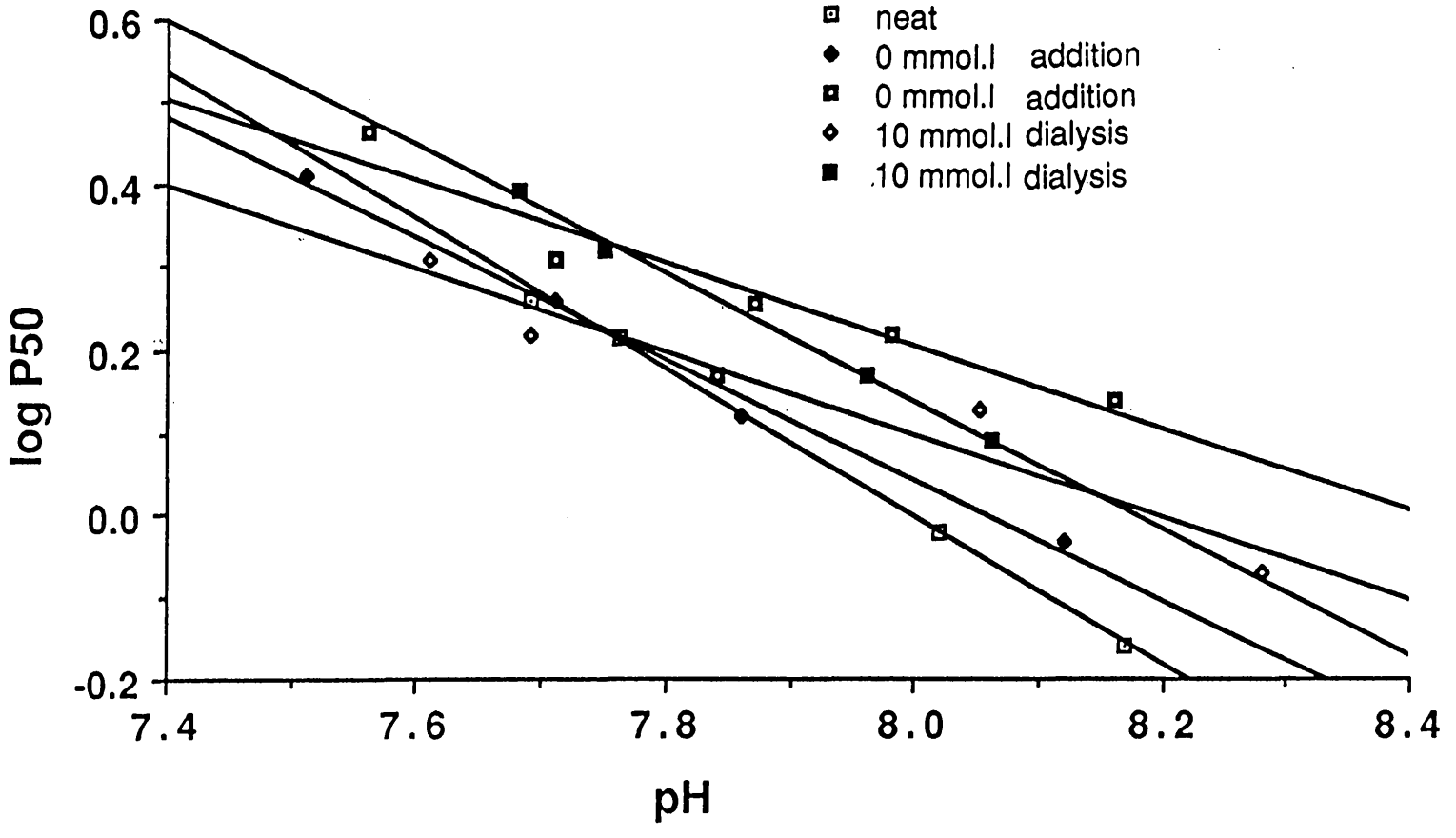

1.0
0.8
0.6
0.4
0.2
0.0
log P50
7.0
7.2
7.4
7.6
7.8
pH
20° C
15° C
10° C
5° C

FIG. 5.12 The effects of L-lactate concentration on oxygen affinity (P50) of the haemolymph of ***Calocaris macandreae***. The pH of the haemolymph was altered by adjusting the proportion of CO_2 in the gas mixtures with which the haemolymph was equilibrated. L-lactate concentration was altered by addition (diamond symbols) or dialysis (square symbols). However, there were no significant effects of L-lactate on oxygen affinity (P50) or Bohr shift (slope) of the haemocyanin.


neat
0 mmol.l addition
0 mmol.l addition
10 mmol.l dialysis
10 mmol.l dialysis
0.6
0.4
0.2
0.0
-0.2
log P50
7.4
7.6
7.8
8.0
8.2
8.4
pH

5.3.5 Carbon dioxide transporting properties of the haemolymph.

The relationship between PCO_2 and pH in *Calocaris* haemolymph is shown in Fig. 5.13, as determined by *in vitro* Astrup titration. There was a significant difference (covariance analysis; $P<0.05$) between the pH of oxygenated and deoxygenated haemolymph at constant PCO_2. This is due to the Haldane effect and is quantified below.

CO_2 equilibrium curves were constructed using haemolymph samples tonometered at several PCO_2's (Fig 5.14). The curves were of hyperbolic form with an initially large CO_2 capacitance coefficient, β, at PCO_2's < 3 Torr (β = 0.91 mmol.l^{-1}.$Torr^{-1}$ and 0.85 mmol.l^{-1}.$Torr^{-1}$ between 1 - 2 Torr in deoxygenated and oxygenated haemolymph respectively). The CO_2 capacitance coefficient decreased to lower values at PCO_2's of 3 - 8 Torr. The absolute CO_2 concentration values within physiological PCO_2 values (usually < 3 Torr, see below) were comparatively low, at approximately 2.5 and 2.0 mmol.l^{-1} at a PCO_2 of 2 Torr in deoxygenated and oxygenated haemolymph respectively.

The functional Haldane coefficient (= $\Delta cCO_2 / cO_2$) was also comparatively low. For example at physiological values of approximately pH 7.8 and PCO_2 2 Torr, the Haldane coefficient = 0.57.

pK' values (calculated from the Henderson-Hasselbalch equation, described above) varied within the pH range measured (Fig 5.15). Calculated pK' in deoxygenated haemolymph decreased from 6.54 at pH 7.99 to 6.37 at pH 7.61, but increased below pH 7.6 to 6.53 at pH 7.44. A similar pattern was evident in oxygenated haemolymph (pK' = 6.76 at pH 7.99; 6.46 at pH 7.61; 6.58 at pH 7.44); although pK' values were consistently higher in oxygenated haemolymph.

FIG. 5.13 The relationship between carbon dioxide tension (pCO_2) and pH in oxygenated and deoxygenated haemolymph of *Calocaris macandreae* equilibrated to varying PCO_2 at $10^{o}C$. The difference between oxygenated and deoxygenated regression lines (the Haldane effect) was significant ($P<0.10$).

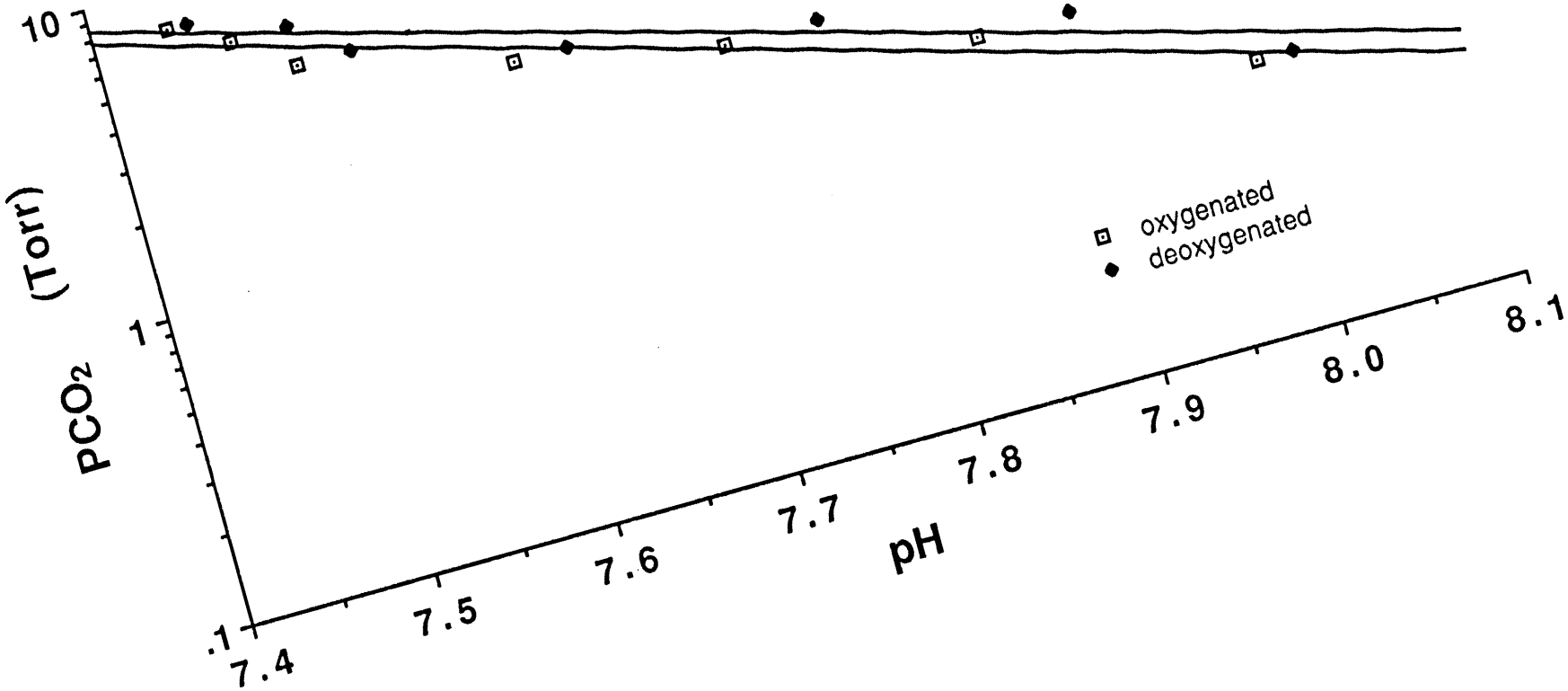

10
1
.1
PCO_2 (Torr)
7.4
7.5
7.6
7.7
7.8
7.9
8.0
8.1
pH
oxygenated
deoxygenated

FIG. 5.14 Carbon dioxide capacity curves for oxygenated and deoxygenated haemolymph of ***Calocaris macandreae***. The curves were constructed at 10°C. The difference between oxygenated and deoxygenated curves is a measure of the Haldane effect (see text for further details).

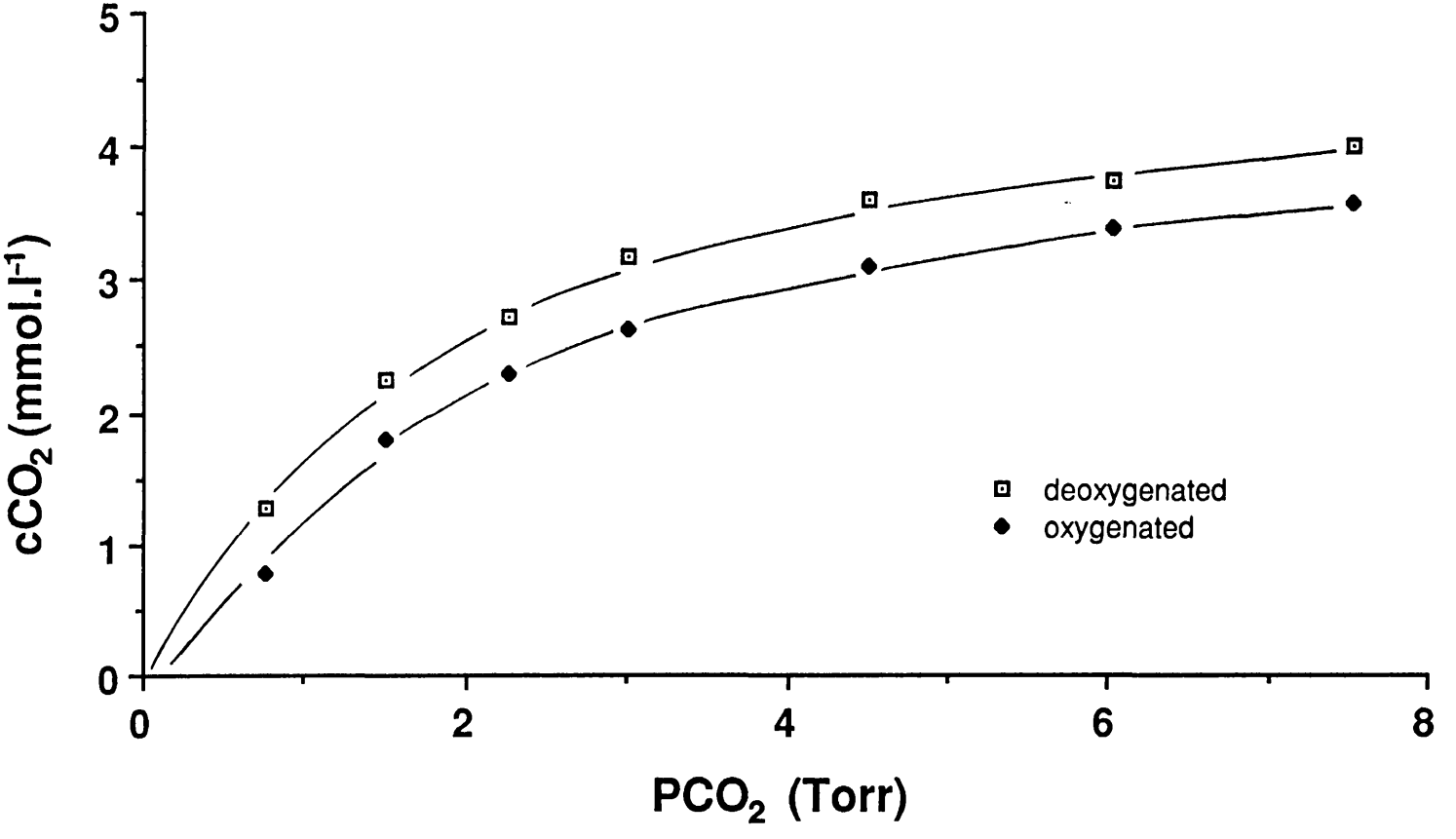
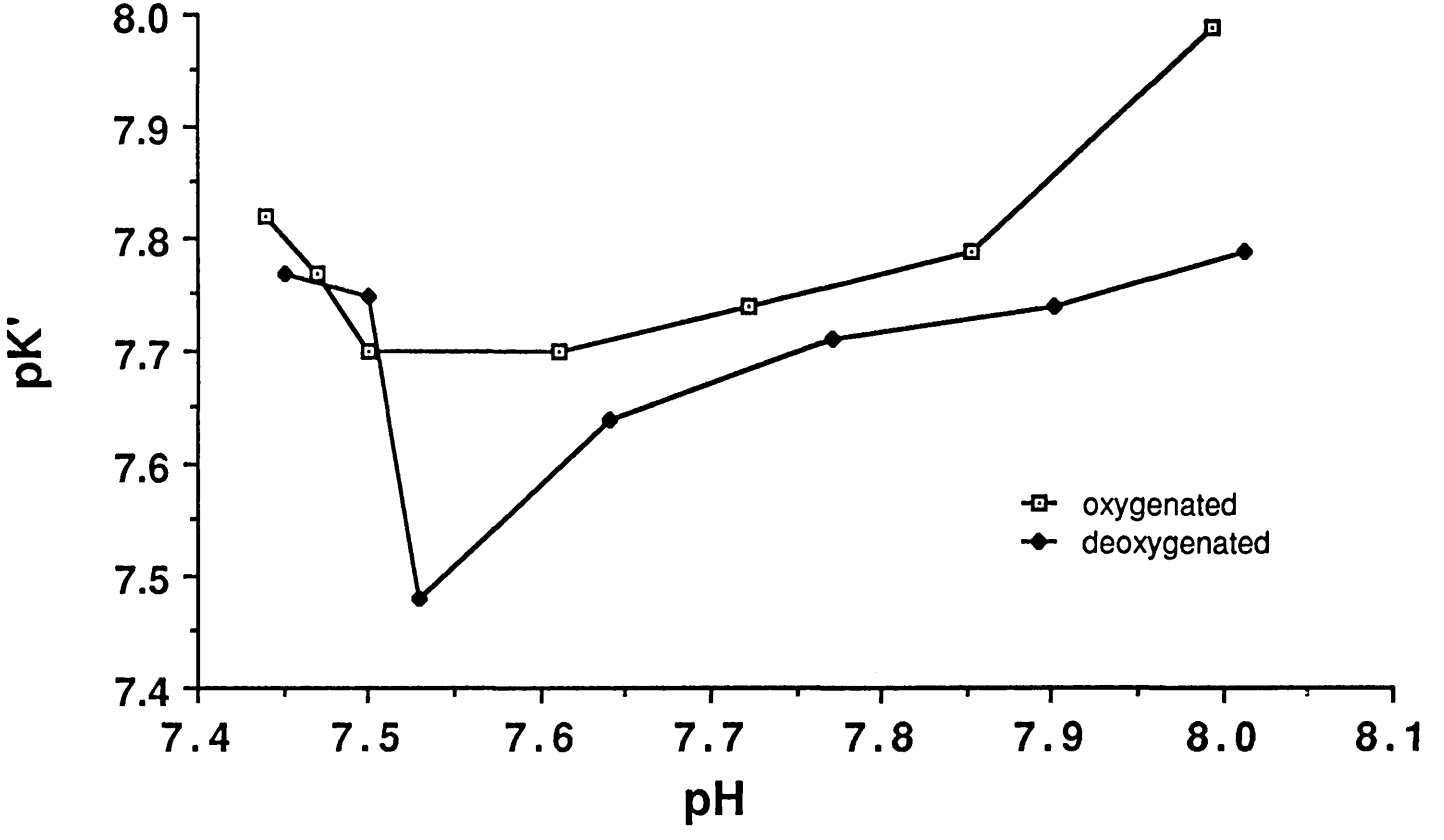

5
4
3
2
1
0
0
2
4
6
8
cCO2 (mmol.l-1)
PCO2 (Torr)
deoxygenated
oxygenated

FIG. 5.15 The relationship between pK' and pH in oxygenated and deoxygenated haemolymph of *Calocaris macandreae* at 10°C. The pH of the haemolymph was altered by adjusting the proportion of CO_2 in the gas mixtures with which the haemolymph was equilibrated.


8.0
7.9
7.8
7.7
7.6
7.5
7.4
pK'
7.4
7.5
7.6
7.7
7.8
7.9
8.0
8.1
pH
oxygenated
deoxygenated

HCO_3 concentrations calculated from measured cCO_2 at known PCO_2 values (as described above), plotted against pH in a form similar to a Davenport diagram, are shown in Fig. 5.16. The non-bicarbonate buffer values (indicated by the slope of the fitted regression line) were similar in oxygenated and deoxygenated haemolymph, at approximately -2.4 $mmol.l^{-1}$ pH $unit^{-1}$.

5.3.6 *In vivo* function of the haemocyanin.

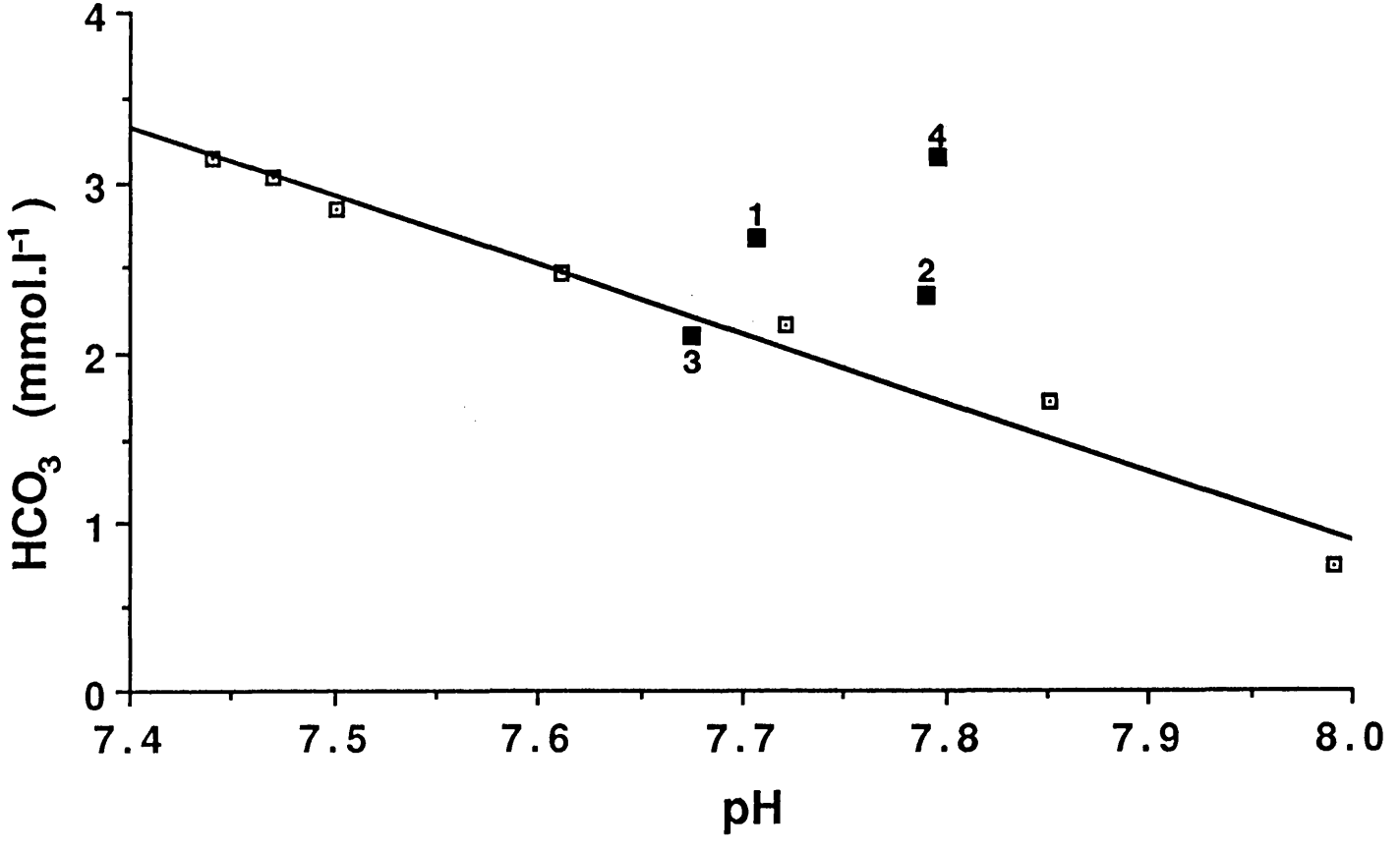
The *in vivo* haemolymph concentrations of L-lactate measured under varying conditions of oxygen availability, in ***Calocaris macandreae*** are described in detail below (Chapter 6).

As previously stated, *in vivo* PO_2 could not be determined for ***Calocaris***. A number of determinations of *in vivo* cO_2 (i.e. the total oxygen carried by the haemolymph) were made from post-branchial haemolymph samples. The $c_{max}O_2$ of further samples from the same animals were also measured (after equilibration with air). The haemolymph saturation (S_{total}) was then calculated as:

$$S_{total} = \frac{in\ vivo\ \ cO_2}{c_{max}O_2}$$

Due to the technical difficulties in obtaining haemolymph samples, only a few replicate determinations could be made. The *in vivo* cO_2 of 3 animals kept in normoxic conditions varied between 0.33 and 0.45 (mean 0.40) $mmol.l^{-1}$ (Fig 5.17A). At an environmental PO_2 of 51 Torr, *in vivo* cO_2 was maintained at similar values. In severe hypoxia, however, *in vivo* cO_2 declined (at 18 Torr: the mean value was 0.26 $mmol.l^{-1}$; 8 Torr: 0.15 $mmol.l^{-1}$; 3 Torr: 0.11 $mmol.l^{-1}$). In Fig. 5.17A, the mean value for $c_{HCY}O_2$ calculated for these samples is also shown. The value

FIG. 5.16 The relationship between calculated bicarbonate concentrations (HCO_3) and pH in oxygenated haemolymph of ***Calocaris macandreae*** at 10°C. The pH of the haemolymph was altered by adjusting the proportion of CO_2 in the gas mixtures with which the haemolymph was equilibrated. The solid points numbered 1 - 4 are *in vivo* measurements (see text, section 5.3.6).


4
3
2
1
0
HCO_3 (mmol.l^{-1})
7.4
7.5
7.6
7.7
7.8
7.9
8.0
pH
1
2
3
4

of *in vivo* cO_2 decreased below the calculated mean $c_{HCY}O_2$ at environmental PO_2's below approximately 20 Torr.

Haemolymph saturation (S_{total}) showed a similar pattern (Fig. 5.17B). The mean value measured in normoxic conditions was 0.82, but decreased in hypoxic conditions:

PO_2(Torr)	mean S_{total}	(S.D.)
158	0.82	0.18
51	0.77	0.01
18	0.56	0.15
8	0.24	0.03
3	0.26	0.14

In vivo pH was measured in post-branchial haemolymph samples taken from a number of quiescent, normoxic animals. The mean post-branchial pH was 7.72 (± 0.09 S.D., n=12). Due to the difficulty of measuring both cCO_2 and pH in the small haemolymph volume available from one animal, a limited number of measurements of acid-base status were made. Successful measurements could be obtained from only 4 animals:

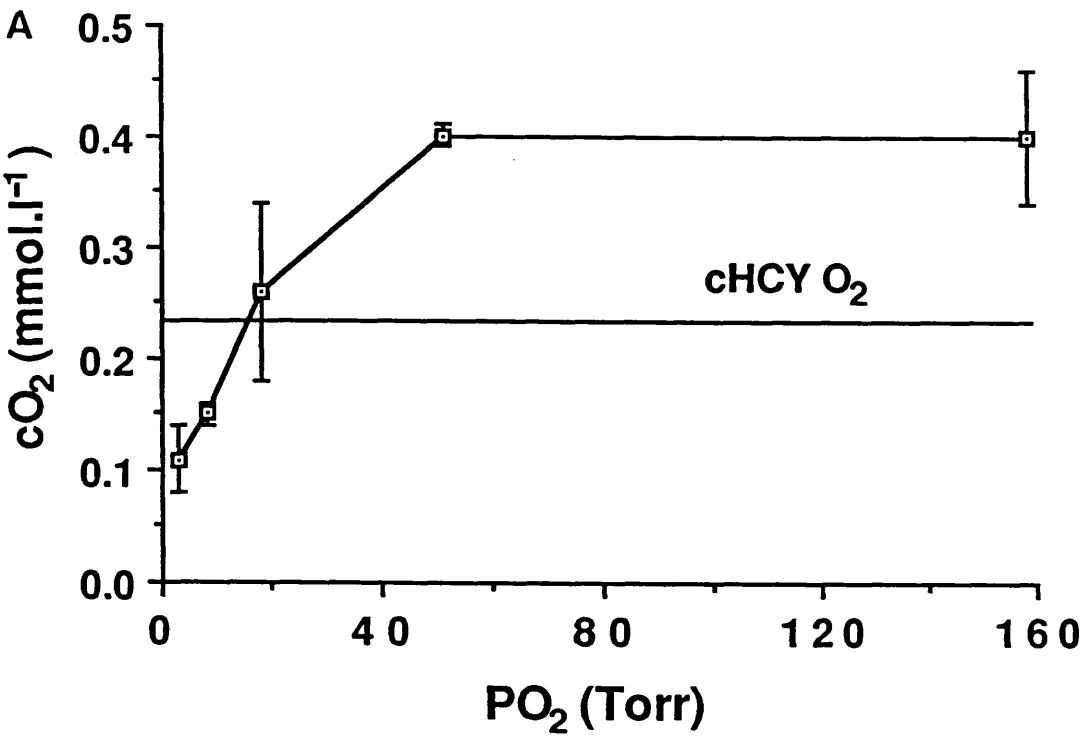
		cCO_2 ($mmol.l^{-1}$)	pH	calc. PCO_2 (Torr)
animal:	1.	2.91	7.71	2.67
	2.	2.53	7.79	1.95
	3.	2.22	7.68	2.18
	4.	3.40	7.80	2.56

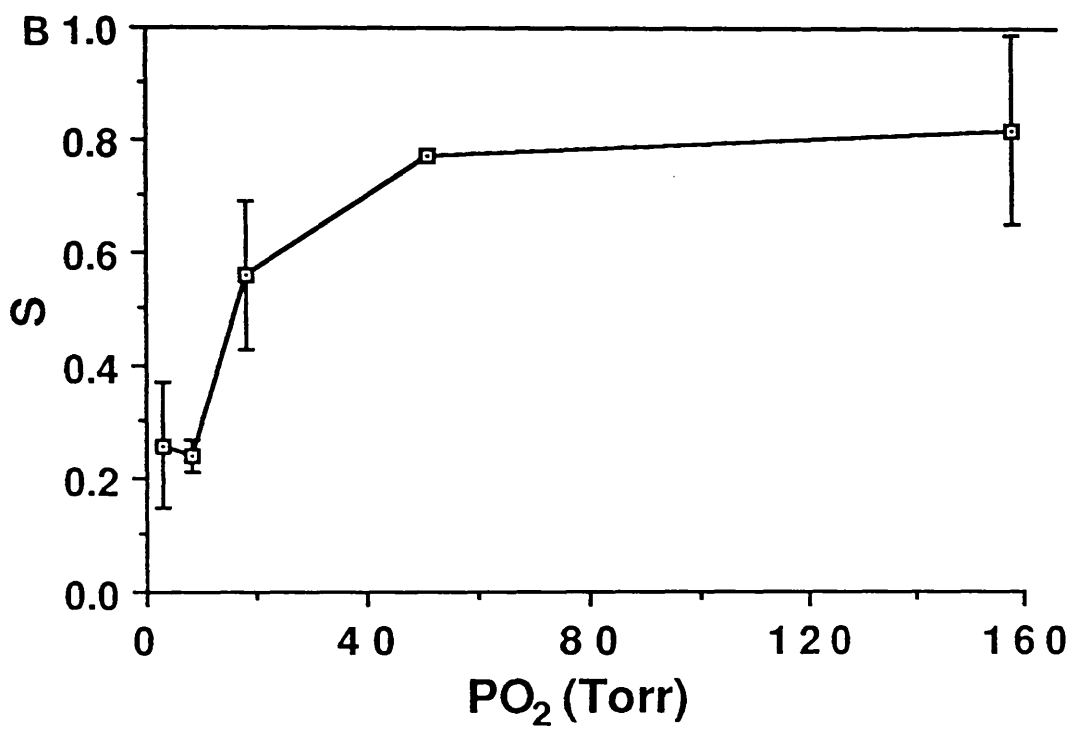
When plotted on the $[HCO_3]$ vs. pH diagram (Fig. 5.16), these recorded values are all close to the calculated *in vitro* non-bicarbonate buffer line. However, this does not necessarily imply that the CO_2/HCO_3 reaction is in equilibrium in the haemolymph, since equilibrium conditions will be attained during measurement in the cell (Cameron, 1986). The mean calculated *in vivo* PCO_2 was 2.34 (± 0.33 S.D.) Torr.

FIG. 5.17 The relationships between *in vivo* measurements of oxygen transport and environmental oxygen tension (PO_2) in the haemolymph of ***Calocaris macandreae***.

A) Oxygen content of the haemolymph (cO_2) in conditions of declining oxygen tension.

B) Haemolymph oxygen saturation (S_{total}) in conditions of declining oxygen tension. See text for further details.


A
0.5
0.4
0.3
0.2
0.1
0.0
cO_2 (mmol.l^{-1})
cHCY O_2
0
40
80
120
160
PO_2 (Torr)


B 1.0
0.8
0.6
0.4
0.2
0.0
S
0
40
80
120
160
PO_2 (Torr)

5.4 DISCUSSION

5.4.1 Ionic composition of the haemolymph.

The concentrations of the major haemolymph ions, with the exception of magnesium, were similar to values recorded in other decapods (Robertson, 1960; Mantel and Farmer, 1983). Magnesium, however, was present at concentrations considerably greater than has been recorded in nearly all other decapod species (and was very similar to the concentration in the medium). Miller and van Holde (1974) measured a value of 48 (± 3) $mmol.l^{-1}$ in the haemolymph of ***Callianassa californiensis***, almost identical to those found in ***Calocaris***.

Robertson (1949, 1953, 1960) has noted that relatively "unresponsive" species (e.g. the crabs ***Lithodes maia***, ***Hyas araneus*** and ***Dromia vulgaris*** have comparatively high Mg^{2+} concentrations (80% of sea water values), wheseas more "active" species have much lower concentrations (typically less than 50%). He also stated that the Ca/Mg ratio is low. The functional basis of this relationship is thought to be related to an anaesthetic effect on the neuromuscular junction. Although several exceptions to this general correlation have now been described, the outline presented by Robertson is still valid (Mantel and Farmer, 1983).

The presence of high Mg^{2+} concentrations (and low Ca/Mg ratios of 0.16 to 0.26, compared with 0.19 - 0.31 in the spider crabs and 0.39 - 2.0 in other decapods; Robertson, 1960), is in accord with the hypothesis that ***Calocaris macandreae*** (and ***Callianassa californiensis***) are sluggish species, adapted to a low level of activity. However, the presence of high Mg^{2+} concentrations may also be related to the aggregation state of the haemocyanin in thalassinids (eikositetramer), which is dependent

on a high concentration of divalent cations (van Holde *et al.*, 1977; Miller and van Holde, 1981; Mangum, 1983a).

5.4.2 Measurement of haemocyanin concentration

As pointed out in the methods and results sections, the oxygen carrying capacity of the haemocyanin ($c_{HCY}O_2$) may be estimated from three different measurements:

1. From direct measurement of the haemolymph carrying capacity, by subtraction of the calculated (or measured in sea water) physically dissolved fraction.
2. From the haemolymph copper concentration (assuming all copper present is associated with haemocyanin; and that haemocyanin copper binds oxygen in the ratio 2:1).
3. From the haemolymph protein concentration (assuming all protein is haemocyanin; and that the functional monomer molecular weight, i.e. protein weight to bind 1 mole oxygen, is 75k).

Although copper concentration may be measured using AAS, and protein using dye-binding spectrophotometric assays (Coomassie blue or Lowry methods), the simplest assay procedures are spectrophotometric measurement of absorbance at 335 and 280 nm respectively. These latter methods require the use of extinction coefficients, either taken from the literature (Nickerson and van Holde, 1971) or previously estimated. The extinction coefficient ($E^{1\%}$) at 280 nm estimated by the present study for *Calocaris* haemocyanin (24.03), is greater than those quoted by Nickerson and van Holde for other decapods (e.g. 14.2 for *Carcinus maenas*). Similarly, the calculated $E^{1\%}_{335}$ was far greater (1702) than that quoted for *Carcinus* by Nickerson and van Holde (2.33); although several workers have found this figure to be open to question when used as a simple extinction coefficient (A.C. Taylor, J.I. Spicer, pers.

comm). It should be noted that the value of Nickerson and van Holde was calculated by subtraction of absorbance values for deoxygenated haemolymph from values for oxygenated haemolymph.

Evidence that these coefficients are accurate for *Calocaris* is provided by the close agreement between the calculated and measured (by the Tucker method) values for $c_{HCY}O_2$. The reasons for interspecific differences in these values are unclear. In the case of $E^{1\%}_{280}$, the absorbance is due to the peptide bond, so that the only relevant factor is the average amino acid molecular weight (i.e. the amino acid composition of the haemocyanin). However, interspecific comparisons of the amino acid sequence of the polypeptide units of haemocyanin do not appear to have been made (Mangum, 1983a).

The close agreement which was noted between three methods of estimating $c_{HCY}O_2$ is good evidence that the basic assumptions were valid. The monomer (or mole-binding) molecular weight predicted from these data is close to 75k (a value similar to that measured by SDS-PAGE, see below).

The haemocyanin concentrations measured during this study were at the low end of the range recorded for decapods (1.0 - 3.5 $ml.100ml^{-1}$: Mangum, 1983a). Total oxygen carrying capacities measured at environmental temperature (10^{o} C) in *Calocaris* had a mean value of 0.52 $mmol.l^{-1}$ (= 1.16 $ml.100ml^{-1}$). The largest oxygen carrying capacities recorded in decapods are from terrestrial and semi-terrestrial crabs (Burggren and McMahon, 1981; Burnett, 1979), although Miller *et al* (1976) calculated a value for *Callianassa californiensis* of 3.4 $ml.100ml^{-1}$ on the basis of protein concentration. Although *Calocaris* had very little non-haemocyanin protein (the regression line intercepts

of Fig. 5.3 are close to the origin), this may not be true of *C. californiensis*.

5.4.3 Structure of the haemocyanin.

The 'native' haemocyanins of the thalassinid species ***Calocaris macandreae*** (Svedberg, 1933), ***Callianassa californiensis*** (Roxby *et al.*, 1974), ***Callianassa gigas*** (Miller *et al.*, 1977) and ***Upogebia pugettensis*** (Miller *et al.*, 1977) have all been reported as eikositetramers (i.e. aggregates of 24 monomers). (N.B. the latter three papers refer to a hexamer aggregate as the 'monomer': see Mangum (1983a) for clarification.) In most other decapods, hexamers and dodecamers are the major fractions. Gel filtration of ***Calocaris*** haemocyanin (present study) produced a haemocyanin fraction with an estimated relative molecular weight (Mr) of 1650k, corresponding to a multiple of 22 monomer units (assuming a monomer molecular weight of 75kd; but see below).

SDS-PAGE slab gels resolved the haemolymph proteins into 4 bands for all the species studied. Previous studies of decapod haemolymph proteins have resolved between 2 and 7 separate bands ascribed to haemocyanin, with up to 3 additional bands of non-respiratory protein (e.g. Markl *et al.*, 1979; review of Mangum, 1983a). These subunits are usually similar in size, between 67 - 90k. Resolution of haemocyanins using PAGE over a wider molecular weight range often produces protein bands thought to represent dimers, hexamers and larger oligomers (e.g. Rochu *et al.*, 1978; Jeffrey, 1979). However, dissociation of haemocyanin is frequently not quantitative (Markl *et al.*, 1979). This is apparently the case in the present study, since the estimated Mr of 'native haemocyanin' is not a simple multiple of the supposed monomer

Mr. Neither are the larger proteins resolved by SDS-PAGE multiples of the smaller 'monomers'. The difficulty of estimating molecular weights of these proteins was noted by Mangum (1983a). The heterogeneity observed in the protein monomers probably results largely from variation in the associated carbohydrate and lipid moieties, as peptide sequences suggest that the protein chains are relatively conservative (e.g. van den Berg *et al.*, 1977). The functional significance of subunit heterogeneity is unclear (see Mangum, 1983a for discussion).

5.4.4 Oxygen transport in the haemolymph.

5.4.4.1 Comparative aspects.

The haemolymph of ***Calocaris macandreae*** contains a high molecular weight haemocyanin, as described above. A major function of the haemolymph is the transport of oxygen from the gills to the respiring tissues. The presence of a respiratory pigment increases the efficiency of transport since the total solubility of oxygen in the haemolymph is increased by the binding of oxygen to the pigment. The transport of carbon dioxide may also be enhanced, since the Haldane effect may link oxygen and CO_2 loading and unloading. An additional respiratory function of the haemolymph is to act as a venous reserve during periods when tissue oxygen demand exceeds oxygen uptake at the gills (e.g. respiratory pauses, exercise). Furthermore, the haemolymph compartment has an important role in acid-base regulation. The physiological functions of the haemolymph are affected by several physical characteristics of the pigment, which can be investigated *in vitro*: the concentration of pigment (carrying capacity); the oxygen affinity and cooperativity of the pigment; and the modulation characteristics of the pigment (by a variety of modulators).

The oxygen affinity (expressed as P50) of ***Calocaris macandreae*** haemocyanin under simulated *in vivo* conditions was approximately 1.6 Torr. The oxygen affinities of haemocyanins from 39 decapod species were reviewed and tabulated by Mangum (1983a). P50 values varied between 5.5 and 25 Torr; in ***Panulirus argus*** (Redmond, 1968) and ***Liocarcinus puber*** (Truchot, 1971) respectively. More recent data for decapod oxygen affinities include P50 values for ***Palaemon elegans*** (3.6 Torr: Morris *et al.*, 1985); ***Atelecyclus rotundatus*** (5.6 Torr), ***Goneplax rhomboides*** (4.1 Torr), ***Liocarcinus depurator*** (24.3 Torr: Taylor *et al.*, 1985); ***Galathea strigosa*** (12.6 Torr), ***Pagurus bernhardus*** (23 Torr), ***Corystes cassivelaunus*** (3.1 Torr), ***Nephrops norvegicus*** (11.5 Torr: Bridges, 1986); ***Holthuisana transversa*** (2.6-11.5 Torr: Morris *et al.*, 1988); and ***Munida rugosa*** (approximately 35 Torr: K. Zainal, pers. comm.). Of these species, the burrowing/burying crabs ***A. rotundatus***, ***G. rhomboides***, ***C. cassivelaunus*** and the burrowing lobster ***N. norvegicus*** have comparatively low P50's of 3.1 - 11.5 Torr.

There have been few studies of the oxygen affinity of thalassinid haemolymph, despite the unusual haemocyanin molecular weight known since Svedberg's (1933) report for ***Calocaris***. Miller and van Holde (1981) reported a P50 of 6.0 Torr (at pH 8.0; 10^{o}C; exogenous buffer Tris-HCl) in ***Callianassa californiensis***. A similarly high affinity was reported for the relatively large, tropical mud-lobster ***Thalassina anomola*** (Mangum, 1982: P50 = 6.0 Torr; pH 7.50; 25^{o}C; exogenous buffer Tris Maleate).

The cooperativity of crustacean haemocyanins is highly conservative, between 2.1 and 4.5 (see the review of Mangum, 1983a). The cooperativity of ***Calocaris macandreae*** haemocyanin under simulated physiological conditions was within this range, and was not modulated

by any of the factors studied. Several recent reports, however, have suggested that n50 values of crustacean haemocyanins may be affected by freezing (Morris, 1988; Spicer and McMahon, in prep.). The haemolymph samples used in this study were frozen for up to several weeks.

The oxygen affinities of the haemocyanins of thalassinids and other burrowing decapods are consistently lower than those of decapods from normoxic habitats (references above), as would be expected on the basis of environmental oxygen availability (see Chapter 2). However, the adaptability of crustacean haemocyanins appears to be limited in comparison with annelid and vertebrate pigments, primarily haemoglobins (Mangum, 1983a). In these taxa, the most reliable correlate with oxygen affinity is the design of the cardiovascular and gas exchange systems, rather than environmental factors (Johansen and Lenfant, 1972; Mangum, 1976).

Exogenous factors known to affect haemocyanin oxygen affinity in decapod crustaceans include temperature, salinity (as it affects the haemolymph ionic concentrations), and carbon dioxide concentration (as it affects *in vivo* PCO_2 and pH). The main endogenous effects which may have functional significance in short-term responses are the Bohr effect (pH); the lactate and urate effects; and probably hormonal effects (possibly mediated by dopamine; Morris, 1988). Exogenous effects on haemocyanin oxygen affinity have been reviewed by Mangum (1983a), with particular emphasis on temperature. In many species, unloading of oxygen at the tissues may be limited at low temperatures since the oxygen affinity increases (compounded by an additional Bohr effect due to a temperature-induced increase in haemolymph pH: Cameron, 1986). However, it is known that partial temperature-acclimation of

oxygen affinity may serve to minimise these maladaptive effects (e.g. Rutledge, 1981; Mauro and Mangum, 1982).

The change in enthalpy concomitant with oxygenation is a measure of the sensitivity of the pigment to temperature. ***Calocaris macandreae*** haemocyanin is highly temperature-sensitive, with a ΔH of -75.6 $kJ.mol^{-1}$. Values of ΔH in crustacean haemocyanins vary widely, from -5 $kJ.mol^{-1}$ in ***Nephrops norvegicus*** (Bridges, 1986) and -7 $kJ.mol^{-1}$ in ***Goneplax rhomboides*** (Taylor *et al.*, 1985), to -130 $kJ.mol^{-1}$ in ***Cancer borealis*** (Mauro and Mangum, 1982). ΔH in the thalassinid ***Callianassa californiensis*** was -42 $kJ.mol^{-1}$ (Miller and van Holde, 1981; re-calculated by Bridges, 1986). There is some evidence that ΔH is inversely correlated with the magnitude of fluctuations in environmental temperature experienced by different species (Jokumsen and Weber, 1982). However, there does not appear to be a simple correlation of ΔH with the burrowing habitat (Bridges, 1986).

The Bohr effect is present in all decapod haemocyanins so far studied, with a magnitude of between approximately -0.13 (***Holthuisana transversa***: Morris *et al.*, 1988) and -1.5 (e.g. ***Carcinus maenas***: Taylor and Butler, 1978; although Truchot, 1971, 1973 reports -0.62). The quantitative value of the Bohr effect in ***Calocaris macandreae*** was close to average (-0.58; see also Table 5.3, below).

The presence of a dialysable modulator of haemocyanin oxygen affinity was first reported by Truchot (1971), who later identified L-lactate as a modulator in ***Carcinus maenas*** (Truchot, 1980). The lactate effect has since been reported from a wide variety of crustaceans (e.g. ***Callinectes sapidus***: Booth *et al.*, 1982; ***Cancer magister***: Graham *et al.*, 1983; ***Palaemon elegans***: Bridges *et al.*, 1984; ***Homarus gammarus***:

Bouchet and Truchot, 1985; ***Ocypode saratan***: Morris and Bridges, 1985; and ***Austropotamobius pallipes***: Morris *et al.*, 1986). However, a lactate effect could not be demonstrated in ***Coenobita clypeatus*** Morris and Bridges (1986), nor in ***Holthuisana transversa*** (Morris *et al.*, 1988). Similarly, the lactate effect was absent in ***Procambarus clarkii*** (Mangum, 1983b). In the thalassinid ***Callianassa californiensis***, a large effect of dialysis, which cannot be attributed to L-lactate, is present (Mangum, 1983a, 1983b). The magnitude of the lactate effect measured in several species is given in Table 5.3:

TABLE 5.3: Lacate and Bohr effects measured in a variety of decapod crustaceans.

Species	Lactate Effect logP50/ log[lactate (mM)]	Bohr Effect logP50/ log pH	Ref.
Callianassa californiensis	0	-1.3	(1,2)
Calocaris macandreae	0	-0.58	(3)
Coenobita clypeatus	0	-0.396	(4)
Holthuisana transversa	0	-0.13	(5)
Carcinus maenas	-0.096	-0.62	(6)
Homarus gammarus	-0.16	-1.1	(7)
Ocypode saratan	-0.16 to -0.24	-0.67	(8)
Goneplax rhomboides	-0.18 to -0.20	-0.62	(9)
Cancer pagurus	-0.21 to -0.24	-1.0	(6)
Cancer magister	-0.25 to -0.29	-	(10)
Liocarcinus depurator	-0.29 to -0.39	-1.40	(9)
Atelecyclus rotundatus	-0.33 to -0.44	-0.92	(9)
Palaemon elegans	-0.56 to -0.63	-1.1 to -1.2 **	(11)

** although -1.7 is given by Morris *et al.*, (1985)

References: (1) Mangum (1983b); (2) Miller and van Holde (1981); (3) present study; (4) Morris and Bridges (1986); (5) Morris *et al.*, (1988); (6) Truchot (1980); (7) Bouchet and Truchot (1985); (8) Morris and Bridges (1985); (9) Taylor *et al.*, 1985; (10) Graham *et al.*, (1983); (11) Bridges *et al.*, (1984).

The relationship between the Bohr effect and the lactate effect is unclear, although there are functional reasons for a correlation (Mangum, 1983c; see below). The present study could demonstrate no significant modulation of the haemocyanin of ***Calocaris macandreae*** by L-

lactate, under physiological conditions. This result is corroborated by the lack of any effect of dialysis, suggesting that dialysable organic modulators are not present in haemolymph samples. It remains possible that a modulating factor (or factors) such as urate was present at low concentrations in the haemolymph samples (so that dialysis did not result in a significant change in concentration). In a preliminary experiment, addition of urate to a final concentration of 20 $mmol.l^{-1}$ had no effect on oxygen affinity of ***Calocaris*** haemolymph.

Modulation by urate (a product of purine catabolism) was described by Morris *et al.* (1985), and reviewed by Bridges and Morris (1986). Urate has also been demonstrated to accumulate *in vivo* to effective (*in vitro*) concentrations as a result of hypoxic, or other stress (Lallier *et al.*, 1987; see below). Modulation of haemocyanin by small, non-dialysable compounds has been demonstrated (e.g. Rutledge, 1981; Mauro and Mangum, 1982). The possibility that haemocyanin may also be modulated *in vivo* by secreted neurohormones has been suggested (Morris, 1988).

5.4.4.2 Functional Aspects.

There are two major aspects of the respiratory function of haemocyanin in decapod crustaceans which may be considered. The efficiency of the oxygen transport system can be assessed using a variety of functional indices, such as equilibration efficiencies (Mangum, 1983a) or figurative models (e.g. Taylor *et al.*, 1985; Bridges, 1986). Secondly, the functional modulation of haemocyanin *in vivo* may be assessed.

In order to relate the *in vitro* characteristics of the haemocyanin to the function of the oxygen transport system *in vivo*, several parameters must be measured *in vivo*. These include the PO_2's of the

inhalant water, and of the pre-branchial and post-branchial haemolymph. In addition, where appropriate it is necessary to ensure that potential modulating factors (e.g. temperature, pH, and inorganic ion, lactate and urate concentrations) were similar in *in vitro* experiments. In the present study of ***Calocaris macandreae***, the determined *in vivo* parameters were limited to temperature, pH, inorganic ion and lactate concentrations, and inhalant and post-branchial PO_2's.

In either quantitative or figurative assessments of oxygen transport in steady-state conditions, the main variables which may be compared are the relative contributions to total oxygen transport of dissolved and pigment-bound oxygen, and the efficiencies of equilibration at the gills and the tissues (E_bO_2 and E_tO_2 respectively, defined by Mangum, 1983a). Unfortunately, steady-state conditions are exceptional (and difficult to apply even in experimental systems) and the performance of transport systems must be considered during changes in motor activity, environmental oxygen availability, and respiratory performance (e.g. due to respiratory pausing). In such conditions the total amount of oxygen delivered to the tissues is not necessarily equal to the amount taken up at the gills, and the function of the venous reserve becomes important.

The efficiency of equilibration of haemocyanin at the gills in decapods is usually high, approximately 40 - 90% (Mangum, 1983a; e.g. 63% in ***Carcinus maenas***: Taylor, 1977; 62% in ***Callinectes sapidus***: Mangum and Weiland, 1975), but may decrease during strenuous activity (to 27% in ***Callinectes***). Although the quantitative parameter E_bO_2 cannot be calculated for ***Calocaris*** in the present study (since pre-branchial PO_2 is unknown), the relatively large values of S_{total} measured in

Calocaris under normoxic conditions suggests that oxygen loading at the gill is effective.

The equilibrium efficiency at the haemolymph-tissue interface is often lower (Mangum, 1983a; e.g. 33% in *Carcinus maenas*: Taylor, 1977). A low efficiency of oxygen unloading at the tissues implies that haemocyanin-bound oxygen acts as a venous reserve. The relative amounts of oxygen transported as physically dissolved, and bound oxygen, together with the amount retained as bound oxygen in pre-branchial haemolymph (the venous reserve) can also be shown figuratively (e.g. Taylor *et al.*, 1985; Bridges, 1986). Typical pre-branchial/post-branchial differences in PO_2 in crustaceans vary within a wide range: 10 - 80 Torr (Mangum, 1983a). As a result of the very high oxygen affinity of the haemocyanin of *Calocaris*, the pigment is unlikely to unload oxygen to the tissues except in conditions when the tissue PO_2 becomes very low (e.g. environmental hypoxia, or during a high level of motor activity). The venous reserve will be large, and of adaptive benefit at low environmental PO_2 (see below). In the larger thalassinid *Callianassa californiensis*, Miller *et al* (1976) found little difference between pre-branchial and post-branchial cO_2 (both of which were large), suggesting that oxygen unloading from the haemocyanin at the tissues is limited (in quiescent animals). This was true in both normoxic and hypoxic (50 Torr) conditions.

Modulation of haemocyanin oxygen affinity may increase the efficiency of oxygen transport to the tissues, particularly during respiratory stress, when haemocyanin modulation represents an adaptive response to short-term perturbations in respiratory performance. Modulation may be beneficial as a response to environmental change (e.g. hypoxia), or to counteract the maladaptive effects of other physiological processes

(for example the Bohr effect resulting from respiratory alkalosis during environmental hypoxia). Modulation may be distinguished from perturbation (which is not necessarily adaptive) by both exogenous and endogenous factors, e.g. temperature, haemolymph pH (Mangum, 1983a).

Modulation of any physiological function requires a gradient in the concentration or magnitude of the modulating parameter, which may be either spatial or temporal. There appear to be few examples of spatial modulation between the gills and tissues in crustaceans. Pre/post-branchial pH differences in resting decapods are usually less than 0.03 units (Truchot, 1983), resulting in a P50 of approximately 0.85 to 1.05 Torr. There is little evidence at present that spatial modulation by organic factors is functionally significant in decapods. Albert and Ellington (1985) measured pre-branchial concentrations of L-lactate in the crab ***Menippe mercenaria*** that are 10 $mmol.l^{-1}$ greater than post-branchial values. However, the magnitude of the lactate effect in this species is unknown. Spatial modulation by organic modulators (mainly organic phosphates) in vertebrate systems may be more significant, since the respiratory pigment is usually intracellular.

The functional significance of the Bohr effect is unclear at present. Morris *et al.* (1985) consider that the large Bohr effect in *Palaemon elegans* may be beneficial in hypoxic, hypercapnic conditions (e.g. intertidal rock pools at night) since a decrease in pH will facilitate oxygen unloading at the tissues. An alternative view is that the Bohr effect is maladaptive in hypoxic situations (e.g. Mangum, 1983c) since oxygen loading at the gills is significantly reduced by metabolic acidosis (neglecting the opposing effect of lactate). In most decapod species which have been studied, the short-term response to

environmental hypoxia is hyperventilation, accompanied by respiratory alkalosis in the haemolymph (e.g. Truchot, 1975; Dejours and Beekenkamp, 1977; McMahon *et al.*, 1978; Sinha and Dejours, 1980; Dejours and Armand, 1980; DeFur *et al.*, 1980). If the pre/post-branchial difference in pH is maintained at a negligible level, an increase in oxygen affinity due to the Bohr effect would result in increased loading at the gills but decreased unloading at the tissues. Only if the pre/post-branchial ΔpH is significant (as in exercised ***Callinectes sapidus***: Mangum and Weiland, 1975) is the Bohr effect clearly adaptive. The true significance of the Bohr effect may be related to the physiological function of the Haldane effect in CO_2 transport, since both effects are aspects of the same process (release of Bohr protons during oxygenation of the haemocyanin).

The adaptive function of the lactate effect is usually interpreted as serving to minimise the maladaptive effects of the Bohr effect during conditions of lactate production and acidosis (e.g. Mangum, 1983c). These conditions are not usually prevalent during the initial stages of environmental hypoxia, but are more typical of functional hypoxia (i.e. motor activity; e.g. Booth *et al.*, 1982). An additional function may be to facilitate unloading of the venous reserve when oxygen uptake ceases and lactate is being accumulated. Recent studies of the time-course of acid-base balance and modulator concentrations during environmental hypoxia suggest that lactate does not increase until very low PO_2's are reached (e.g. Lallier *et al.*, 1987; present study). However, since the effect of lactate on haemocyanin oxygen affinity is usually exponential over the physiological range (Bridges and Morris, 1986), the lactate effect may operate even at the onset of lactate accumulation.

Urate may be more likely to act as a true modulator of haemocyanin oxygen affinity during environmental hypoxia. Urate has been shown to accumulate during moderate hypoxia in two species which also show a urate effect on haemocyanin oxygen affinity (***Carcinus maenas***: Lallier *et al.*, 1987; ***Hemigrapsus nudus***: Morris *et al.*, 1988). At present, however, it is difficult to interpret the significance of *in vitro* urate modulation.

Although the acid-base status in ***Calocaris macandreae*** during environmental hypoxia was not studied, the presence of a pronounced hyperventilation response (see Chapter 4) suggests that the Bohr effect may increase loading at the gills during moderate hypoxia (PO_2 above 10 Torr). At these PO_2's, lactate is not produced, so a compensatory metabolic acidosis is unlikely. At PO_2's below 5 Torr, hyperventilation is reduced, and lactate is accumulated in the haemolymph. A metabolic acidosis (unopposed by a lactate effect) in these conditions would facilitate unloading at the tissues, and exploitation of the venous reserve.

Direct measurement of the oxygen content of post-branchial haemolymph in conditions of declining oxygen tension supports the view that regulatory mechanisms operate to maintain oxygen uptake. Both measured cO_2 and calculated S_{total} were maintained at values close to those observed in normoxic conditions, at an environmental PO_2 of 51 Torr, before declining. In contrast, Miller *et al* (1976) found that cO_2 in ***Callianassa californiensis*** decreased from 1.48 ml.100 ml^{-1} (0.66 $mmol.l^{-1}$) at 160 Torr to 0.83 ml. 100ml^{-1} (0.37 $mmol.l^{-1}$) at 50 Torr. Although the relative proportions of dissolved and bound oxygen cannot be estimated, the cO_2 does not drop below the value at which

haemocyanin unloading definitely occurs (i.e. below the mean $c_{HCY}O_2$), until a PO_2 of approximately 20 Torr (i.e. around the Pc). However, the rate of oxygen delivery to the tissues is not simply correlated with post-branchial cO_2, but with the pre/post-branchial cO_2 difference.

In general terms, the pattern of cO_2 decrease during progressive hypoxia might be expected to conform with one of three models (Fig. 5.18). If *in vivo* PO_2 (post- or pre-branchial) is not regulated, then initially, cO_2 would decline in proportion to environmental PO_2 (as the dissolved component decreases). In more severe hypoxia an internal PO_2 is reached (related to the haemocyanin P50 and n50) at which the haemocyanin begins to unload (Pc'', Fig. 5.18A). In this case the pattern is biphasic. In contrast, if *in vivo* PO_2 is regulated (by changes in ventilation, perfusion etc.) then a triphasic pattern is produced, since cO_2 is initially regulated until a critical environmental PO_2 (Pc', Fig 5.18B). Finally, if the values of Pc' and Pc'' are similar, a biphasic pattern is produced in which *in vivo* cO_2 is regulated even in relatively severe hypoxia (Fig. 5.18C). Note that this model does not indicate changes in oxygen delivery rates (dependant on cO_2) but in relative changes in amounts of haemolymph oxygen. In order to determine delivery rates, this analysis could be extended to indicate both pre- and post-branchial cO_2's (Fig. 5.18D).

The results obtained in the present study are not sufficiently detailed to resolve which model category could be applied to ***Calocaris macandreae.*** Further work in this area would therefore be of interest. Nevertheless, it is suggested that this type of approach may be usefully applied to small animals (in which PO_2 cannot be measured), in which it is difficult to interpret *in vitro* characteristics of haemocyanin function using the quantitative and figurative models

FIG. 5.18 Models of oxygen transport in declining oxygen tension in the medium (PO_2).

A) *In vivo* PO_2 is not regulated; haemocyanin-bound oxygen is unloaded below Pc''.

B) *In vivo* PO_2 is regulated above Pc'; haemocyanin-bound oxygen is unloaded below Pc''.

C) *In vivo* PO_2 is regulated at all external PO_2's above Pc''.

D) The amount of oxygen delivered to the tissues can be calculated (hatched area) from the pre-branchial/post-branchial PO_2 difference. Note that the respective positions of Pc' and Pc''may differ in pre- and post-branchial haemolymph. See text for further details.

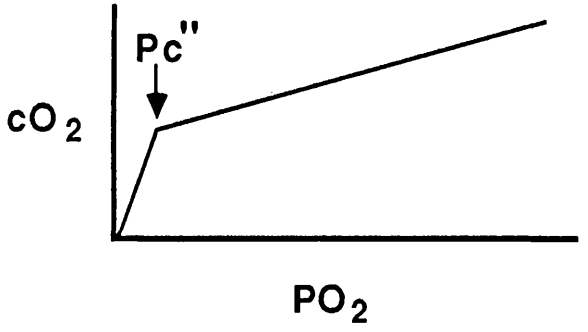


Fig. 18A

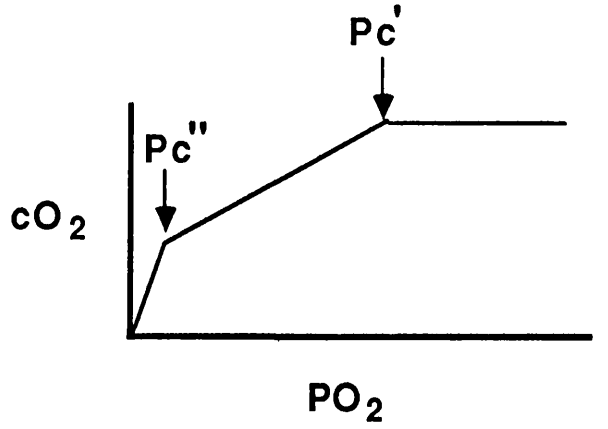


Fig. 18B

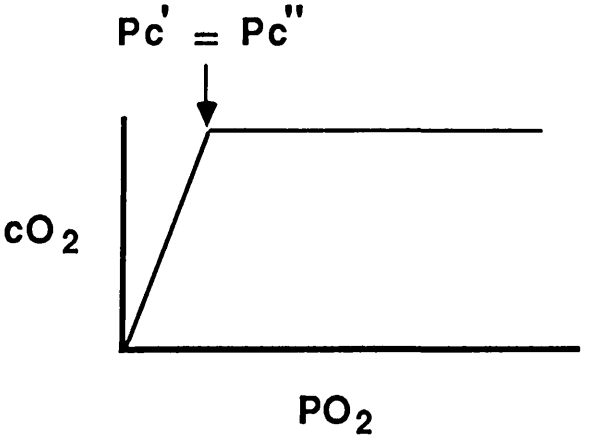


Fig. 18C

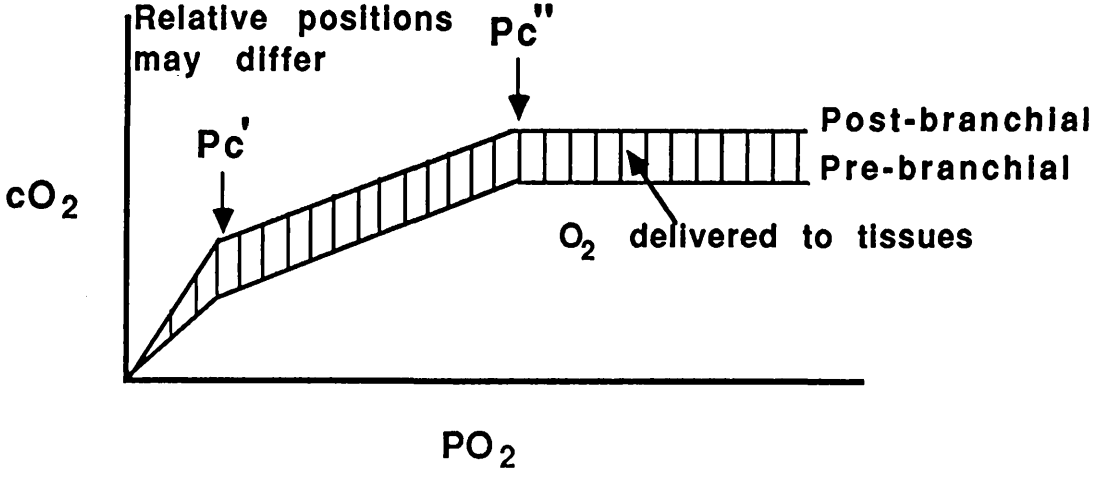


Fig. 18D

reviewed above.

In summary, the oxygen transport system of *Calocaris macandreae* appears to be adapted to maintain a comparatively low rate of oxygen delivery to the tissues, in conditions of prolonged hypoxia, and possibly even during occasional brief anoxic periods. In normoxic and probably moderately hypoxic (down to 10 - 20 Torr) conditions, the high affinity of the haemocyanin results in effective oxygen loading at the gills. A large proportion of the oxygen delivered at the tissues will probably come from physical solution, and the relative venous reserve will be maintained at a high level. In very severely hypoxic conditions (2 - 10 Torr) hyperventilation ceases and the venous reserve may be unloaded as a result of the Bohr effect.

A speculative calculation of the duration of the utilisation of the venous reserve during environmental or functional anoxia is possible. With the assumptions that haemolymph volume = 150 $\mu l.g^{-1}$ (estimated from the average sample volume from individual *Calocaris*: probably an underestimate), measured values of *in vivo* cO_2 (0.4 $mmol.l^{-1}$) and of tissue oxygen consumption (0.75 $\mu mol.g^{-1}.h^{-1}$), it appears that the venous reserve may suffice for approximately 1.6 minutes of anoxia. During long-term anoxia (more than 6 hours; PO_2 < 2 Torr), it is shown in the next chapter that anaerobic metabolic pathways are utilised, and lactate is accumulated.

5.4.5 Carbon dioxide transport and acid-base balance.

There are two major processes involving carbon dioxide in the haemolymph of animals: the transport and excretion of CO_2 produced by aerobic respiration, and the regulation of acid-base balance in the extracellular and intracellular compartments. Understanding of the operation of both these processes in crustaceans is developing very rapidly. However, the technical difficulties of measurement in small animals (such as ***Calocaris macandreae***) still limits the investigation of CO_2 transport and acid-base balance *in vivo*.

CO_2 dissociation curves have been constructed *in vitro* for a number of crustacean species since the original description of Truchot (1976b) for ***Carcinus maenas***. In all cases, the curves have a similar form to those found in the present study, with initially large capacitance coefficients over the physiological PCO_2 range. Maximum total CO_2 concentrations have been found to vary from 2 to 10 $mmol.l^{-1}$. The conclusions of Truchot (1976b) that CO_2 curves were highly variable between individuals (depending particularly on the moult cycle and associated changes in haemolymph protein concentration) have been verified. The presence and magnitude of the Haldane effect have also been studied (e.g. Truchot, 1976b; Randall and Wood, 1981; Taylor *et al.*, 1985).

The presence of a Haldane effect suggests that oxygen loading of the haemocyanin will be linked to carbon dioxide unloading (and *vice versa*). The Haldane effect probably results from dissociation of Bohr protons from the haemocyanin on oxygenation, and is quantitatively linked to the Bohr effect (Wyman, 1948). The physiological significance of the Haldane effect is related to the relative values of the Haldane effect and the oxygen carrying capacity (i.e. the ratio

$\Delta CO_2/c_{HCY}O_2$; Truchot, 1976b), which is comparable in ***Calocaris*** with literature values (e.g. 0.5 $molCO_2 . molO_2^{-1}$; ***Carcinus maenas***: Truchot, 1976b). Due to the small partial pressure gradient between pre- and post-branchial haemolymph, the Haldane effect probably has an important role in *in vivo* CO_2 transport (Truchot, 1976b, 1983; Taylor *et al.*, 1985).

There are few direct measurements of *in vivo* PCO_2 even from relatively large decapods, due to the low PCO_2 values present, and the difficulty of obtaining anaerobic samples of sufficient volume. Most studies have measured cCO_2 and pH values *in vivo*, and pK' values *in vitro*. PCO_2 is then calculated from the Henderson-Hasselbalch equation assuming that the bicarbonate system is at equilibrium in the haemolymph. However, carbonic anhydrase (CA) is absent from the haemolymph of decapods (Aldridge and Cameron, 1979; Randall and Wood, 1981; Henry and Cameron, 1982; McMahon *et al.*, 1984), although it is present in the gills (see below). The non-catalysed hydration/dehydration rate constants for the $CO_2/HCO_3/CO_3$ equilibrium are low (Edsall, 1969). There is evidence from comparisons of calculated and measured PCO_2 *in vivo* (Defur *et al.*, 1980), and from studies of the time-course of equilibration of sampled haemolymph (Cameron, 1986), that disequilibrium conditions are present throughout most of the circulation. These studies are supported by model calculations (Aldridge and Cameron, 1979; Cameron, 1986). Model calculations are tentative, however, since haemolymph residence times in various parts of the circulation remain largely unknown (Truchot, 1983; Cameron, 1986).

The functions of carbonic anhydrase in the gills of decapods have been the subject of debate (Aldridge and Cameron, 1979; Burnett *et al.*,

1981; McMahon and Burnett, 1981; Henry and Cameron, 1983; McMahon *et al.*, 1984; Burnett *et al.*, 1985). Various treatments of animals with CA inhibitors (usually acetazolamide) have demonstrated that CA is involved in both respiratory excretion of CO_2 (by dehydration of HCO_3^- to molecular CO_2, which is permeable) and ionic regulation (by hydration of CO_2 to HCO_3^- and H^+, to supply counterions for Cl^- and Na^+ uptake). Recently, Henry (1988) has proved that the dehydration and hydration reactions catalysed by CA are functionally separated, and occur in the gill epithelial membrane and cytoplasm respectively.

Cameron (1986) has suggested that in normoxic conditions, the gills of decapod crustaceans are hyperventilated with respect to CO_2. Evidence for this theory is provided by model calculations of CO_2 parameters in water and haemolymph at the gills, and by the apparently excessive CO_2 capacity rate ratio (i.e. the CO_2 capacity of the volumes of water and haemolymph flowing over/through the gills). The model calculations, however, rely on the assumptions of equlibrium conditions in both water and haemolymph, and on the presence of perfect countercurrent exchange. Both of these assumptions must be regarded with caution at present. There is evidence that the gills are not hyperventilated, since hyperoxia and hyperventilation consistently result in respiratory acidosis (Truchot, 1983).

The major parameters which contribute to the regulation of haemolymph pH are the total weak acid activity, PCO_2 and the strong ion difference (SID) (Stewart, 1978). The weak acid buffering of crustacean haemolymph is due mainly to the proteins present (Truchot, 1983) and is usually constant during short term perturbations of acid-base balance (which occur, for example, due to hypoxia, hypercapnia, ventilatory changes, air exposure and exercise). The amount of buffering due to

weak acids is usually termed "non-bicarbonate buffering" and is represented by the regression line of a Davenport diagram (see above). Bicarbonate buffering results from changes in the relative concentrations of CO_2, HCO_3^- and CO_3^{2-} and is, therefore, a function of PCO_2 and the pK' value of the haemolymph. Changes in acid-base balance resulting from bicarbonate buffering (i.e. respiratory compensation) result in changes in pH and HCO_3^- concentration along the non-bicarbonate buffer line. The influence of SID on acid-base balance (metabolic compensation) is shown as movement of the buffer line relative to the HCO_3 concentration axis of a Davenport diagram. SID is defined as the activity difference between anions and cations which do not change their dissociation in the physiological pH range, and is extremely difficult to measure directly due to formation of weak complex ions (Heisler, 1986). However, factors which contribute to changes in SID include the major haemolymph ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-}) and metabolic acids such as L-lactate.

Although there is some interspecific variation, the acid-base responses of crustaceans to several types of stress are relatively stereotyped (see review of Truchot, 1983). As described in Chapter 2, hypercapnia was not observed in the burrows of ***Calocaris macandreae*** although high PCO_2's have been observed in the burrows of fish (***Lumpenus lampretaeformis*** and ***Cepola rubescens***: Atkinson *et al.*, 1987). Probably the most frequent sources of acid-base disturbance in ***Calocaris*** result from regulation of oxygen uptake. For example, respiratory acid-base disturbance results from hyperventilation in hypoxia; metabolic metabolic disturbances may be due to SID variations (e.g. metabolic L-lactate production).

Although Cameron (1986) disputes the point, by far the majority of

studies of acid-base balance during respiratory hyperventilation have noted a respiratory alkalosis, i.e. CO_2 excretion rates are increased, HCO_3^- concentration decreases and pH increases (references cited above). Hypoxia-induced metabolic alkalosis may also occur (Truchot, 1975; McMahon *et al.*, 1978). The response to hypoxia-induced alkalosis is variable; metabolic compensation (probably release of bicarbonate) occurs in ***Carcinus maenas*** (Truchot, 1975; Johnson and Uglow, 1987) but not in ***Astacus*** (Dejours and Beekenkamp, 1977). Metabolic compensation (i.e. SID variation) may be achieved by alterations in organic acid production, carbonate mobilisation or by ionic exchange mechanisms at the antennary gland and gills (Truchot, 1983). Several studies have shown that carbonate mobilisation occurs. Carbonate concentrations may be increased by uptake from the medium (suggested by decreases in the counterion Cl^-: Hagerman and Uglow, 1981, 1982; Johnson and Uglow, 1987), or by mobilisation of carbonate from the hepatopancreas and exoskeleton (suggested by increased Ca^{2+} concentration: e.g. Defur *et al.*, 1980; Henry *et al.*, 1981; Truchot, 1983; Johnson and Uglow, 1987). Cameron (1985) found that the proton sink in compensation of hypercapnic acidosis in ***Callinectes sapidus*** was external sea water. The quantitative importance of the contribution of carapace carbonate was small. Acidosis may also result from production of lactate during anaerobiosis.

The measured cCO_2 and pH, and calculated PCO_2, HCO_3^- and non-bicarbonate buffer slope values obtained for ***Calocaris macandreae*** in the present study are all within the ranges previously determined for decapod crustaceans (references above). The acid-base responses of ***Calocaris*** to perturbations resulting from respiratory processes are unknown, but may represent a limiting factor in regulation of oxygen uptake, and therefore merit further attention.

6.1 INTRODUCTION

The major theme of this thesis has been to examine the behavioural, morphological and physiological mechanisms by which ***Calocaris macandreae*** maintains oxygen uptake in the hypoxic environment of the burrow. It has been shown that the environmental 'critical oxygen tension' below which oxygen uptake rates cannot be maintained corresponds to the oxygen tension at which several of these mechanisms break down (i.e. 10 -20 Torr). However, several studies have shown that thalassinids (especially ***Callianassa*** species) can survive extended periods of anoxia, 50 - 60 hours at 10°C (Felder, 1979; Mukai and Koike, 1984; Zebe, 1982; see below).

Following early studies of anaerobic metabolism in vertebrates, it has been established that L-lactate is an end-product of carbohydrate catabolism in many animals. In the 1940's, however, von Brand discovered that volatile fatty acids were accumulated in endoparasitic nematodes. In the last 20 years, a good understanding of the general characteristics of anaerobic metabolism in several phyla has developed. In particular, it is now recognised that a variety of end-products of anaerobic metabolism may be accumulated in different animals. For example, alanine, propionate and succinate are accumulated in annelids and bivalve molluscs (Gäde, 1980; de Zwaan and Zurburg, 1981) whereas the major end-product accumulated by the sipunculid worm ***Sipunculus nudus*** is octopine (Pörtner *et al.*, 1984). In decapod crustaceans, however, the only metabolic end-product accumulated during anoxia is L-lactate (e.g. Teal and Carey, 1967; Pritchard and Eddy, 1979; Gade, 1984; Albert and Ellington, 1985; Taylor and Spicer, 1987).

The accumulation of lactate, and depletion of carbohydrate reserves during environmental anoxia have been studied in the thalassinids ***Callianassa californiensis*** and ***Upogebia pugettensis*** (Hawkins, 1970; Pritchard and Eddy, 1979; Zebe, 1982). Metabolic responses to anoxia in ***Calocaris macandreae*** were therefore studied in order to provide comparative data, and as one aspect of an integrated study of the respiratory ecology and physiology of this species.

To provide background information to the metabolic studies, the rate of mortality of ***Calocaris*** in anoxic conditions was estimated. The rates of depletion of carbohydrate reserves, and accumulation of metabolic end-products (L-lactate), during exposure to anoxia were estimated. The time course of changes in metabolite concentrations during recovery from anoxic exposure was also investigated. In order to confirm that L-lactate was the major end-product of anaerobic carbohydrate catabolism (as reported for other crustaceans, see below), the concentrations of a range of organic acids were compared using High Performance Liquid Chromatography (HPLC) before and after anoxic exposure. Finally, as a preliminary investigation of the biochemical basis of comparative differences in anaerobic metabolism, the activities of lactate dehydrogenase (LDH) in tissues of ***Calocaris*** were measured.

6.2 MATERIALS AND METHODS.

6.2.1 Effects of anoxia and subsequent recovery.

Calocaris macandreae (fresh weight range from 1.5 to 2.5g) were caught in the Firth of Clyde using a ring-dredge between July and September 1988. As this period included the moulting period for this *Calocaris* population, both pre- and post-moult animals were used in experiments. The results obtained from both sets of animals were compared. Animals were maintained in aquaria at 10°C for several days before experiments were carried out.

During each experimental exposure to anoxia, 10-25 animals were placed in an enclosed aquarium (5 l volume) containing fresh sea water in a temperature-controlled room at 10°C. Gas mixtures containing N_2, CO_2 and air were supplied by a needle-valve gas mixing system and bubbled through the water at a rate of 500 $ml.min^{-1}$. An oxygen electrode connected to a Strathkelvin meter was inserted into the experimental water, in which the PO_2 was continuously monitored. Initially, pH and PCO_2 were also measured until it was found that these did not change significantly during the experiments. A PO_2 of < 2 Torr was reached 1 hour after commencing the experiment. Control individuals were maintained under normoxic conditions in separate aquaria. When appropriate, at the end of the experimental anoxic period, animals were gently transferred to aquaria containing aerated sea water for the duration of the recovery period.

In one experiment, the survival of *Calocaris macandreae* in prolonged anoxia was examined by exposing 18 individuals to anoxia. The animals were examined at regular intervals (until all had died) and classed as dead when no movement could be detected after gentle prodding. Cumulative survival curves could then be constructed.

Due to the limited availability of *Calocaris*, several experiments were carried out to investigate the metabolic consequences of anoxia exposure, and the results combined. In each case several sampling times were duplicated to ensure the compatibility of the data. Two groups of experiments were carried out: initially the animals were exposed to anoxia for 18 hours, then allowed to recover for 48 hours in normoxic conditions. Subsequently, it became apparent that *Calocaris* could survive far longer than 18 hours in total anoxia and experiments were carried out to investigate the effects of extended anoxia exposure.

Animals to be sampled for tissue metabolites were gently removed from the experimental aquarium, blotted dry and quickly frozen in liquid N_2 before being stored at -20°C. Animals sampled for haemolymph metabolites were removed and blotted dry. The haemolymph sample was quickly removed from the pericardium with a 1 ml plastic syringe and frozen at -20°C. Due to the small volume of the haemolymph samples (approximately 100µl) it was necessary to combine haemolymph from two individual animals to provide sufficient material for biochemical assays.

6.2.2 Analytical methods.

Total tissue extracts were prepared by grinding the frozen animals into a fine powder using a pestle and mortar containing liquid N_2. Following evaporation of the N_2, a weighed amount (approximately 50 mg) of the frozen powder was suspended in 500 µl of 0.3 $mol.l^{-1}$ perchloric acid (PCA). After centrifugation at 10,000Xg for 10 min, the supernatant was removed and a further 500 µl PCA wash of the

homogenised tissue was taken. The two washes were combined and neutralised by the addition of 4 mmol.l^{-1} K_2CO_3. After further centrifugation (10,000Xg for 10 min) the supernatant was removed and stored at -20°C.

Initial experiments showed that due to the low protein concentration of *Calocaris* haemolymph, deproteinization of haemolymph samples with PCA was unnecessary. Therefore in subsequent experiments, haemolymph samples were simply centrifuged to remove haemocytes and cellular debris (10,000Xg for 5 min).

The concentration of L-lactate in the haemolymph and tissue extracts was measured using the method of Gutmann and Wahlefeld (1974) with the modifications suggested by Graham *et al.* (1983). This method follows the production of NADH concomitant with the conversion of L-lactate to pyruvate by lactate dehydrogenase (LDH). EDTA was added to the assay to prevent interference with the reaction end-point caused by Cu^{2+} ions present in the haemolymph (Engel and Jones, 1978). Details of this assay are given in Appendix 4.

The concentration of D-glucose in the haemolymph was assayed using an enzymatic method (Slein, 1965) in which hexokinase catalyses the production of glucose-6-phosphate (G6P), which is further oxidised by G6P dehydrogenase with the proportional conversion of NADP to NADPH. The increase in NADPH is determined spectrophotometrically or spectrofluorimetrically. Details are given in Appendix 5.

An anthrone method (Carrol *et al.*, 1956) was used for the determination of total hexose/pentose sugars (i.e. total glycogen + oligosaccharides + monosaccharides). This method uses acid hydrolysis to break all

glycosidic bonds and dehydrate the resulting glucosyl units to furfural and its derivitives. These react with anthrone (10 keto-9,10 dihydroanthracene) to form a complex which is assayed spectrophotometrically. This assay is described in Appendix 6.

After initial experiments had shown the presence of high levels of carbohydrates in the haemolymph, and in order to confirm the nature of the carbohydrates present in the tissues, haemolymph and tissue samples from several individuals were assayed for glycogen and oligo/monosaccharide components separately. Glycogen was precipitated from the samples using ethanol, as described in Appendix 6.

6.2.3 Effects of exposure to hypoxia.

In order to investigate the range of hypoxia over which anaerobiosis is initiated, groups of *Calocaris* were exposed to various degrees of hypoxia using the experimental system described above. The animals were sampled after 24 hours exposure to sea water equilibrated to 0, 2.7, 6.7, 10.0, 20.0 and 157 Torr (± 0.3 Torr). Total tissue lactate was assayed, and results expressed as the total-tissue lactate concentration present after 24 hours exposure.

6.2.4 HPLC Analysis.

Changes in organic acid metabolites during anoxia were investigated using High Performance Liquid Chromatography (HPLC). Animals exposed to control conditions and 24 hours anoxia, were immersed in liquid nitrogen and homogenised as described above. Most of the frozen homogenate (approximately 1.5 g) was suspended in 1 ml PCA and centrifuged at 10,000Xg for 2 minutes. 200µl of supernatant was then added to 40 µl 18N sulphuric acid (HPLC grade), 1 ml di-ethyl ether and

0.12 g NaCl in an Eppendorf tube. After mixing for 1 minute, the mixture was centrifuged, transferred to another tube (using a glass pipette), 100 ul 0.1N NaOH was added and the mixture was mixed and centrifuged again. The ether was then removed with a Pasteur pipette, and the extract was allowed to stand for 15 minutes (to allow residual ether to evaporate) before being injected into the HPLC column.

The samples were then eluted by isocratic HPLC using a Polypore H column at 70°C, with 0.01 mol.l^{-1} H_2SO_4 as the mobile phase. Organic acids were detected by absorbance at 210 nm. The standards used (in order of retention time) were tartrate, malate, malonate, pyruvate, succinate, ketobutyrate, oxaloacetate, D-lactate, L-lactate, fumarate, formate, ketoisocaproate, acetate, hydroxybutyrate, propionate, isobutyrate, butyrate, hydroxyisocaproate, isovalerate, methylbutyrate, n-valerate, methylvalerate and n-caproate.

6.2.5 Lactate dehydrogenase activity.

The activity of L-lactate dehydrogenase (LDH; EC 1.1.1.28) in several tissues of a single ***Calocaris macandreae*** was examined.

Samples of abdominal muscle tissue and gill tissue were dissected from an individual which had been sacrificed by rapid chilling in a 4°C fridge. 50 - 100 mg of tissue was immediately weighed into 1ml cold Tris buffer (also containing 10 µl mercaptoethanol + 1 µl 0.1% Triton) and homogenised in a 3 ml homogeniser. Triton was used to ensure complete homolysis of all cells; mercaptoethanol was present to denature protein disulphide bonds. The homogenate was washed into a microcentrifuge tube and centrifuged (10,000X g for 5 mins).

The assay mixture comprised:

Tris buffer (100 mmol.l^{-1} Tris-HCl, pH 7.5)

KCN (2 mmol.l^{-1}) to inhibit NADH oxidation by mitochondrial cytochromes

mercaptoethanol (1/10 absolute)

NADH (0 - 0.5 mmol.l^{-1})

pyruvate (0 - 4 mmol.l^{-1})

The sample size was 50 µl in a total reaction mixture of 1060 µl.

The reaction was followed at 340 nm for 2 minutes using the rate mode of a Phillips PU8700 spectrophotometer. Within this time-scale the reaction was linear. Km and Vmax values for the LDH present in the extracts were estimated using Lineweaver-Burke plots. Total activity was calculated from Vmax assuming the extinction coefficient of NADH to be 6.2×10^3 $l.mol^{-1}.cm^{-1}$.

6.3 RESULTS

6.3.1 Mortality in anoxia.

A cumulative mortality curve for 18 *Calocaris* maintained under anoxic conditions is shown in Fig. 6.1. Mortality remains low for approximately 25 hours before rapidly increasing. The LT_{50} was 43 hours. Animals maintained in control conditions showed a low mortality (< 10 % over 50 hours).

6.3.2 HPLC analysis.

The only organic acid detected which changed significantly in concentration after 25 hours anoxia, was L-lactate (Fig. 6.2). The traces shown in Fig. 6.2 have been selected to show clearly the observed changes in lactate concentration. Only one other peak could be detected, with a retention time of 13.58 minutes. The identity of this peak is unclear, since it does not co-elute with any of the standards used. This peak was smaller in the samples from animals exposed to anoxia and would therefore appear not to represent an accumulated end product of anaerobic metabolism.

6.3.3 Effects of anoxia on tissue and haemolymph metabolites.

6.3.3.1 Metabolite concentrations in control conditions.

Total tissue carbohydrate concentrations of individual *Calocaris* in normoxic control conditions varied between 35.0 to 116.3 $umol.g^{-1}$ (glucosyl units; fresh weight) with a mean value of 97.3 $\pm$ 38.9 (S.D.). Haemolymph carbohydrate concentrations varied from 8.3 to 10.3 with a mean of 9.4 $\pm$ 1.1 $mmol.l^{-1}$. Glycogen comprised approximately 87% of the total tissue carbohydrate, and approximately 73% of the haemolymph carbohydrate. The remaining fractions probably represent mono- and oligosaccharides.

FIG. 6.1 Cumulative mortality (%) recorded during exposure of ***Calocaris macandreae*** to anoxia. The experiment was carried out at $10^{o}C$.

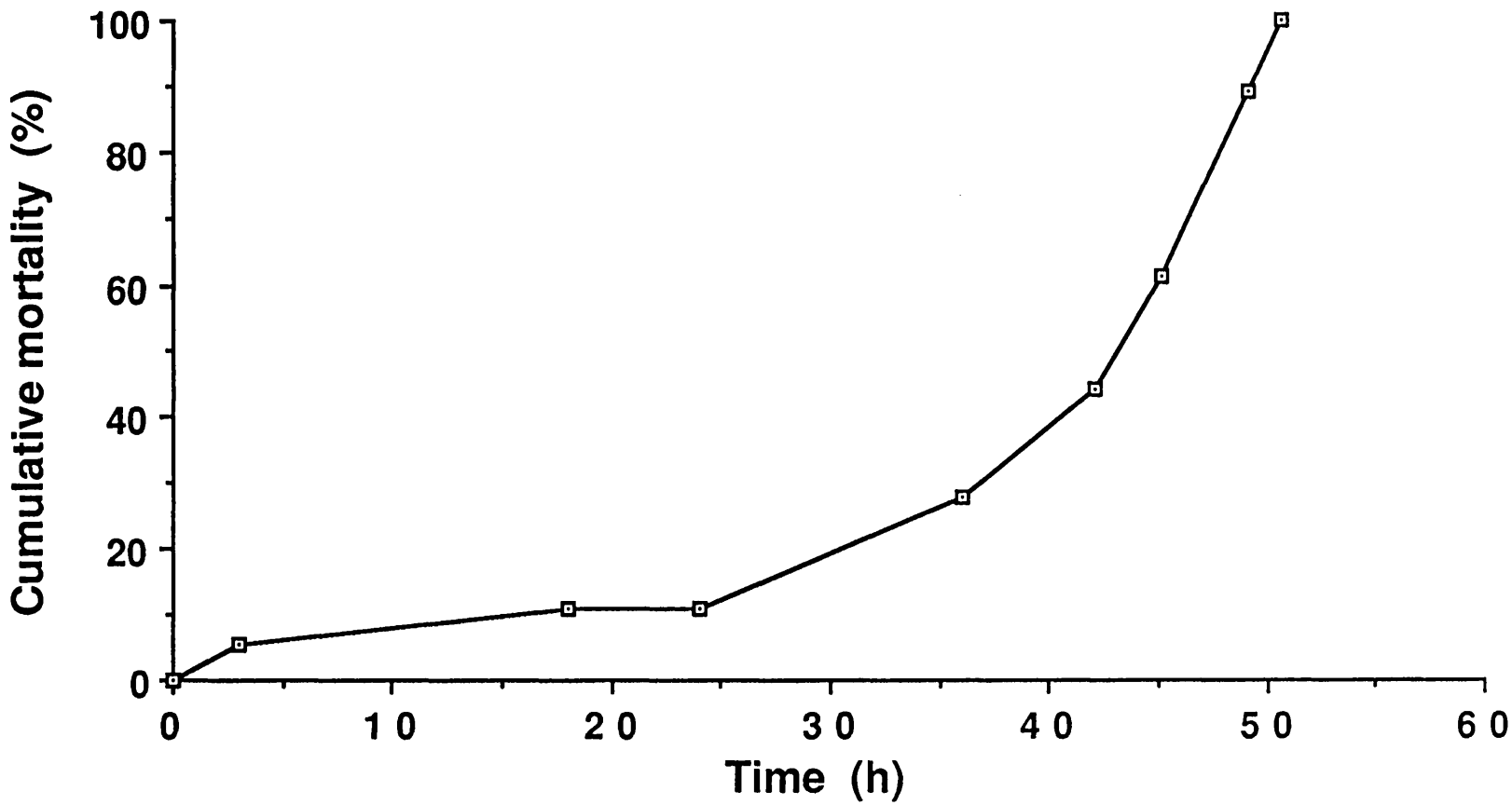
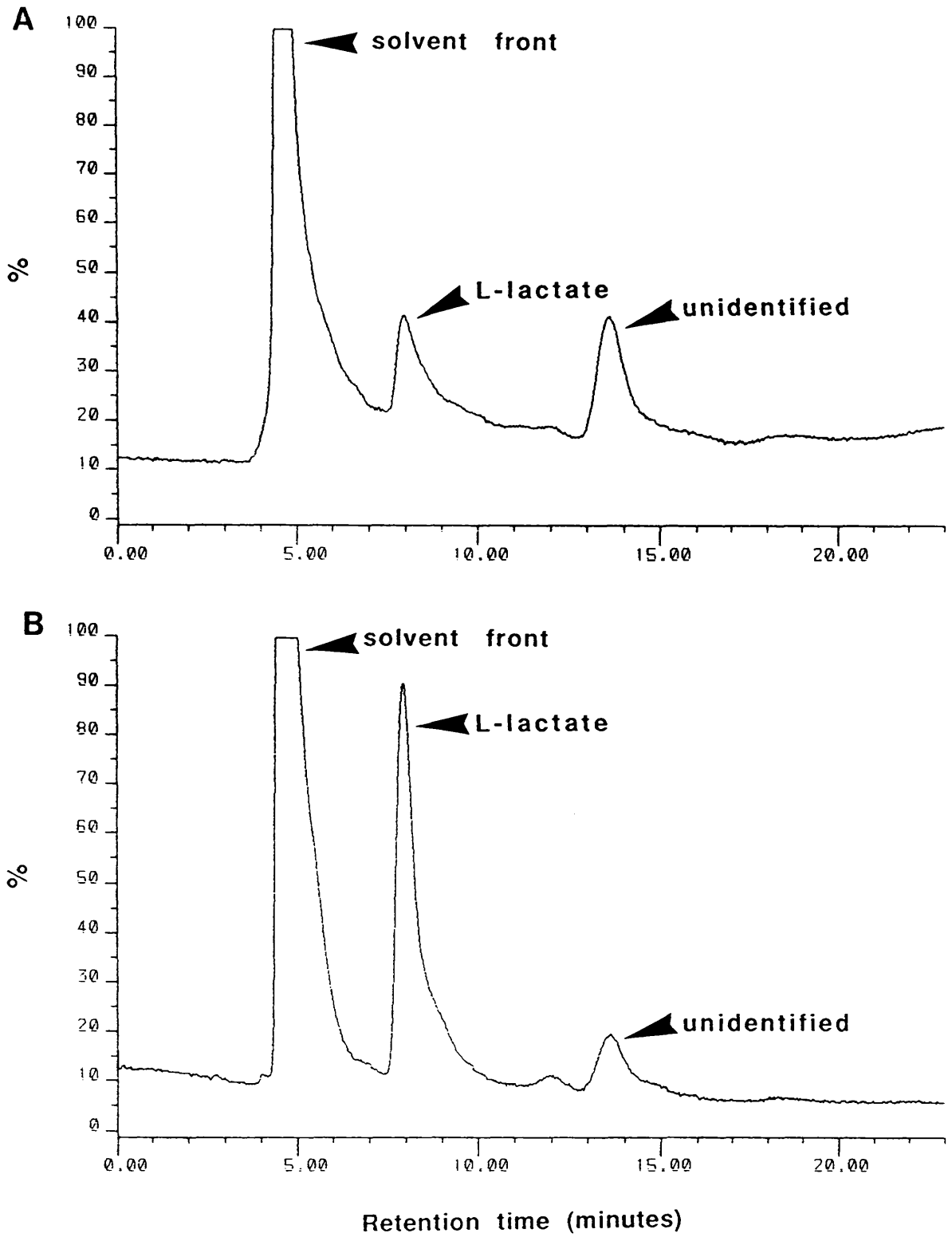

Cumulative mortality (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (h)

FIG. 6.2 HPLC traces of resolved organic acids from ***Calocaris macandreae*** exposed to A) normoxic conditions; B) 24 hours anoxia. L-lactate increased significantly in 3 replicates (t-test, $P<0.001$). The identity of the peak at RT 13.6 minutes is unknown. The y-axis scale is arbitrary.


A
100
90
80
70
60
50
40
30
20
10
0
%
solvent front
L-lactate
unidentified
0.00
5.00
10.00
15.00
20.00
B
100
90
80
70
60
50
40
30
20
10
0
%
solvent front
L-lactate
unidentified
0.00
5.00
10.00
15.00
20.00
Retention time (minutes)

Tissue L-lactate concentrations in normoxic conditions varied from <0.5 to 3.4 (mean 2.2 ± 1.6) $\mu mol.g^{-1}$. Control concentrations of haemolymph lactate were 0.9 to 3.4 (mean 1.9 ± 1.3) $mmol.l^{-1}$.

D-glucose was present in the haemolymph in concentrations ranging from 0.15 to 0.19 (mean 0.17 ± 0.02) $mmol.l^{-1}$.

No significant differences in control or experimental metabolite concentrations were noted between pre- and post-moult individuals.

6.3.3.2 Metabolite concentrations during anoxia

There was no significant change (t-test, P>0.1) in carbohydrate concentration in either total tissue samples (Fig. 6.3) or haemolymph samples (Fig. 6.4), from animals maintained for 36 hours in anoxia.

Tissue lactate concentrations showed no significant increase (P>0.1) during the initial 8 hours of anoxia, but increased significantly (P<0.001) after 8 to 36 hours. The maximum mean lactate concentration after this time was 30.2 ± 1.6 $\mu mol.g^{-1}$ (Fig. 6.5). The rate of tissue lactate accumulation from 8 to 36 hours anoxia was approximately linear (correlation coefficient, r = 0.985). The highest individual tissue lactate concentration recorded was 32.0 $\mu mol.g^{-1}$.

Haemolymph lactate concentrations similarly showed no increase over the initial (6 hour) period of anoxia (Fig. 6.6) but increased significantly (P<0.001) to a maximum of 40.2 ± 1.9 $mmol.l^{-1}$ after 36 hours.

There was a pronounced hyperglycaemic reaction in the haemolymph,

FIG. 6.3 Concentrations of carbohydrate (measured by the anthrone method) in the tissues of *Calocaris macandreae* during 36 hours anoxia. Values are means ± S.D..

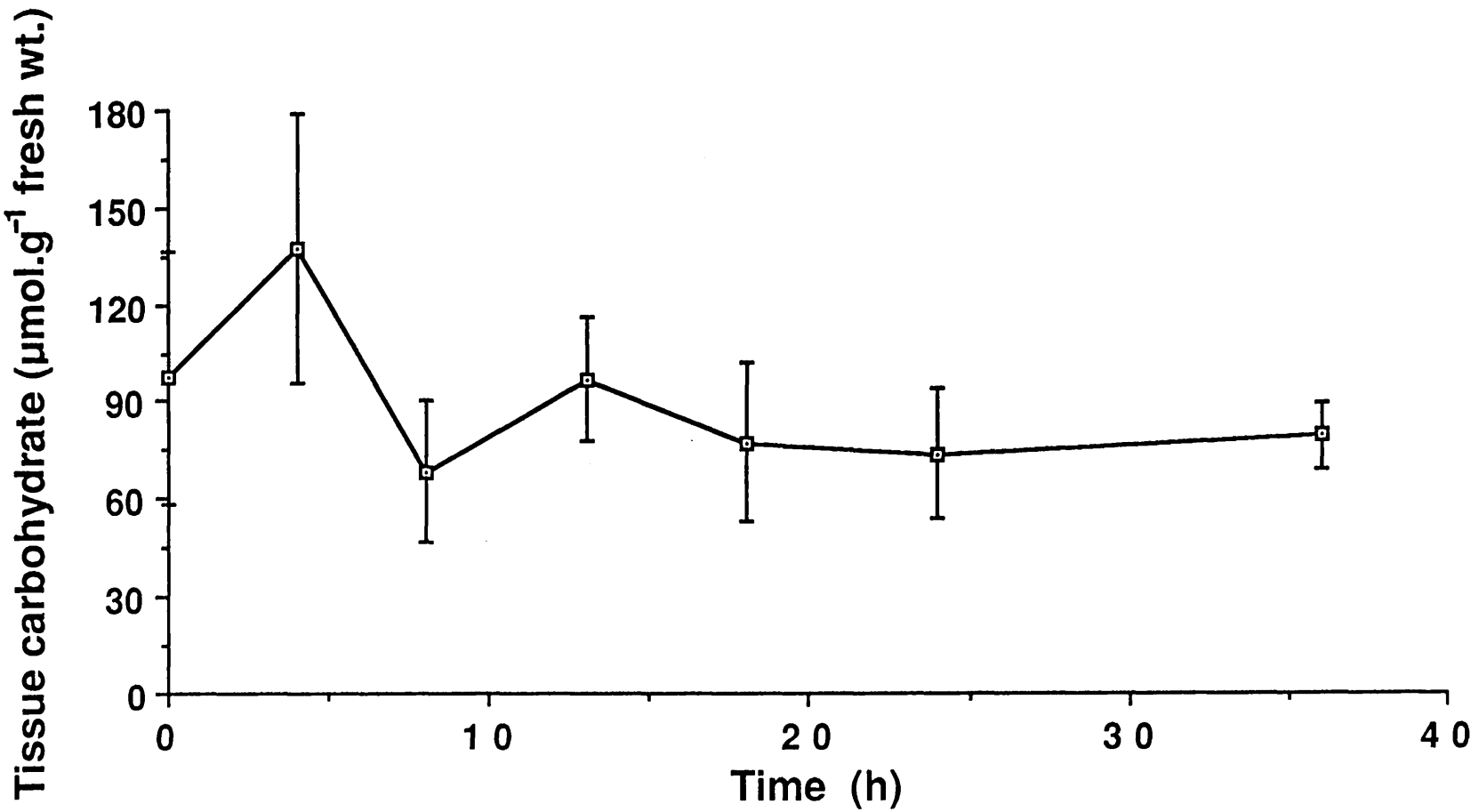

Tissue carbohydrate (µmol.g-1 fresh wt.)
180
150
120
90
60
30
0
0
10
20
30
40
Time (h)

FIG. 6.4 Concentrations of carbohydrate (measured by the anthrone method) in the haemolymph of *Calocaris macandreae* during 36 hours anoxia. Values are means ± S.D..

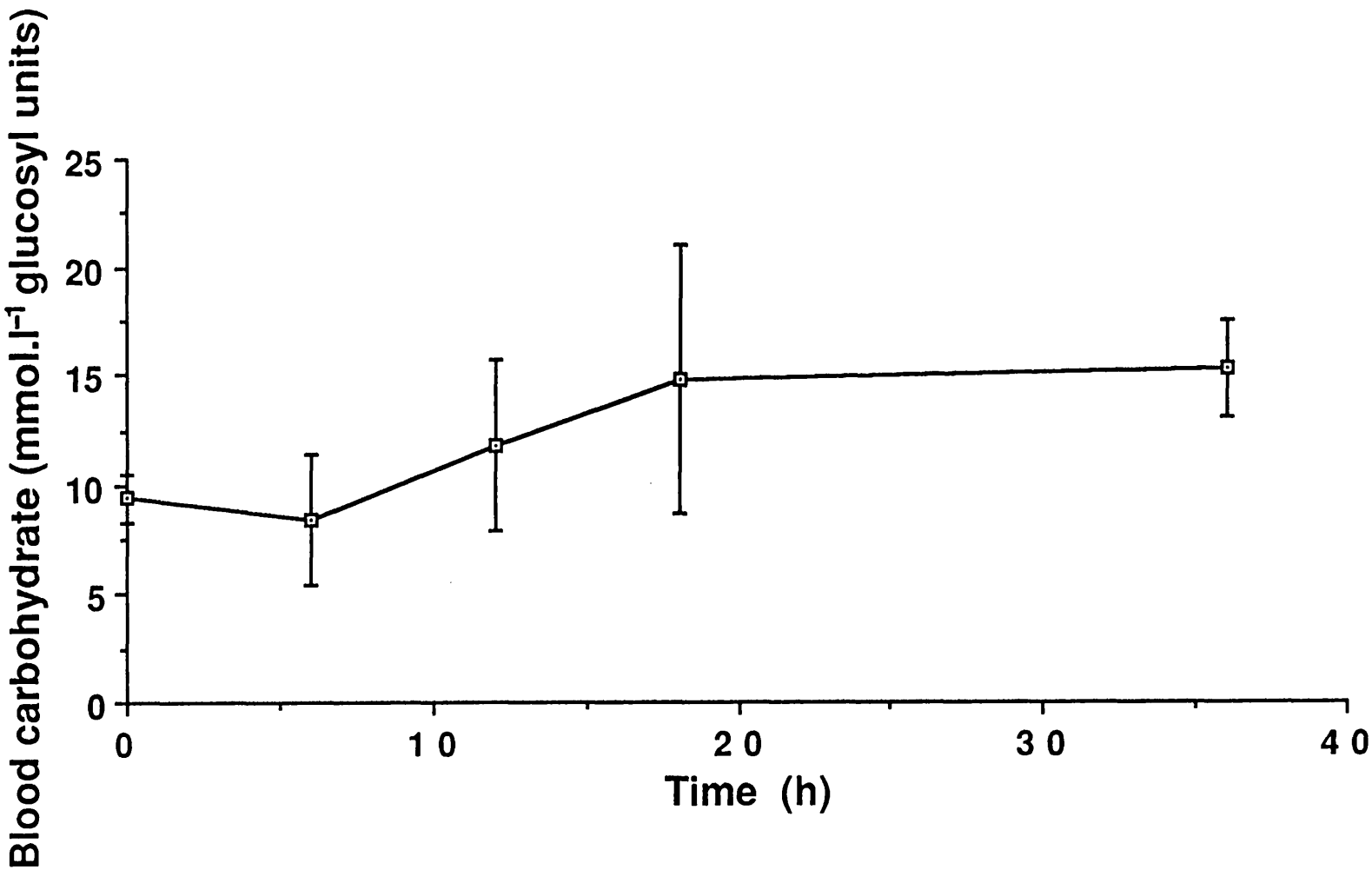

Blood carbohydrate (mmol.l-1 glucosyl units)
25
20
15
10
5
0
0
10
20
30
40
Time (h)

FIG. 6.5 Changes in the concentration of L-lactate in the tissues of *Calocaris macandreae* during 36 hours anoxia. Values are means ± S.D..

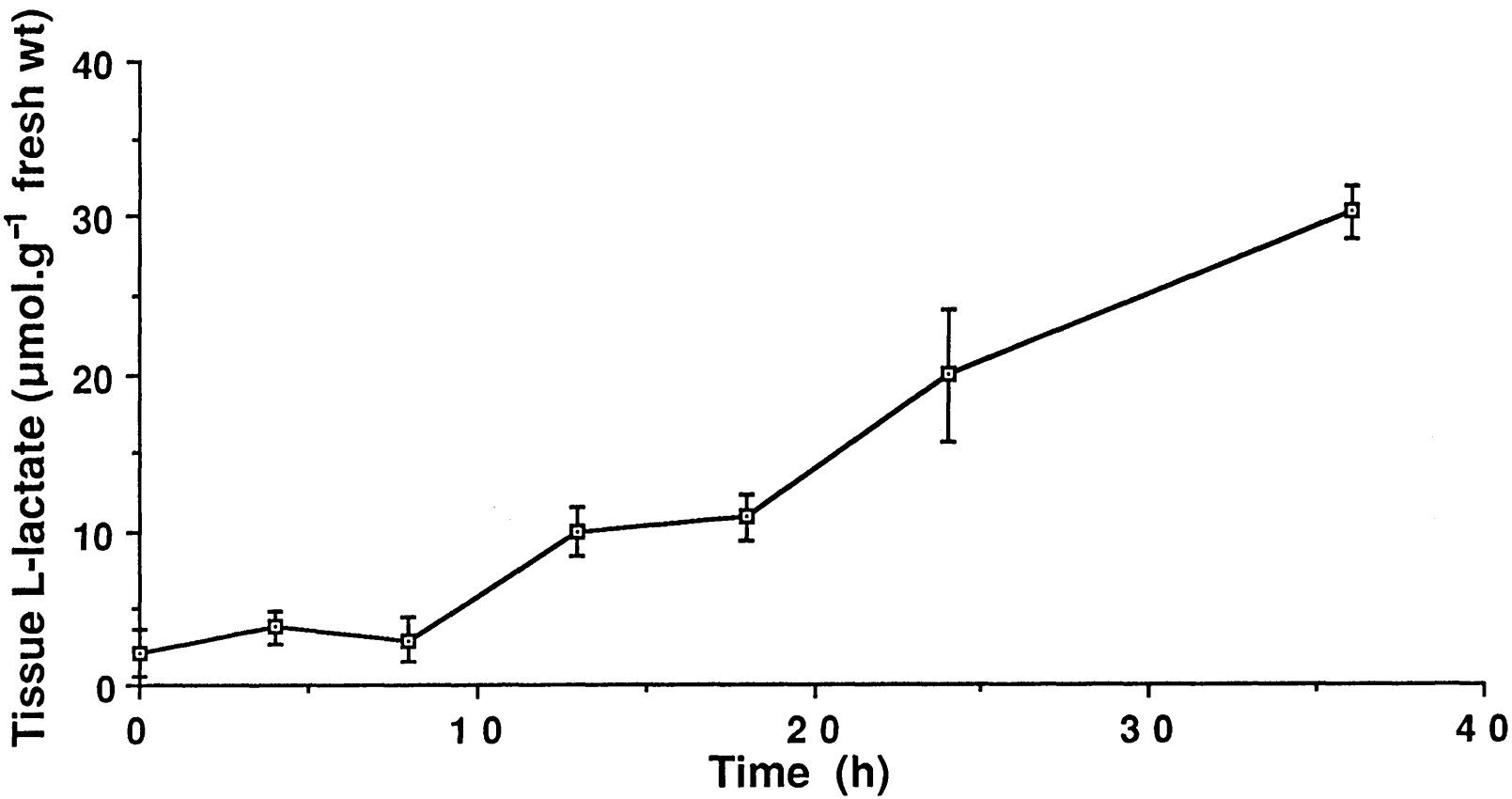

Tissue L-lactate (µmol.g^{-1} fresh wt)
40
30
20
10
0
0
10
20
30
40
Time (h)

FIG. 6.6 Changes in the concentration of L-lactate in the haemolymph of ***Calocaris macandreae*** during 36 hours anoxia. Values are means ± S.D..

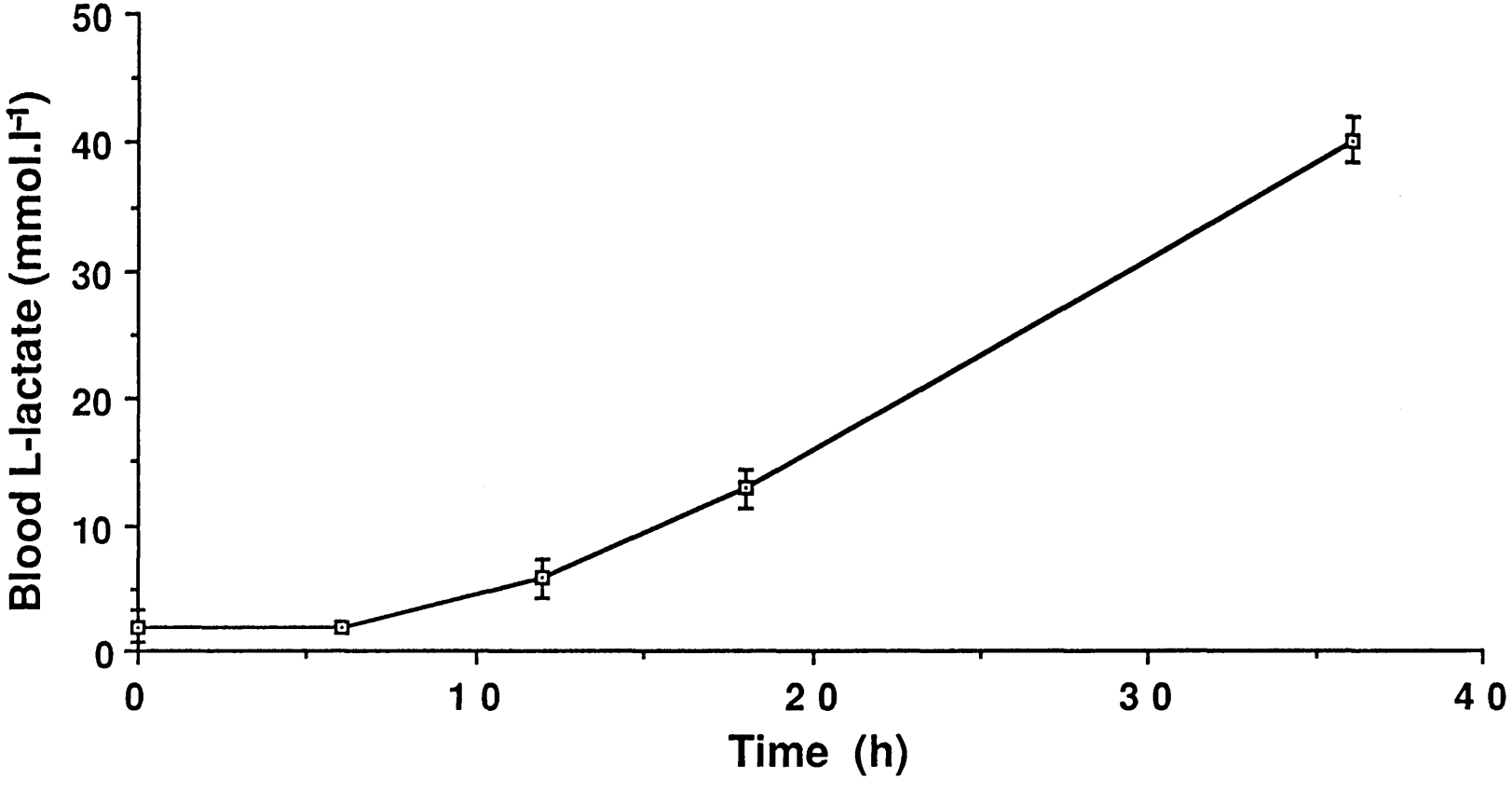
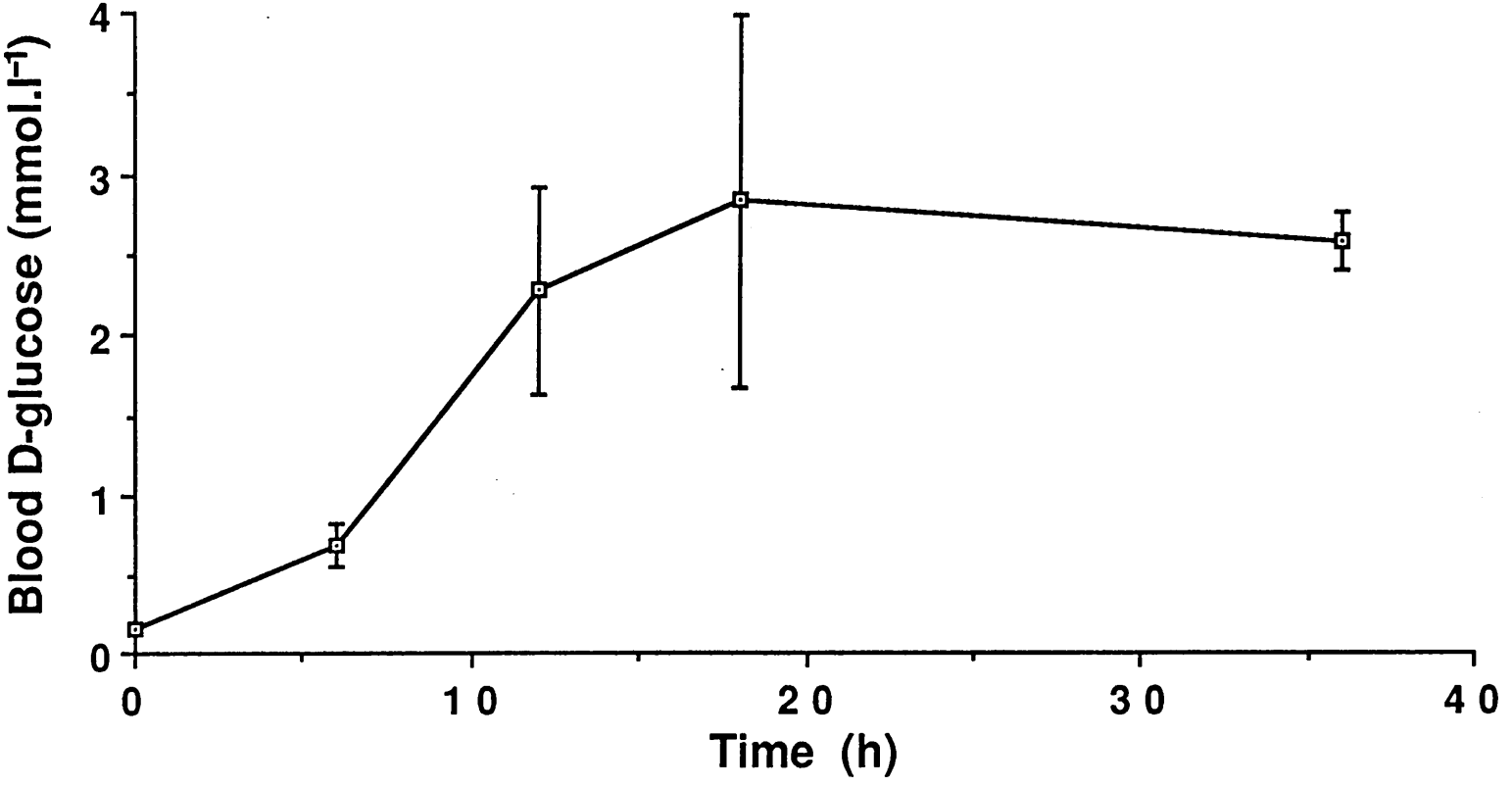

Blood L-lactate (mmol.l-1)
50
40
30
20
10
0
0
10
20
30
40
Time (h)

FIG. 6.7 Changes in the concentration of D-glucose in the haemolymph of *Calocaris macandreae* during 36 hours anoxia. Values are means ± S.D..


Blood D-glucose (mmol.l-1)
4
3
2
1
0
0
10
20
30
40
Time (h)

resulting in glucose concentrations increasing significantly ($P<0.001$) to 2.83 $\pm$ 1.15 $mmol.l^{-1}$ after 18 hours of anoxia (Fig. 6.7). This appeared to be the maximum response, however, since the glucose concentration did not change significantly ($P>0.1$) during a further 18 hours of anoxia.

6.3.3.3 Metabolite concentrations during recovery from anoxia.

Figures 6.8 and 6.9 show no significant ($P>0.1$) changes in tissue and haemolymph carbohydrate concentrations during exposure to 18 hours anoxia and subsequent 48 hours recovery in normoxic conditions.

Tissue lactate concentrations recovered from a value of 10.8 $\pm$ 1.5 $\mu mol.g^{-1}$ (18 hours anoxia) to values similar to controls after 48 hours (Fig. 6.10). Initial recovery of haemolymph lactate concentrations was more rapid with a 40% decrease (12.9 $\pm$ 1.5 to 7.8 $\pm$ 0.5 $mmol.l^{-1}$) during the first hour. The subsequent recovery occurred more slowly with concentrations reaching control values ($P>0.1$) after about 40 hours (Fig. 6.11).

There was a dramatic decrease in glucose concentration in the haemolymph during the first hour of recovery, when the glucose concentration decreased by 68% to 0.84 $\pm$ 0.14 $mmol.l^{-1}$ (Fig. 6.12). Recovery to values not significantly different from controls had occurred within 24 hours in normoxic water.

6.3.4 Lactate accumulation during anoxia.

The total-tissue lactate concentration after 24 hours of exposure to various degrees of hypoxia is shown in Fig. 6.13. There was no significant increase in lactate when PO_2 decreased from normoxic levels

FIG. 6.8 Changes in the concentration of carbohydrate (measured by the anthrone method) in the tissues of ***Calocaris macandreae*** during 18 hours anoxia followed by 48 hours recovery in normoxic conditions. Values are means ± S.D..

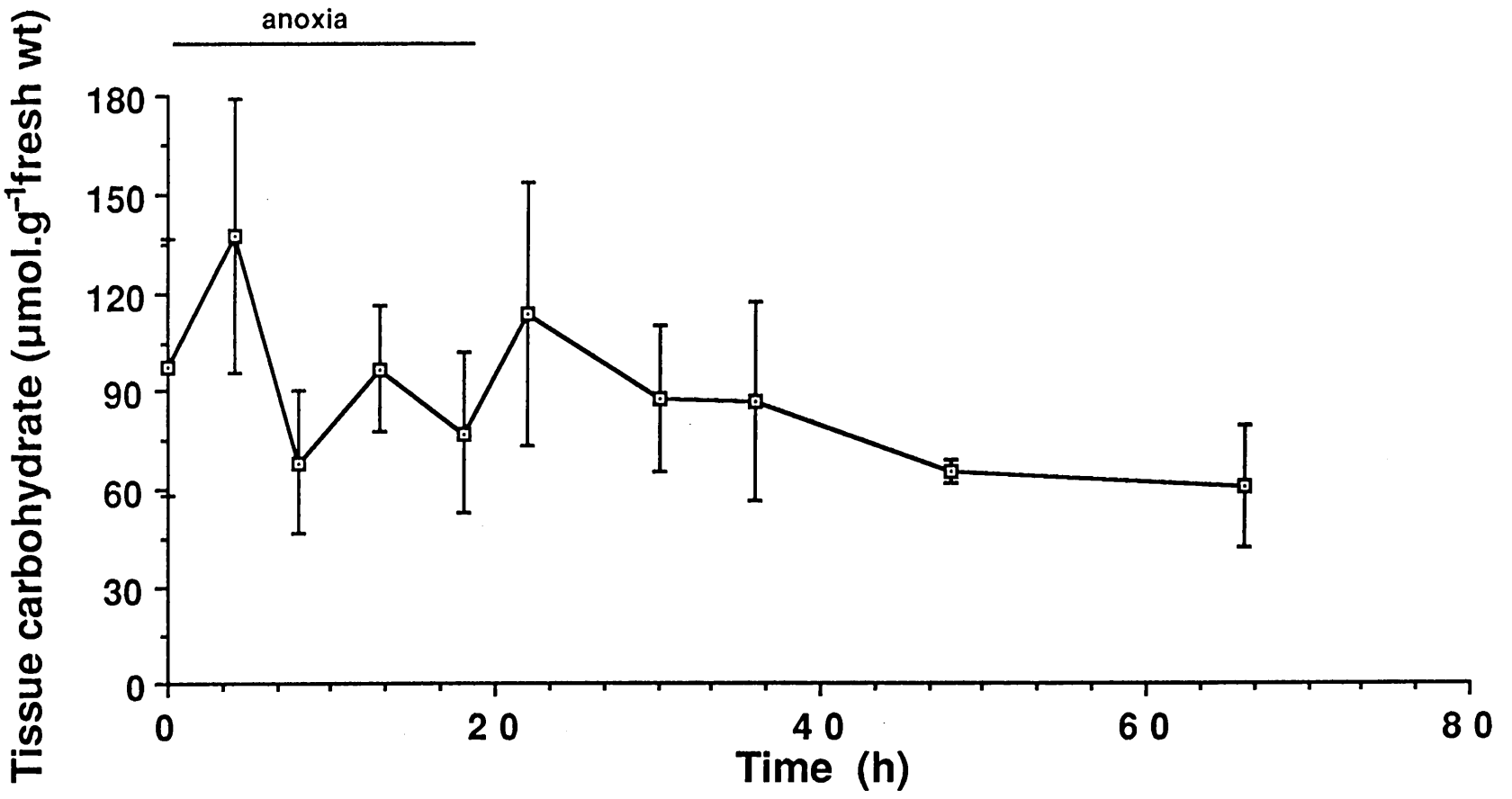

anoxia
Tissue carbohydrate (µmol.g-1fresh wt)
180
150
120
90
60
30
0
0
20
40
60
80
Time (h)

FIG. 6.9 Changes in the concentration of carbohydrate (measured by the anthrone method) in the haemolymph of ***Calocaris macandreae*** during 18 hours anoxia followed by 48 hours recovery in normoxic conditions. Values are means ± S.D..

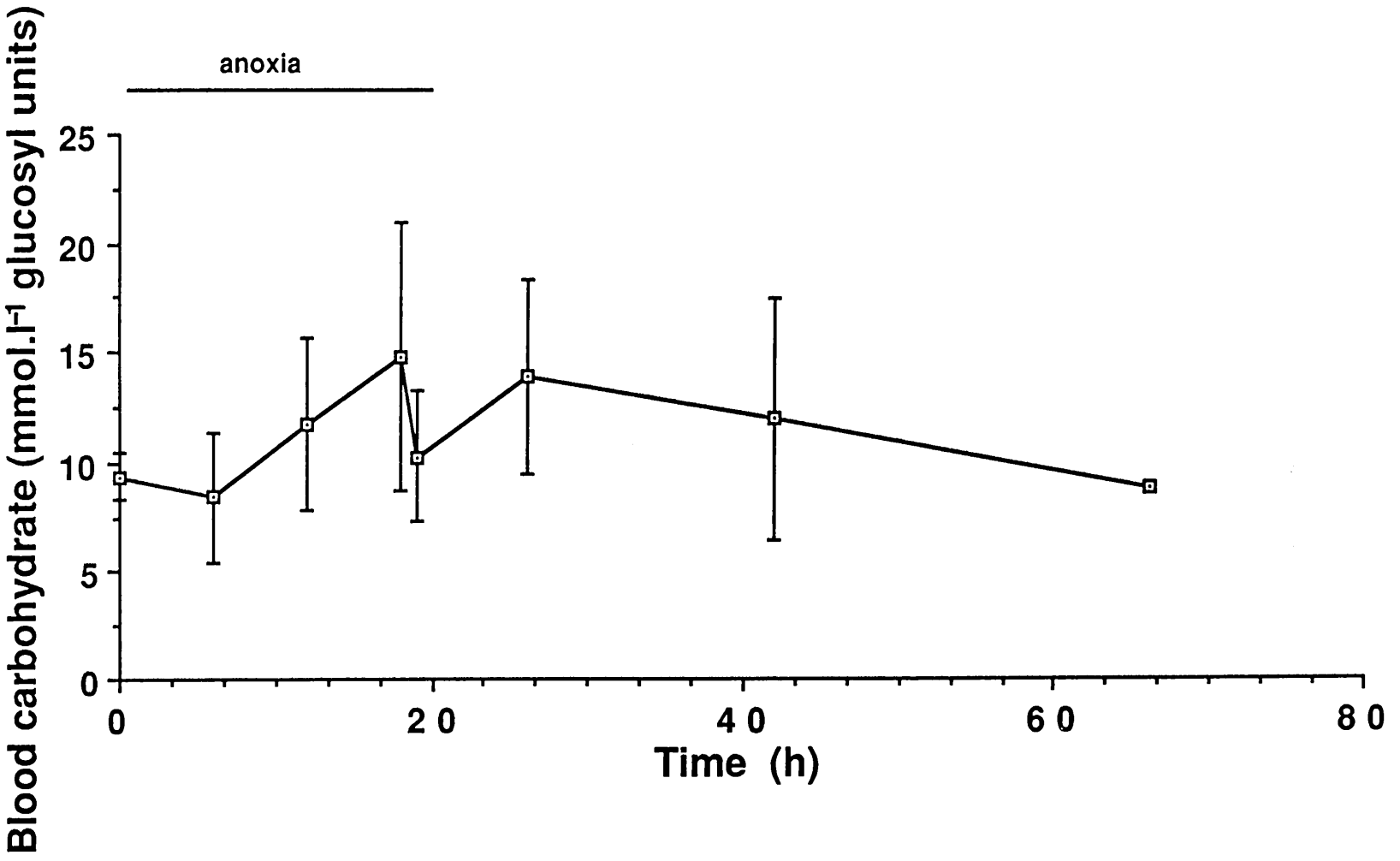

anoxia
Blood carbohydrate (mmol.l-1 glucosyl units)
25
20
15
10
5
0
0
20
40
60
80
Time (h)

FIG. 6.10 Changes in the concentration of L-lactate in the tissues of ***Calocaris macandreae*** during 18 hours anoxia followed by 48 hours recovery in normoxic conditions. Values are means $\pm$ S.D..

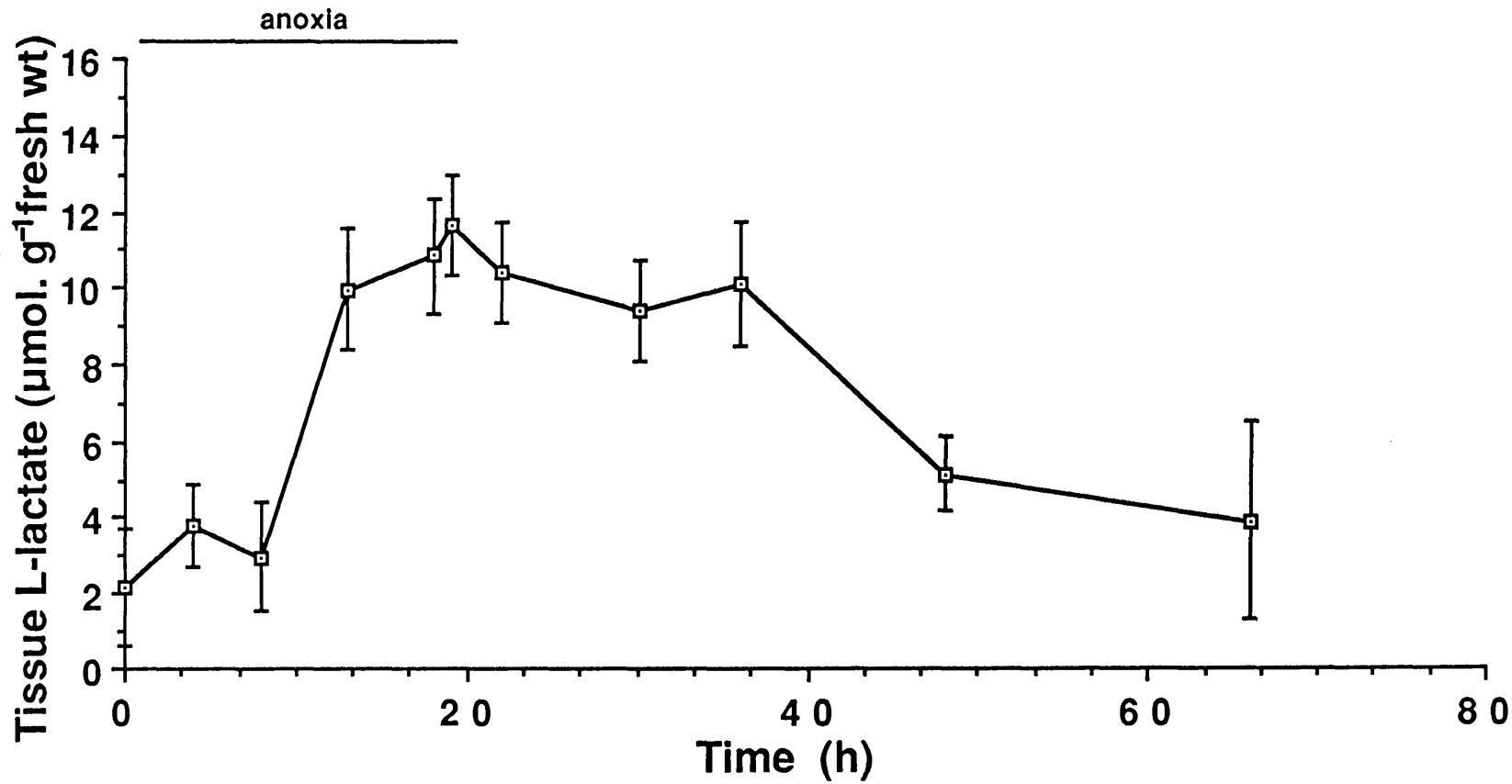

anoxia
Tissue L-lactate (µmol. g-1fresh wt)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
Time (h)

FIG. 6.11 Changes in the concentration of L-lactate in the haemolymph of *Calocaris macandreae* during 18 hours anoxia followed by 48 hours recovery in normoxic conditions. Values are means ± S.D..

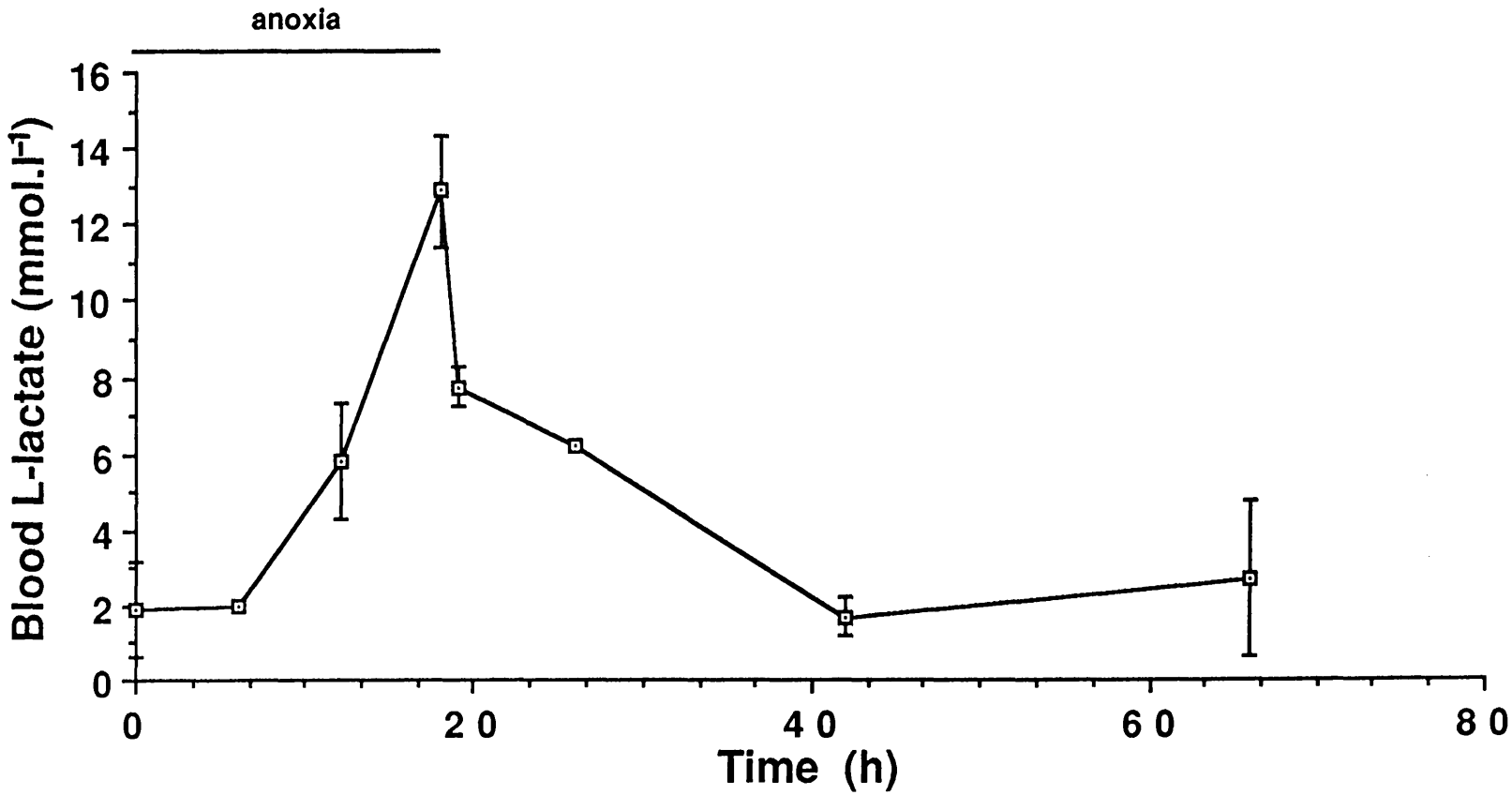
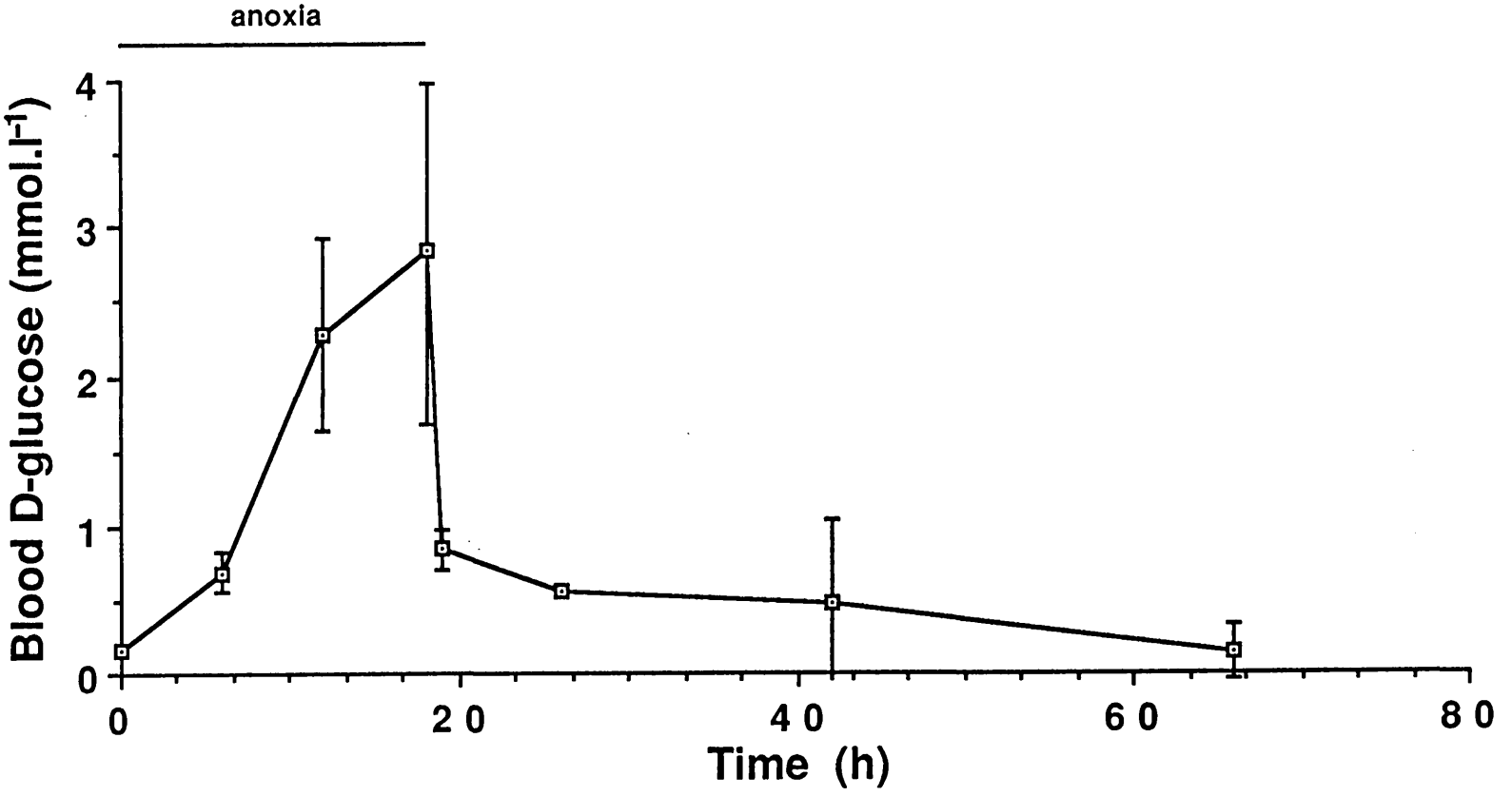

anoxia
Blood L-lactate (mmol.l-1)
16
14
12
10
8
6
4
2
0
0
20
40
60
80
Time (h)

FIG. 6.12 Changes in the concentration of D-glucose in the haemolymph of *Calocaris macandreae* during 18 hours anoxia followed by 48 hours recovery in normoxic conditions. Values are means ± S.D..


anoxia
Blood D-glucose (mmol.l-1)
4
3
2
1
0
0
20
40
60
80
Time (h)

to 6.7 Torr. Below this PO_2, lactate increased significantly (t-test, $P<0.001$). However, the lactate concentration was significantly less at 2.7 Torr (6.82 $\mu mol.g^{-1}$) than at 0 Torr (19.85 $\mu mol.g^{-1}$).

6.3.5 Lactate dehydrogenase activity.

The effects of substrate concentration on LDH activity of abdominal muscle homogenate are shown as a Lineweaver-Burke plot in Fig. 6.14. K_m values for pyruvate and NADH are 0.50 and 0.19 $mmol.l^{-1}$ respectively. V_{max} can be estimated from Fig. 6.14 to be 43.8 $umol.min^{-1}.g^{-1}$ (fresh weight) (= 43.8 $units.g^{-1}$). No LDH activity could be detected in homogenates of gill tissue.

FIG. 6.13 The relationship between lactate accumulation (over 24 hours) and environmental oxygen tension in ***Calocaris macandreae***. See text for experimental details.

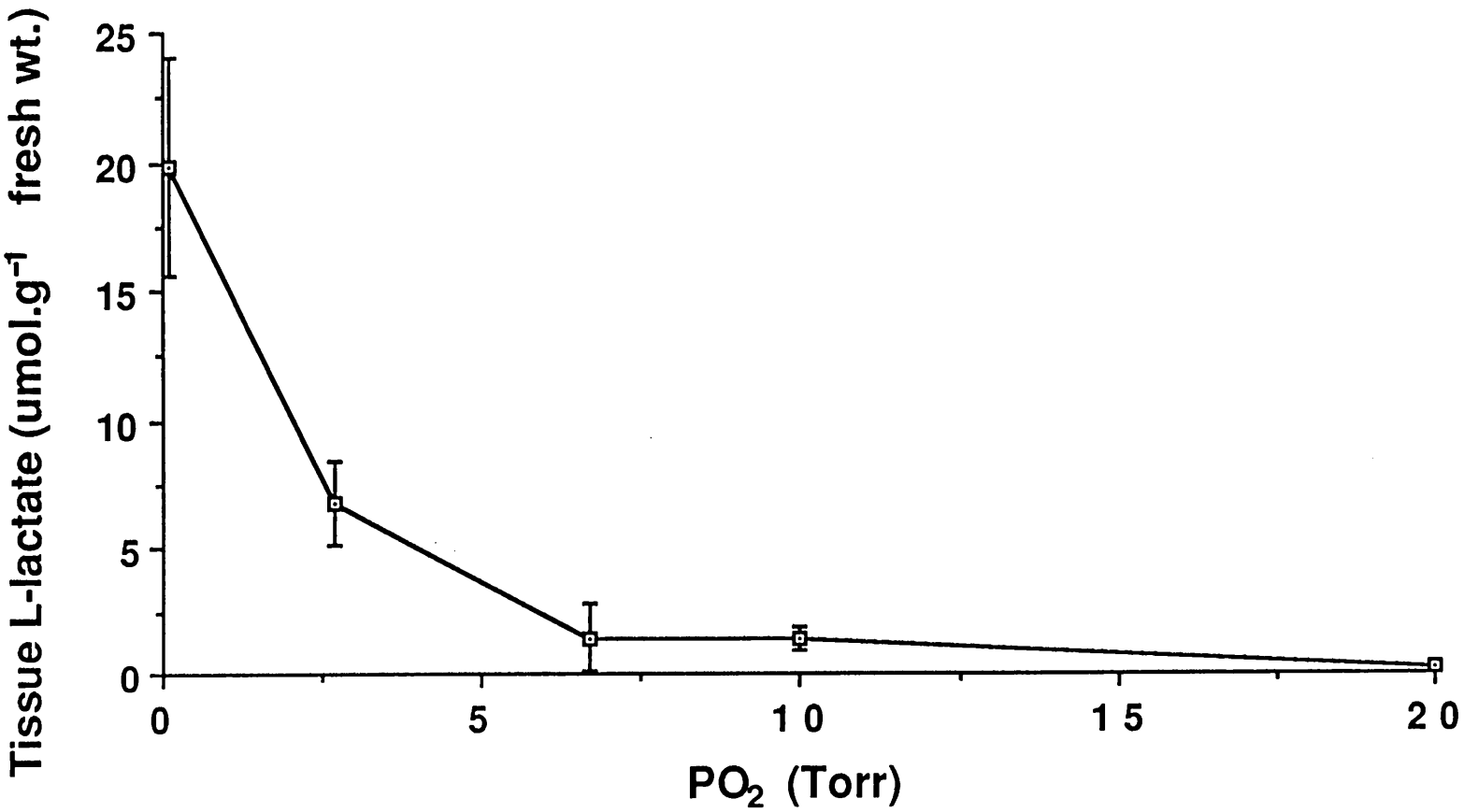

Tissue L-lactate (umol.g-1 fresh wt.)
25
20
15
10
5
0
0
5
10
15
20
PO2 (Torr)

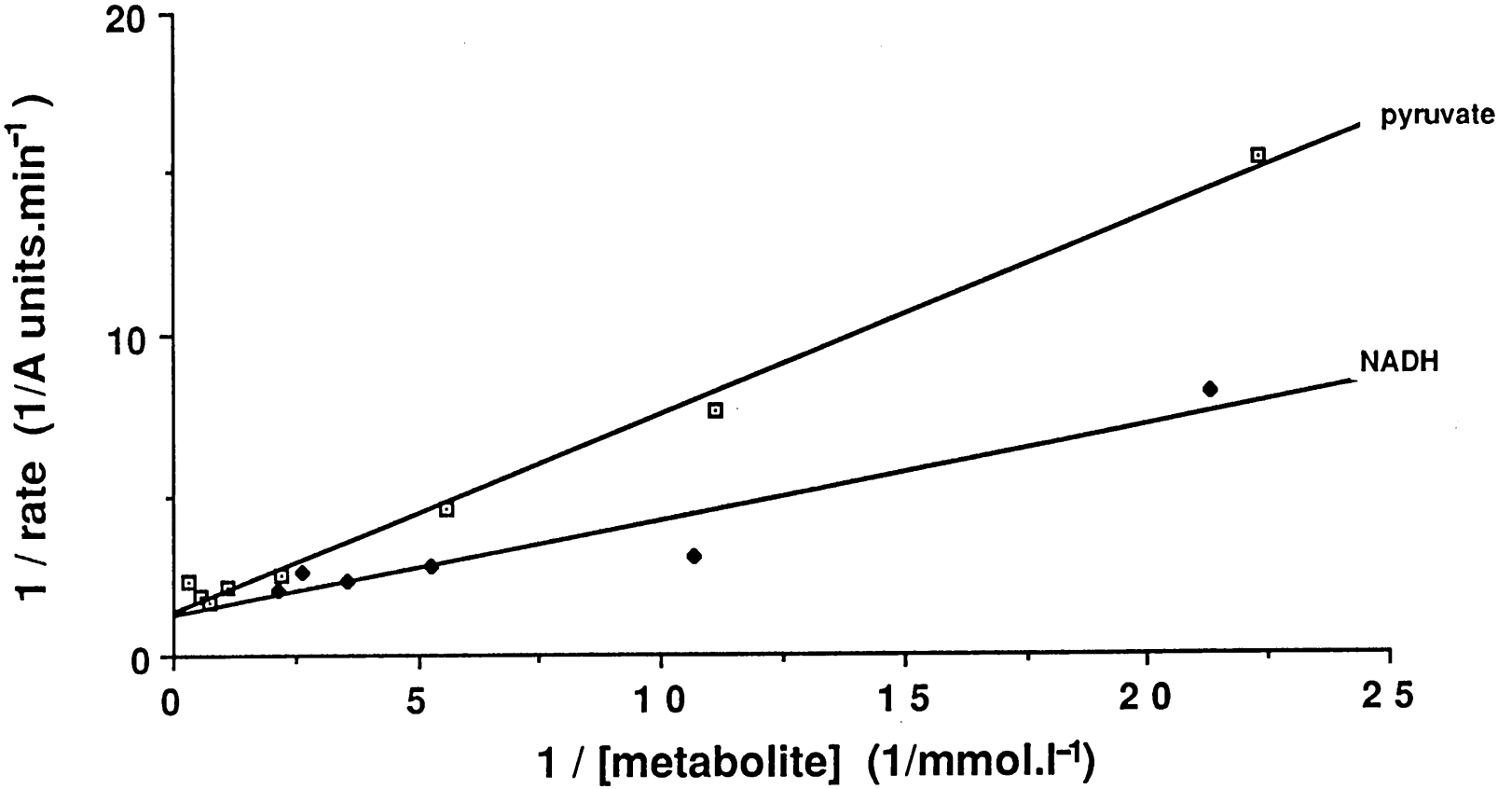
FIG. 6.14 Lineweaver-Burke plot of the relationship between substrate concentration and rate of NADH oxidation by lactate dehydrogenase in homogenised abdominal muscle of ***Calocaris macandreae***. The equations of the regression lines are:

pyruvate: $y = 1.43 + 0.61x$ ($r = 1.00$; $P<0.001$)

NADH: $y= 1.29 + 0.29x$ ($r = 0.95$; $P<0.01$)

Calculated $V_{max} = 43.8\ \mu mol.min^{-1}.g^{-1}$.

K_m values for pyruvate and NADH are 0.50 and 0.19 $mmol.l^{-1}$ respectively.


20
10
0
0
5
10
15
20
25
1 / rate (1/A units.min^{-1})
1 / [metabolite] (1/mmol.l^{-1})
pyruvate
NADH

6.4 DISCUSSION

6.4.1 Comparative aspects of anaerobic metabolism in Calocaris macandreae.

6.4.1.1 Survival in anoxia.

The survival time of ***Calocaris macandreae*** (expressed as LT_{50}) was determined to be 43 hours at 10°C, although individual animals survived for up to 49.5 hours. Previously reported survival times in anoxia of ***Callianassa californiensis*** are 138 and 52 - 60 hours (Thompson and Pritchard, 1969 and Zebe, 1982 respectively). The survival of ***Callianassa jamaicense*** in anoxia was estimated at 80 hours (Felder, 1979); while ***C. japonica*** was studied by Mukai and Koike (1984: maximum survival < 63 hours; LT_{50} 40 h). There are very few comparative data for other decapods in the literature, although the LT_{50} for ***Carcinus maenas*** is much shorter, at approximately 14 h (A.D. Hill, unpub. obs.).

6.4.1.2 Anaerobic metabolism in *Calocaris macandreae* and other thalassinids.

Only two previous studies of anaerobic metabolism in thalassinid species have been published (Pritchard and Eddy, 1979; Zebe, 1982), in addition to an unpublished MS thesis (Hawkins 1970). All three studies have considered the American species ***Callianassa californiensis*** and ***Upogebia pugettensis***. There is, however, a larger literature concerning anaerobiosis in brachyuran crabs and to a lesser extent other decapods. Both environmental (i.e. resulting from environmental anoxia or hypoxia) and functional (i.e. resulting from exercise beyond the capacity of the aerobic system) anaerobiosis have been extensively studied.

Pritchard and Eddy (1979) measured haemolymph L-lactate and tissue glycogen concentrations in ***Callianassa californiensis*** and ***Upogebia***

pugettensis during exposure to 20 hours anoxia. Hawkins (1970) measured the same metabolites in *C. californiensis* together with haemolymph glucose concentration (over a time period of 30 hours). Zebe (1982) measured a variety of tissue metabolite concentrations (including glycogen, lactate and glucose) during 24 hours exposure to anoxia in both species. In the present study, lactate and glycogen concentrations were measured in the tissue and haemolymph of *Calocaris macandreae*, together with the concentration of haemolymph glucose, during 36 hours of anoxia, and 18 hours of anoxia followed by 48 hours of recovery in normoxic conditions.

The pattern of total tissue lactate accumulation during 24 hours anoxia described by Zebe (1982) in *Callianassa californiensis* is very similar to that found in *Calocaris*. However, the rate of accumulation was slightly lower (0.7 $\mu mol.g^{-1}.h^{-1}$; calculated from Zebe's data) in *C. californiensis*. The final (24 hour) lactate concentration in *C. californiensis* was 16.8 µmol.g-1 compared with 19.85 $\mu mol.g^{-1}$ in *Calocaris*. In contrast, the rate of lactate accumulation in *Upogebia pugettensis* was considerably higher (1.52 $\mu mol.g^{-1}.h^{-1}$ over 24 hours) resulting in a final concentration of 36.4 $\mu mol.g^{-1}$. Zebe (1982) also quotes individual lactate values after "maximum periods of anoxia" (presumably 24 hours) of 20 and 50-60 $\mu mol.g^{-1}$ in *C. californiensis* and *U. pugettensis* respectively. Most studies of lactate accumulation in decapods have found an approximately linear increase during anoxia. From Zebe's values for lactate accumulation rate and survival time in *C. californiensis*, the final lactate concentration after lethal exposure to anoxia would be approximately 35-40 $\mu mol.g^{-1}$. The mean tissue lactate levels accumulated during a near-lethal exposure to anoxia in all three thalassinid species would then be very similar (*C.*

californiensis 35-40 $\mu mol.g^{-1}$ /60 h; *U. pugettensis* 35 $\mu mol.g^{-1}$ /24 h; *Calocaris* 30 $\mu mol.g^{-1}$ / 36 h).

The accumulation of lactate in the haemolymph of *C. californiensis* and *U. pugettensis* during exposure to anoxia has been measured by Hawkins (1970) and by Pritchard and Eddy (1979). The results given by these authors have been re-calculated from the authors' original units (i.e. mg lactate/ 100 ml haemolymph) to $mmol.l^{-1}$, in order to allow comparison with results from this study. Hawkins found that haemolymph lactate concentration increased linearly over 30 hours to a final value of 30.3 $mmol.l^{-1}$ in *C. californiensis*, although Pritchard and Eddy reported a lower rate of accumulation, with concentrations of only 5.5 $mmol.l^{-1}$ in the same species after 20 hours (compared with 18.8 $mmol.l^{-1}$ interpolated from Hawkins' data). Pritchard and Eddy also measured haemolymph lactate concentration in *U. pugettensis* and reported higher values of 39.2 $mmol.l^{-1}$ after 20 hours. These values compare to interpolated levels of 17 $mmol.l^{-1}$ / 20 h and 32 $mmol.l^{-1}$ / 30 h in *Calocaris macandreae*. Thus the rate of haemolymph lactate accumulation in *Calocaris* appears very similar to that obtained by Hawkins for *C. californiensis*, but intermediate between those recorded for *C. californiensis* and *U. pugettensis* by Pritchard and Eddy. These comparisons are summarised in the following table:

TABLE 6.1: Rates of accumulation of L-lactate ($mmol.l^{-1}.h^{-1}$) in the haemolymphs of *Calocaris macandreae*, *Callianassa californiensis* and *Upogebia pugettensis*.

Species	20h	30h
Calocaris (present study)	0.85	1.06
Callianassa (Hawkins 1970)	0.94	1.01
Callianassa (Pritchard and Eddy 1979)	0.28	-
Upogebia (Pritchard and Eddy 1979)	1.96	-

There is no evidence from the study of Hawkins (1970), or from the present study, for a saturation effect of haemolymph lactate as was found by Pritchard and Eddy (1979) in *C. californiensis*. This effect has been found in other decapods (*Palaemon elegans* and *P. serratus*: Taylor and Spicer, 1987; *Carcinus maenas* and *Nephrops norvegicus*: A.D. Hill, pers.comm.). In addition, there is no consistent 'lethal limit' concentration of lactate in the haemolymph, as was apparent in total tissue lactate between the three species (above).

Both Hawkins (1970) and Pritchard and Eddy (1979) found considerable individual variation in tissue glycogen concentrations in *C. californiensis* and *U. pugettensis*, and had some difficulty in demonstrating glycogen utilisation during anoxia. Pritchard and Eddy concluded that "there is no net utilization of glycogen under short-term anoxic conditions", although Hawkins had previously found significant glycogen depletion in *C. californiensis* hepatopancreas and chela muscle after 31 hours anoxia. Zebe (1982) also found utilisation of total tissue glycogen although he used very small samples (n = 4) and gave no indication of individual variation. The present study failed to demonstrate any significant change in tissue or haemolymph carbohydrate during anoxia or recovery. This can be partly attributed to the large variation in glycogen concentration measured in different individuals.

Zebe (1982) found that total tissue D-glucose increased from 0.25 to 0.64 µmol.g-1 in *C. californiensis* and 0.29 to 1.39 in *U. pugettensis* after 12 hours exposure to anoxia. There was a subsequent decrease during the following 12 hours. In *Calocaris*, concentrations of D-glucose in the tissue were below the reliable limits of spectrophotometric assay (i.e. < 0.5 µmol.g^{-1}). There was a

hyperglycaemic response in the haemolymph, however, which was very similar to the pattern of hyperglycaemia found by Hawkins (1970) in *C. californiensis*. Hawkins found a significant increase over 13 hours of anoxia to a maximum value of 2.36 mmol.l^{-1}, with no subsequent increase after 30 hours (although his results are based on only two sampling times). A similar increase in haemolymph glucose concentrations was found in *Calocaris*, with an initial accumulation of 2.83 mmol.l^{-1} after 18 hours not followed by a further increase (over 36 hours). All the studies also recorded a large individual variation in haemolymph glucose concentrations and this appears to be typical of crustacean haemolymph (e.g. Dean and Vernberg, 1965; Telford, 1968; Taylor and Spicer, 1987).

The only previous study of metabolic recovery from anoxic stress in a thalassinid species is Hawkins (1970), for *C. californiensis*. Both haemolymph lactate and glucose concentrations show different recovery patterns in *C. californiensis* from those observed in *Calocaris*, although the concentrations were similar during anoxic exposure. Lactate concentrations in *C. californiensis* showed a non-significant increase during the first 1.5 hours of recovery followed by a slow decrease, without reaching control levels after 10 hours (the limit of Hawkins' study). In contrast, there was a rapid (39.9%) recovery of lactate concentrations in *Calocaris* over the first hour, with lactate reaching control concentrations after c.40 hours. An almost linear recovery of haemolymph glucose concentrations in *C. californiensis* takes place over approximately 10 hours, whereas recovery in *Calocaris* is very rapid, being 70% complete within 1 hour. In both species, however, recovery of haemolymph metabolites is essentially complete within a recovery time period only slightly longer than the anoxic

exposure. This is in contrast to recovery of total tissue lactate concentrations in *Calocaris*, which is not complete until over 2.5 times the anoxic period.

The overall pattern of anaerobic metabolism in *Calocaris macandreae* therefore appears consistent with the limited existing data for other thalassinid species. There is close similarity between the results of the present study and those of Hawkins (1970) and Zebe (1982) for *C. californiensis*, in terms both of the concentrations and the rates of accumulation/ depletion of metabolites (with the possible exception of glycogen depletion). If the results of Pritchard and Eddy (1979) are accepted, however, they would indicate that *C. californiensis* has a considerably greater capacity for anaerobiosis. Both Pritchard and Eddy, and Zebe found much less anaerobic ability in *Upogebia pugettensis* than in either *Callianassa californiensis* or *Calocaris*. These results will be discussed in terms of functional and ecological significance below.

6.4.1.3 Comparison with other decapod Crustaceans.

There are several studies which have investigated environmental anaerobiosis in decapod Crustacea. These studies have measured total tissue metabolites (Teal and Carey, 1967; Spotts, 1983); haemolymph metabolites (Burke, 1979; Bridges and Brand, 1980; Mauro and Malecha, 1984; Lowery and Tate, 1986; van Aardt and Wolmarans, 1987); or both (Gäde, 1984; Albert and Ellington, 1985; Taylor and Spicer, 1987).

The following table summarises data taken from the literature, for concentrations and rates of accumulation of lactate in the tissue and haemolymph of several crustacean species. Although experimental procedures (particularly temperatures) varied between examples, there

is general consistency between the results. It should be noted that tissue lactate concentration may be measured in dissected tissues (Gäde, 1984; Albert and Ellington, 1985) or in homogenised total tissue (Teal and Carey, 1967; Zebe, 1982; Spotts, 1983; Taylor and Spicer, 1987; present study). Although lactate concentrations in dissected tissues are likely to be greater than in homogenised total tissue, the results of these two methods appear to be comparable.

TABLE 6.2: Maximum concentrations and rates of accumulation of L-lactate in the tissues and haemolymphs of selected decapod Crustacea.

TISSUE Species	Maximum conc.*	Rate**	Reference
Macrobrachium rosenbergii	5.32/4	1.29	Spotts, 1983
Menippe mercenaria	16-20/12	1.5	Albert and Ellington, 1985
Orconectes limosus	19.3/12	1.5	Gäde, 1984
Palaemon elegans	16.7/3	3.97	Taylor and Spicer, 1987
Palaemon serratus	9.6/1	5.6	"
Uca pugnax	40/25	2.2	Teal and Carey, 1967
THALASSINIDS			
Upogebia pugettensis	6.4/24	1.52	Zebe (1982)
Callianassa californiensis	16.8/24	0.7	"
Calocaris macandreae	30.2/36	0.9	Present study

HAEMOLYMPH Species	Maximum conc.*	Rate**	Reference
Corystes cassivelaunus	8.63/5	1.73	Bridges and Brand, 1980
Galathea strigosa	7.13/5	1.46	"
Macrobrachium rosenbergii	12/2	5.85	Mauro and Malecha, 1984
Orconectes limosus	60/16	3.75	Gäde, 1984
Menippe mercenaria	30-50/12	3.33	Albert and Ellington, 1985
Potamon warreni (aerial)	34.78/6	5.7	van Aardt and Wolmarans, 1987
Palaemon elegans	13/4	2.98	Taylor and Spicer, 1987
Palaemon serratus	6.93/1	6.35	"
THALASSINIDS			
Upogebia pugettensis	39.2/20	1.96	Pritchard and Eddy (1979)
Callianassa californiensis	30.3/30	0.94	Hawkins (1970)
Calocaris macandreae	40.18/36	0.85	Present study

* maximum recorded mean value / experimental period ($\mu mol.g^{-1}$ / h)
** rate of increase ($\mu mol.g^{-1}$ (fresh wt.).h^{-1} or $mmol.l^{-1}.h^{-1}$)
(nb. takes account of initial concentrations)

The rates of tissue lactate accumulation during environmental anaerobiosis (i.e. in anoxia or severe hypoxia, below the Pc) varied from 1.29 to 5.6 $\mu mol.g^{-1}.h^{-1}$ in non-thalassinid decapods. The maximum lactate concentration recorded (not necessarily the maximum produced) was between 9.6 and 20 $umol.g^{-1}$. ***Callianassa californiensis*** and ***Calocaris macandreae*** are characterised by slow rates of accumulation of lactate, and high final concentrations. However, lactate accumulation rates and final concentrations in ***Upogebia pugettensis*** are similar to those recorded in non-thalassinids. A similar comparison is evident if haemolymph lactate is considered. It should be noted, however, that most of the species studied have been selected because of their ecological tolerance of hypoxic/anoxic environments; thus ***Menippe mercenaria*** and ***Orconectes limosus*** probably represent the known adaptive limits of the brachyuran crabs and astacid crayfish respectively, and both occupy habitats which experience regular hypoxic events (Albert and Ellington, 1985; Gäde, 1984). Possibly the only crustacean species so far studied which does not experience environmental oxygen depletion, is ***Palaemon serratus*** (Taylor and Spicer, 1987); this species shows a rapid accumulation of lactate and a low maximum concentration on death.

Glycogen depletion has been demonstrated in several crustacean species during environmental anaerobiosis; e.g. ***Uca pugnax*** (Teal and Carey, 1967); ***Callianassa californiensis*** (Hawkins, 1970; Zebe, 1982); ***Upogebia pugettensis*** (Zebe, 1982); ***Orconectes limosus*** (Gäde, 1984); ***Palaemon elegans*** and ***P. serratus*** (Taylor and Spicer, 1987). The functional significance of glycogen depletion will be discussed below.

Although haemolymph glucose has been measured under resting conditions in a few crustacean species (McWhinnie and Saller, 1960; Dean and

Vernberg, 1965; Telford, 1968; Hawkins, 1970; Taylor and Spicer, 1987), the effects of anoxic stress on haemolymph glucose levels have only been recorded in *Palaemon elegans* and *P. serratus* (Taylor and Spicer, 1987). Hyperglycaemia also seems to be a general stress response in Crustacea (Telford, 1968; Johnson and Uglow, 1985).

6.4.2 Functional aspects of anaerobic metabolism in Calocaris macandreae.

6.4.2.1 Compartmentalisation of metabolites.

Relatively few studies of anaerobic metabolism in decapods have considered the distribution of metabolites in the body compartments. Metabolic intermediates and end-products may be localised in any of several body compartments: in particular in the intracellular, extracellular and haemolymph compartments. In addition, metabolites may be localised in particular tissues. Albert and Ellington (1985) have measured lactate concentrations in excised tissue samples from the stone crab *Menippe mercenaria* during severe hypoxia. Although lactate concentrations varied between heart, leg socket muscle and cheliped closer muscle the relative increases in concentration during 12 hours hypoxia were similar (15.43 - 18.75%).

Albert and Ellington (1985) also measured lactate levels in pre- and postbranchial haemolymph, finding a 10 $mmol.l^{-1}$ greater concentration in prebranchial samples which was ascribed to haemolymph flow patterns. The functional significance of a pre/post-branchial lactate gradient to haemocyanin oxygen unloading is unknown in *Menippe*, although a gradient of this magnitude might be expected to favour oxygen delivery to the tissues in other species (see Chapter 5).

A comparison of total tissue and haemolymph L-lactate concentrations is possible for only a few species at present; ***Palaemon elegans*** and ***P. serratus*** (Taylor and Spicer, 1987); ***Callianassa californiensis*** and ***Upogebia pugettensis*** (Pritchard and Eddy, 1979; Zebe, 1982) and ***Calocaris macandreae*** (present study) although a comparison of muscle and haemolymph lactate is also possible in ***Orconectes limosus*** (Gäde, 1984) and ***Menippe mercenaria*** (Albert and Ellington, 1985).

Assuming that the haemolymph volume in a 1g ***Calocaris*** is 150ul (Chapter 5), the following distribution of lactate can be calculated during 36 hours anoxia:

TABLE 6.3: Calculated amounts (μmol) of L-lactate in haemolymph and tissue of *Calocaris macandreae* during environmental anoxia.

Time	Haemolymph	Tissues *	% Haemolymph **
0 hours	0.29 umol	1.87 umol	13.4
6	0.30	2.70	10.0
12	0.87	6.03	12.6
18	1.93	8.91	17.8
36	6.03	24.15	20.0

* total tissue - calculated haemolymph
** calculated haemolymph / total tissue

It is evident that the proportion of L-lactate present in the haemolymph increased from approximately 10% of the total lactate pool during normoxia, to a maximum of 20% in anoxia.

The relative concentrations of lactate in the tissues and haemolymph can be compared if a tissue concentration of 1 $\mu mol.g^{-1}$ is assumed to be equivalent to a concentration of 1 $mmol.l^{-1}$ in the fluid compartments of the tissue (as apparently assumed by Gäde, 1984 and Albert and Ellington, 1985). During normoxic conditions, the concentrations of lactate in ***Calocaris*** haemolymph and tissue are

similar (1.91 and 2.16 mmol.l^{-1} respectively) probably reflecting equilibrium conditions although the distribution will also be dependant on membrane potentials (Albert and Ellington, 1985) and possibly active transport (a lactate-hydronium symport is present in mammals: Johnson *et al.*, 1980). During anoxia, however, the increase in the concentration of lactate in the haemolymph was greater than the equivalent increase in tissue concentration (40.2 and 30.2 mmol.l^{-1} respectively).

Compartmentalisation of the lactate pool is reflected in the increase in the contribution of the haemolymph lactate pool calculated above. A similar gradient between the tissues and haemolymph has been noted by Gäde (1984) and Albert and Ellington (1985). However, tissue and haemolymph concentrations are apparently similar in ***Palaemon elegans*** and ***P. serratus*** (Taylor and Spicer, 1987) and in ***Upogebia pugettensis*** (Hawkins, 1970; Zebe, 1982). In contrast, Phillips *et al.* (1977) report greater lactate concentrations in muscle than haemolymph in the freshwater (astacid) yabbie ***Cherax destructor.***

The high concentration of lactate in the haemolymph has been interpreted by Gäde (1984) as serving to enhance lactate (and ATP) production by channelling the end product of the equilibrium reaction catalysed by lactate dehydrogenase out of the cellular compartment. He also notes that the relative Km values of LDH for lactate (38 mmol.l^{-1}: Urban, 1969) and pyruvate (0.055 mmol.l^{-1}; although this study found 0.5 mmol.l^{-1} in ***Calocaris***) are such that high lactate levels can be tolerated before the reaction is shifted to lactate oxidation. Other functional effects of high haemolymph lactate concentrations may be related to the modulation of haemocyanin oxygen affinity by lactate

(see Chapter 5), or to a direct toxicity effect resulting from high intracellular lactate concentrations. In addition, the accumulation of lactate in the haemolymph is an important component of the metabolic acidosis often noted during environmental hypoxia in crustaceans (Truchot, 1983), and may be functional in opposing the respiratory alkalosis which results from hyperventilation. Gade (1984) and Albert and Ellington (1985) noted that haemolymph lactate might be buffered *in vivo* by mobilisation of skeletal $CaCO_3$ and complex formation between cations and lactate. Albert and Ellington in fact noted an increase in Ca^{2+} and Mg^{2+} levels during hypoxia. This subject was considered in more detail in the previous chapter.

The total amount of carbohydrate present in the haemolymph comprises approximately 1.5 μmols (assuming a haemolymph volume of 150 μl), or approximately 1.5% of the total carbohydrate pool. As described above, glycogen comprised 73% of this carbohydrate. Glycogen is probably present in the haemocytes (Johnston and Spencer Davies, 1972). The remainder of the haemolymph carbohydrate may comprise monosaccharides and oligosaccharides in solution.

A large increase in haemolymph glucose has been noted as a response to anoxia and other stresses in several crustacean species (see above). In *Calocaris*, 18 hours exposure to anoxia resulted in a 16-fold increase in haemolymph glucose concentration. For a 1g (150μl haemolymph volume) animal the resultant 0.42 umols glucose comprises less than 0.5% of the total animal carbohydrate but approximately 30% of haemolymph carbohydrate (compared with 1.6% in normoxia). The slight (non-significant) increase in total haemolymph carbohydrate (of about the same magnitude over the same time course) may imply hydrolysis of tissue glycogen to haemolymph glucose. The source of

haemolymph glucose is unclear with at least two possibilities suggested in the literature. Hydrolysis of glycogen to glucose and resynthesis of glycogen from free haemolymph oligosaccharides has been suggested (Meenakshi and Scheer 1961; Scheer and Meenakshi 1961). Hydrolysis of haemolymph oligosaccharides to glucose is an alternative source. In either case no utilisation of carbohydrate reserves can be detected in ***Calocaris*** or would be expected from the relative amounts involved. The functional significance of anoxic hyperglycaemia is presumably related to the Pasteur effect (see below).

6.4.2.2 Depletion of carbohydrate reserves.

Teal and Carey (1967) suggested that a ratio of lactate formation to glycogen (glucosyl unit) utilisation (L:G ratio) of approximately 2 would indicate that glycogen and lactate were the sole substrate and end-product respectively of anaerobic energy metabolism. This hypothesis assumes that two lactate molecules are produced per glucosyl unit derived from glycogen, and that alternative metabolites would perturb the ratio. Taylor and Spicer (1987) found ratios of approximately 2 in ***Palaemon elegans*** and ***P. serratus***. Similarly, Zebe (1982) measured ratios of exactly 2 in ***Callianassa*** and 1.93 in ***Upogebia***. However, Hawkins (1970) found a ratio of 0.63 in ***Callianassa***. Gäde (1984) also concluded that glycogen was the sole substrate in ***Orconectes limosus***, although in dissected muscle tissue L:G was about 0.4.

In the case of ***Calocaris*** total lactate production was 30.18 $\mu mol.g^{-1}$ over 36 hours, whereas no significant change could be demonstrated in total carbohydrate from concentrations of 70-100 $\pm$ 20-40 $\mu mol.g^{-1}$. This is not surprising in view of the expected glycogen depletion of 15

$\mu mol.g^{-1}$. In conclusion, the consensus view is that anaerobiosis in crustaceans utilises glycogen as the sole substrate (A.D. Hill, pers. comm.) and this study provides no evidence to the contrary.

6.4.2.3 End-products of anaerobiosis.

There have been very few studies of changes in a variety of carbohydrate metabolites during anoxia. The development of HPLC has allowed measurement of the concentration of a large range of organic compounds (for example 23 in the present study) in a single small sample. However, there have been few applications of this technique to studies of crustacean metabolism. Although van Aardt and Wolmarans (1987) used gas chromatography, they measured only L- and D-lactate and succinate. The anaerobic metabolism of ***Carcinus maenas*** has recently been studied using HPLC (A.D. Hill, pers. comm.).

There is little doubt that L-lactate is the main end-product accumulated during anoxia in decapod Crustacea (see references above). However, it is interesting to note that D-lactate is accumulated during hypoxia in the horseshoe crab ***Limulus polyphemus*** (Long and Kaplan, 1968; Carlsson and Gade, 1986; Gade *et al.*, 1986). Succinate and alanine are minor end-products in the isopod ***Cirolana (=Natatolana) borealis*** (de Zwaan and Skjoldal, 1979). This contrasts with the situation in molluscs, polychaetes and sipunculids, some species of which are known to accumulate other compounds (see Introduction). The possible advantages to lactate are discussed by Fields (1983).

6.4.2.4 Comparison of aerobic and anaerobic metabolism.

The main functional result of anaerobic fermentation of glycogen to lactate, or aerobic oxidation of glycogen to CO_2, in any animal is the production of ATP. From the results of this study it is possible to

compare the rate of ATP production in *Calocaris* in normoxic and anoxic environmental conditions. It should be noted, however, that the analysis is simplistic, in particular regarding the assumption that carbohydrate catabolism is the sole source of ATP.

The mean rate of oxygen consumption of a quiescent 1g *Calocaris* in normoxia is 0.75 μmol $O_2.h^{-1}$ (Chapter 4). Aerobic catabolism via the Krebs cycle utilises 6 molecules of oxygen and produces 39 ATP molecules per glucosyl unit (G1P): the resultant rate of ATP production in *Calocaris* is therefore 4.88 $\mu mol.h^{-1}$.

During environmental anaerobiosis a 1g *Calocaris* produces about 30.18 umol of lactate in 36 hours i.e. 0.84 $\mu mol.h^{-1}$. This corresponds to a glycolytic flux of 0.42 $\mu mol.h^{-1}$ and an ATP production of 1.26 $\mu mol.h^{-1}$ (3 ATP per glucose unit).

Calocaris shows a distinct behavioural response to anoxia, with an almost complete cessation of the locomotory behaviour shown under normoxic conditions (Chapter 2, and personal observations of animals held in tanks). Animals in burrows show a cessation in the burrow irrigatory behaviour displayed during hypoxia (Chapter 2). Scaphognathite function also ceases during anoxia (Chapter 3) although the heart continues to beat. Thus a 3-fold decrease in energy demand seems plausible especially if anabolic demands are also decreased. Behavioural responses to hypoxia and anoxia have been reported in other Crustacea (avoidance: *Carcinus maenas* (Taylor and Butler, 1973); *Austropotamobius pallipes* (Wheatly and Taylor, 1980); *Palaemon elegans* (Taylor and Spicer, 1987); lethargy: e.g. *Callianassa californiensis* (Thompson and Pritchard, 1969; Hawkins, 1970); *Carcinus maenas* (A.D.

Hill, pers. comm.)).

The Pasteur effect has been defined by Storey (1985) as "the effect of oxygen deprivation in increasing the rate of carbohydrate uptake and catabolism". The Pasteur effect has been demonstrated in a variety of unicellular organisms, in locust flight muscle (Ford and Candy, 1972) and in isolated muscle tissues (Lowry *et al.*, 1964; Ramaiah, 1974) and is thought to reflect the increased demand for glycolytic substrate as a result of the lower efficiency of anaerobic catabolism. Many facultative anaerobes do not show a Pasteur effect presumably because the metabolic rate is greatly depressed (De Zwaan and Wijsman, 1976; Ebberink and De Zwaan, 1980; Gade, 1983; Storey, 1985).

A comparison of calculated rates of glycolytic flux in *Calocaris* shows that during normoxia, 0.125 $\mu mol.g^{-1}.h^{-1}$ glucosyl units are catabolised compared with 0.49 $\mu mol.g^{-1}.h^{-1}$ during anaerobiosis. This 4-fold Pasteur effect is comparable with those found in the studies cited above. The functional significance of this response is unclear in view of the lack of demonstrable depletion of carbohydrate reserves during anaerobiosis in *Calocaris*.

6.4.2.5 Recovery from anaerobiosis in Calocaris.

Recovery from a period of anoxia has been studied in the crustaceans *Callianassa californiensis* (Hawkins, 1970; see above); *Corystes cassivelaunus*, *Atelecyclus rotundatus*, *Nephrops norvegicus*, *Carcinus maenas*, *Homarus gammarus* and *Galathea strigosa* (Bridges and Brand, 1980); *Orconectes limosus* (Gade, 1984); *Macrobrachium rosenbergii* (Mauro and Malecha, 1984); *Menippe mercenaria* (Albert and Ellington, 1985); *Palaemon elegans* and *P. serratus* (Taylor and Spicer, 1987) and a variety of other invertebrates (Ellington, 1983). In all cases,

recovery of lactate concentrations (haemolymph or tissue) was relatively slow when compared with the duration of the original stress.

Ellington (1983) has identified two basic processes which occur during recovery: recharging of phosphagen and ATP pools (fairly fast); and disposal of accumulated end products by excretion, oxidation or metabolic conversion (fairly slow). Since the status of the phosphagen and adenylate pools were not measured in the present study, this aspect of recovery will not be considered. In addition to the above processes, recovery of metabolic intermediates (specifically glucose) to control values might be expected to reflect recovery.

Although recovery of the haemolymph lactate pool is initially rapid in ***Calocaris*** after 18 hours anoxia, complete recovery requires at least 48 hours. In contrast, tissue lactate recovery occurs at an apparently steady rate over the same time period. Similar responses are evident in other crustacean species which have been studied, although the relationship between tissue and haemolymph lactate is somewhat varied (Gade, 1984, Albert and Ellington, 1985). Taylor and Spicer (1987) found that recovery in ***Palaemon elegans*** was relatively rapid, recovery from 6 hours of hypoxia being complete within 6 hours, whereas in *P. serratus* complete recovery required longer than 12 hours.

The metabolic fate of lactate is at present unclear but it appears likely that lactate is slowly reconverted to glycogen via gluconeogenesis (Phillips *et al.*, 1977). Sites suggested for this process are the midgut gland (Munday and Poat, 1971), gills (Thabrew *et al.*, 1971) and haemocytes (Johnston *et al.*, 1971). This subject is under study in ***Carcinus maenas*** (A.D. Hill, pers.comm.). Although

excretion of lactate has been recorded in a few crustaceans (*Natatolana* (as *Cirolana) borealis*: De Zwaan and Skjoldal, 1979; *Upogebia pugettensis*: Zebe, 1982), no lactate excretion was detected in *Calocaris macandreae*.

6.4.2.6 The initiation of anaerobiosis in *Calocaris*.

It was shown above that significant quantities of L-lactate are produced in *Calocaris macandreae* only at PO_2's less than 2 Torr (well below the Pc). This is in contrast to the patterns observed in *Palaemon elegans* (Taylor and Spicer, 1987), and *Carcinus maenas* (Lallier *et al.*, 1987) in which lactate is accumulated at higher PO_2's (10 - 30 and 10 Torr respectively). The significance of this observation is unclear since the responses of animals may vary greatly in different experimental systems (J. I. Spicer, pers.comm.). In the experiment described above, the apparent time-lag between presumed cessation of ventilation and oxygen uptake (at 10 - 20 Torr) and the initiation of lactate accumulation, exceeded the calculated time for which the venous reserve could supply oxygen to the tissues (see Chapter 5).

6.4.2.7 Lactate Dehydrogenase Activity.

Lactate dehydrogenase activities were measured in *Callianassa californiensis* and *Upogebia pugettensis* by Pritchard and Eddy (1979). Maximum rates of pyruvate reduction in chela muscle (*C. californiensis*) and abdominal muscle (*U. pugettensis*) were 52 and 40 $umol.min^{-1}.g^{-1}$ respectively. LDH activities in the gills of *C. californiensis* and *U. pugettensis* were much lower than muscle activities, as was found for *Calocaris macandreae*. There are few data in the literature for other crustacean species, although LDH activity in several bivalve molluscs has been estimated (Hammen, 1969).

In theory, the characteristics of the LDH found in different species should be related to the patterns of lactate accumulation observed during anaerobiosis. In practice, the situation is considerably more complex. The sites of lactate production remain unclear, so that LDH activities measured in isolated tissue homogenates are not representative of the whole animal system. The rates and substrate affinities of LDH are different for the alternative reaction directions (i.e. pyruvate reduction and lactate oxidation). Although the ratio of maximal activity in each direction has been proposed as an index of the tendancy to form lactate (Hammen, 1969), there are methodological problems since reaction rates are dependant on many variables (e.g. pH), *in vivo* values of which may vary. Finally, the lactate/pyruvate equilibrium cannot be considered as independant of other metabolic processes which involve pyruvate, NAD and NADH (R.H.C. Strang, pers. comm.).

6.4.3 Ecological considerations.

The thalassinids ***Callianassa californiensis*** and ***Calocaris macandreae*** are amongst the most tolerant crustaceans of environmental anoxia so far studied. In general, the greater ability of thalassinids to survive anoxic events results from quantitative rather than qualitative metabolic and behavioural adaptive differences (i.e. the physiological mechanisms are similar).

As was pointed out above, most of the crustacean species so far studied in this context have been selected because of preconceived ideas about their anaerobic ability, based mainly on ecological observations. Thus the subject species have inhabited rockpool/intertidal habitats (e.g.

Carcinus maenas, *Palaemon elegans*, *Menippe mercenaria*); stagnant freshwater (*Macrobrachium rosenbergii*, *Orconectes limosus*) or mud-burrows (thalassinids, *Nephrops norvegicus*, *Uca pugnax*). A review of metabolic adaptation in relation to habitat in crustaceans is therefore biased by a lack of studies on 'normoxia-adapted' species.

Although conditions within the burrows constructed by mud-burrowing thalassinids (discussed in Chapter 2) are frequently severely hypoxic, completely anoxic conditions appear to be experienced only rarely. Intertidal thalassinids are likely to experience severe hypoxia on a regular tidal basis, while subtidal species (such as *Calocaris macandreae*) may experience chronic hypoxia in the 20 - 40 Torr range. The results of this study indicate that *Calocaris* will rarely, if ever, experience complete anoxia. Any anoxic events are likely to be of short duration (a few hours) before burrow oxygen exchange is re-established.

In view of the oxygen regimes likely to be experienced, the adaptive responses shown by *Calocaris* and other thalassinids seem appropriate. There do not appear to be fundamental differences between the metabolic mechanisms of anoxia tolerance shown by thalassinids and other decapods, i.e. lactate is accumulated (and presumably an oxygen debt is repaid during recovery, although this was not demonstrated). As with many of the physiological characteristics of these animals, the significant aspect is probably a low metabolic rate during both aerobic and anaerobic periods. This results in a low rate of lactate accumulation, and a long survival in the absence of oxygen. As was pointed out previously, speculations concerning adaptive significance require data (or at least hypotheses) in two areas: the nature of the selective pressures which are presumed to act, and the evolutionary history of the species in question. With regard to thalassinid metabolism, neither of these fields is sufficiently developed.

CHAPTER 7. GENERAL DISCUSSION.

The experimental results presented in this thesis include data for physiological characteristics (which may represent adaptive responses) at several levels of biological complexity (the 'hierarchical levels' of Feibleman, 1954). Some general data on the biology and ecology of megafaunal burrower communities in Loch Sween, and ***Calocaris macandreae*** in particular, were generated (i.e. population structure, burrow morphology). In addition, the ecology of mud-burrowing in thalassinids was reviewed, with reference to those aspects which affect burrow structure and behaviour and hence respiratory conditions (feeding strategy, predation, spatial competition etc.). These aspects provide background to the general theme of the thesis (ecological and respiratory physiology) and will not be considered further.

The physiological ecology of ***Calocaris macandreae*** was studied in Chapter 2. It was concluded that oxygen availability in the burrow was limited, and that a pronounced gradient of oxygen tension was always present (this is also true of other thalassinids). Under 'normal' conditions, ***Calocaris*** spends relatively little time actively irrigating the burrow (in contrast to previous data for other thalassinids). When the PO_2 of the overlying water column is experimentally reduced, however, burrow irrigation is increased. The results of this study indicate that complete anoxia is a rare event in ***Calocaris*** burrows under 'normal' conditions. There is now evidence, however, that severe hypoxia may occur in the water column overlying field populations in the Clyde Sea Area (Clyde River Purification Board, unpublished data). The effect of this on burrow conditions is at present unknown, but is clearly likely to result in even greater severity of hypoxia in the burrows of ***Calocaris***.

A more comparative approach was taken in the investigation of branchial morphology (Chapter 3), since living animals were not required (or available) for most of the work. There are pronounced differences in the branchial morphologies of different thalassinids, with a trend towards simplification of the gill formulae, and towards a phyllobranchiate condition, in the laomediid, upogebiid and callianassid families. Although there are a few references in the literature which note thalassinid gill structure, these trends have not previously been studied using a consistant, comparative approach. Unfortunately, the functional implications of these differences remain unclear, since the efficiency of the primary role of the gills (presumed to be the extraction of oxygen from the medium to the haemolymph) cannot be studied directly in these small animals. An additional structural characteristic of thalassinid gills (at least those of ***Calocaris macandreae***, ***Upogebia stellata*** and ***U. deltaura***) appears to be a small surface area (in comparison with other decapods). It was speculated that gill surface area in decapods may be more correlated with endogenous behavioural activity rates (low in thalassinids) than with exogenous environmental factors.

A mainly functional approach to respiratory physiology was made in Chapter 4. The rate of oxygen uptake in ***Calocaris macandreae*** was found to be maintained at a constant, low, level throughout a wide range of external oxygen tensions. These characteristics were related to the burrowing lifestyle of ***Calocaris***. The main physiological mechanisms resulting in this ability are thought to be a pronounced hyperventilatory response, and the extremely high oxygen affinity of the respiratory pigment, haemocyanin. Since most decapods show a similar hyperventilation when exposed to hypoxia, but considerably less ability to maintain oxygen uptake, the characteristics of the

haemocyanin were examined in some detail.

The interpretation of *in vivo* physiological function of the haemocyanin in Crustacea, requires detailed *in vivo* data. Functional studies are therefore severely limited in thalassinids by technical difficulties (resulting from small animal size). As a result, the present study was essentially limited to a comparative investigation of quantitative physiological parameters of the respiratory pigment and haemolymph. The main features of ***Calocaris*** noted were:

1. The concentration of haemocyanin in the haemolymph is low, resulting in low carrying capacities for oxygen and carbon dioxide. It is postulated that haemocyanin concentration is related to the amount of oxygen consumed (in turn to behavioural characteristics), rather than environmental conditions of oxygen availability. (N.B. The presumed adaptation to a sluggish lifestyle is also correlated with a comparatively low Ca/Mg ratio.)
2. The structure of the haemocyanin molecule in thalassinids is comparatively unusual. The results of the present study confirm that ***Calocaris*** conforms to the general pattern previously noted for thalassinids (i.e. an eikositetramer aggregation state).
3. The oxygen affinity of the haemocyanin is extremely high (favouring maintenance of oxygen uptake from the medium, even under hypoxia), but modulatory potential is limited.

The limited data in the literature suggest that these characteristics are common to other thalassinids which have been studied. The oxygen affinity of the pigment is probably related to environmental conditions, however, it is argued (in contrast to much of the previous literature) that modulation of haemocyanin oxygen affinity in decapod

crustaceans is either maladaptive, or adaptive to endogenous (behavioural) factors. *In vivo* function of the oxygen transport can only be interpreted for ***Calocaris macandreae*** by speculation. The relationships between structural and functional characteristics of thalassinid haemocyanin remain unclear.

A final comparative study of the physiological characteristics of ***Calocaris macandreae*** was made at the metabolic level, viz. energy metabolism during anoxia. In common with ***Callianassa californiensis***, ***Calocaris*** has a pronounced ability to tolerate anoxia (LT_{50} approximately four-fold greater than most decapods). However, the mechanisms by which this tolerance is achieved appear to be similar to those in other Crustacea (and vertebrates); thus L-lactate is the sole end-product, and is accumulated rather than excreted.

Both the comparative and functional (adaptational) approaches utilised above have limitations. The parameters which are directly measured by comparative ecological and physiological techniques (e.g. 'Pc' and haemocyanin P50) have neither direct nor simple relationships with the functional processes which (presumably) are the subject of selection (at the genetic level) and evolutionary change. Neither are they simply associated with single structural characteristics (or single genetic alleles; although some parameters, e.g. P50, may be relatively simply related to protein structures, in turn dependant on nucleic acid base sequences). Therefore, the usefulness of interspecific comparisons to an overall understanding of biological systems (ecosystems, species or physiological processes) is limited by the difficulties in relating parameters studied at different hierarchical levels (i.e. it is impossible to relate 'mechanism', 'organisation' and 'purpose' as proposed by Feibleman, 1954).

Most recent studies of respiratory physiology in Crustacea have at least attempted to draw adaptational conclusions. In the present study, it has been proposed that the major adaptive response to the hypoxic burrow environment has been the adoption of a sedentary behavioural regime. This has allowed a variety of structural and physiological responses (low gill area, low rates of oxygen consumption, low haemocyanin concentration (i.e. oxygen carrying capacity), low rates of lactate accumulation in anoxia etc.). It should be noted that the actual causal selective pressure which must be assumed for these responses to make adaptive sense, is energy (or resource) economy. The most pronounced physiological parameter which is directly adaptive to low oxygen availability, is the high oxygen affinity of the haemolymph. As noted several times above, however, it is impossible to conclude which characteristic (burrowing or sedentary behaviour, or physiological process) was the primary causal factor.

In the recent development of crustacean physiology, there have been difficulties in producing consistent adaptational interpretations of some physiological characteristics. For example, it might be expected that animals which regularly experience hypoxia would have comparatively greater gill surface areas; this does not appear to be the case. Problems (and inconsistent interpretations) are particularly apparent in recent developments in understanding of haemocyanin characteristics *in vitro* and function *in vivo* (see Chapter 5). Although there appears to be a general (comparative physiological) correlation between environmental conditions and haemocyanin oxygen affinity, the interpretation of functional modulation of affinity is difficult. It is clear that the Bohr shift is maladaptive in many circumstances (Mangum, 1983c).

The major assumptions (fallacies) inherent in a purely adaptational interpretation of function are outlined by Gould and Lewontin (1979). They make two fundamental objections:

1. The approach depends on atomisation of an organism (or physiological function) into independent 'traits' (or unitary processes). This neglects the interactions which are always present (for example, between osmotic balance, oxygen uptake, carbon dioxide excretion, acid-base balance etc.). "Organisms are integrated entities" (Gould and Lewontin, 1979).

2. "Natural selection [is] the main, but not the exclusive means of modification" (Darwin, 1872). Alternative explanations to immediate adaptation for patterns of form, function and behaviour are reviewed by Gould and Lewontin (1979). In the context of the present study, many physiological characteristics (in particular, of haemocyanin) have been interpreted as adaptive responses to environmental selective pressures. Gould and Lewontin (1979) describe the mechansims by which traits can result from no adaptation and no selection at all; no adaptation and no selection of the trait in question; decoupling of adaptation and selection; adaptation and selection but no selective basis for differences among adaptations; and secondary adaptation of pre-selected traits.

Gould and Lewontin advocate an alternative approach to interpretation of form and function, which may be applied at all hierarchical levels of ecology, anatomy and physiology. They state that "organisms must be analysed as integrated wholes, with *Bäuplane* so constrained by phyletic heritage, pathways of development and general architecture that the constraints themselves become more interesting and more important in delimiting pathways of change than the selective force that may mediate

change when it occurs". The term '*Bäuplan*' used here (and also in the recent phylogenetic literature, see discussion of Schram, 1983) means the basic structural plan of an organism as constrained by evolutionary history and species-specific ontogeny.

If the levels of constraint imposed on a species' *Bäuplan* are extended to include ecological and physiological factors, this approach could be a useful method of interpreting the ecological physiology of thalassinid Crustacea. If, as suggested by the experimental evidence and literature reviews above, the burrowing habit and low level of behavioural activity are correlated with many physiological characteristics of thalassinids (low gill areas, low rates of oxygen consumption, low haemocyanin concentration, low rates of lactate accumulation during anaerobic metabolism etc.), then a pluralistic (holistic) view of thalassinid (and ***Calocaris macandreae***) biology is necessary. The thalassinid *Bäuplan* would then include constraints resulting from the physiological ecology of burrowing, and the evolutionary history of physiological functions in ancestral thalassinids. As a particular example, the function of haemocyanins in thalassinids and other decapods should be interpreted taking into account the constraints imposed by phyletic heritage and concurrent physiological processes. It is hoped that the present study has established an ecological physiology *Bäuplan* for ***Calocaris macandreae*** (and more generally, thalassinids) to which future studies may be related.

APPENDIX 1. METHOD FOR THE DETERMINATION OF ORGANIC CARBON CONTENT OF SEDIMENT.

This method is taken from Buchanan (1984) and follows that of Walkley and Black (1934). The sediment sample is digested with a chromic acid-sulphuric acid mixture and the excess of chromic acid not reduced by the organic matter is titrated with a ferrous salt.

REAGENTS.

N Potassium dichromate: dissolve 49.04 g of reagent grade $K_2Cr_2O_7$ in water and dilute to 1 litre.

Sulphuric acid: not less than 96% with 1.25 g of silver sulphate added for every 100 ml of acid. (The silver sulphate removes the interference of chlorides.)

Phosphoric acid: at least 85%.

Diphenylamine: dissolve 0.5 g diphenylamine in 20 ml water and add 100 ml of concentrated sulphuric acid.

N Ferrous sulphate: dissolve 278 g of reagent grade $FeSO_4.7H_2O$ in water, add 15 ml of concentrated sulphuric acid and dilute to 1 litre. Standardise by titrating against 10.5 ml potassium dichromate (as described below).

The sediment sample was ground to pass a 0.5 mm seive. A weighed quantity of sediment (c. 5 g) was placed in a 500 ml conical flask, 10 ml of N potassium dichromate and 20 ml of conc. sulphuric acid were added. After shaking for one minute, the flask was placed in a boiling water bath for 30 minutes. The flask was cooled, then 200 ml water, 10 ml phosphoric acid and 1 ml diphenylamine (indicator solution) were added. The mixture was titrated with ferrous sulphate until the

solution turned green (since this occurs with no warning, an excess of dichromate was restored by adding 0.5 ml potassium dichromate, and the titration completed drop by drop).

The amount of organic carbon (chromic oxidation value) is given by the expression:

$$\frac{V1 - V2}{W} \times 0.003 \times 100$$

where V1 equals the volume of normal potassium dichromate (10.5 ml), V2 equals the volume of ferrous sulphate in ml, W equals the amount of sediment taken.

APPENDIX 2. SELECTED PHYSICO-CHEMICAL DATA FOR UPPER LOCH SWEEN.

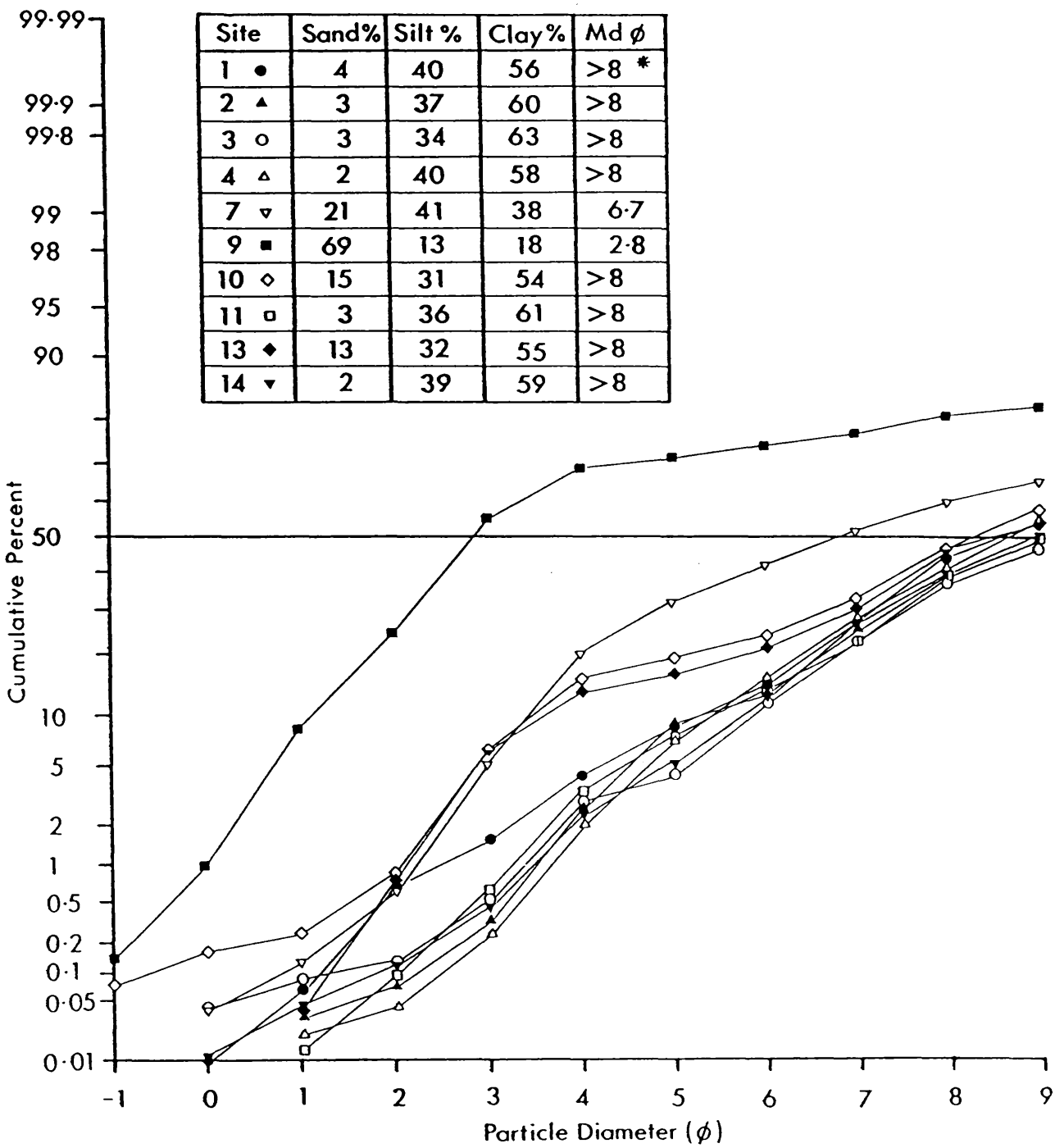
(TAKEN FROM ATKINSON, 1987)

Table A2.1 Organic carbon, chromic oxidation values for sites in Loch Sween.

SITE	Organic C (%)					
		depth in sediment				
	Homogenised top 10cm	surface	5cm	10cm	20cm	30cm	40cm
1	9.675						
2	6.650						
3	4.875	4.425	4.125	4.275	3.975	3.975	4.200
4	5.025						
5		6.075		6.225	5.925		
7	4.650						
9	2.700						
10	3.900						
11	4.350						
13	3.150						
14	4.725						

FIG. A2.1 Cumulative probablity curves illustrating the particle size distributions of upper Loch Sween sediments. Md - median particle diameter (* - >8 phi = <3.9 μm).

Site	Sand%	Silt %	Clay %	Md ϕ
1 ●	4	40	56	>8 *
2 ▲	3	37	60	>8
3 ○	3	34	63	>8
4 △	2	40	58	>8
7 ▽	21	41	38	6·7
9 ■	69	13	18	2·8
10 ◇	15	31	54	>8
11 □	3	36	61	>8
13 ◆	13	32	55	>8
14 ▼	2	39	59	>8



APPENDIX 3. PROTOCOL FOR SCANNING ELECTRON MICROSCOPE PREPARATIONS.

Specimens which were used for Scanning Electron Microscopy (S.E.M.) were either fresh or had been preserved in 5 % formalin in sea water. Specimens were prepared for S.E.M. as follows:

1. Glutaraldehyde/Sodium cacodylate/sea water fixative.....1 h;
2. Sodium cacodylate/sea water rinse.....3 x 10 min;
3. 1 % Osmium tetroxide stain.....1 h;
4. Distilled water rinse.....3 x 10 min;
5. 0.5 % Uranyl acetate.....30 min (dark);
6. Acetone series dehydration: 30 % acetone.....15 min;
   50 % .....15 min;
   70 % .....15 min;
   90 % .....15 min;
   Analar absolute acetone.....2 x 20 min;
   dried Analar absolute acetone.....20 min;

Specimens were then critical point dried in liquid carbon dioxide. The dried specimens were mounted on stubs using quick-drying conductive silver paint and gold-coated using a sputter-coater (750V; 25mA; 8 min).

The specimens were observed and photographed using a Phillips microscope.

APPENDIX 4. METHOD FOR L-LACTATE DETERMINATION.

This method is used by A.D. Hill (pers. comm., based on that of Gutmann and Wahlefeld (1974) and can be used on both tissue and blood samples. L-lactate is oxidised to pyruvate in a reaction catalysed by lactic dehydrogenase (EC.1.1.1.28). It is assumed that the formation of NADH is proportional to the amount of L-lactate present in the sample.

REAGENTS.

Glycine-hydrazine buffer:	3.75 g of glycine, 0.75g of EDTA and 2.0 ml of hydrazine hydrate were mixed with 98 ml of distilled water. The pH was adjusted to 9.0, using NaOH (1 M).
Lactic acid	The following standards were used: 2 mM, 1mM, 0.5 mM and 0.25 mM (38.4 mg/200 ml = 2 mM).
NAD^+	26.5 $mg.ml^{-1}$ (40 mM).
LDH	Dilution of 1 in 2 (i.e. 600 $U.mg^{-1}$ protein).

PROCEDURE.

The following reagents were mixed in a 1.5 ml Eppendorf tube:

Glycine-Hydrazine buffer:	1000 µl
NAD^+:	50 µl
sample/standard:	50 µl
LDH:	5 µl

The reaction mixture was mixed thoroughly and incubated in a water bath at 37°C for 2 hours. The absorbance at 340 nm was then measured. Blanks were run by substituting 50 µl of distilled water for the sample. Sample concentrations were interpolated from a calibration curve constructed using the standards stated above.

APPENDIX 5. METHOD FOR D-GLUCOSE DETERMINATION.

This method (adapted by A.d. Hill from that of Slein, 1965) involves a two-step reaction using the enzymes hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC.1.1.149). The hexokinase catalyses the oxidation of glucose-6-phosphate (G6P) to 6-phosphogluconate. The method assumes that the formation of NADPH is proportional to the amount of G6P and D-glucose present.

REAGENTS.

Tris buffer: 2.42 g of Tris and 0.240 g of magnesium sulphate (100 mM and 10 mM respectively) were added to 200 ml of distilled water. The pH was adjusted to 7.4, using HCl (5 mM).

D-glucose: The following standards were used: 1 mM, 0.5 mM, 0.25 mM and 0.1 mM (18 mg/100 ml = 1 mM).

Hexokinase: A dilution of 1 in 25 (i.e. 180 $U.mg^{-1}$ protein).

G6P-DH: A dilution of 1 in 25 (i.e. 300 $U.mg^{-1}$ protein).

ATP: 121 mg/10 ml (22 mM).

$NADP^+$: 168 mg/10 ml (22 mM).

The following reagents were added to a 1.5 ml semi-micro cuvette:

Tris buffer	700 µl
ATP	100 µl
$NADP^+$	100 µl
sample	100 µl
G6P-DH	10 µl
Hexokinase	10 µl

The absorbance at 340 nm was measured before either enzyme was added (E1); 5 minutes after the addition of G6P-DH (E2); and 5 minutes after the further addition of hexokinase (E3). Then,

E2 - E1 = change proportional to the concentration of G6P present in the sample

E3 - E2 = change proportional to the concentration of D-glucose present in the sample.

APPENDIX 6. METHOD FOR THE DETERMINATION OF TOTAL HEXOSE/PENTOSE SUGARS (THE 'ANTHRONE METHOD').

This method (adapted by A.D. Hill from that of Carroll *et al*, 1956) involves the acid hydrolysis of glycosidic bonds to give monosaccharide units, using anthrone reagent. These in turn are hydrated to furfural and its derivitives. The furfural reacts with anthrone (10-keto-9,10-dihydro-anthracene) to give a blue-green complex, which is assayed spectrophotometrically at 620 nm.

REAGENTS.

Anthrone reagent: 72 ml of concentrated sulphuric acid was added (carefully!) to 28 ml distilled water. 50 mg of anthrone was then dissolved into the 72% H_2SO_4, allowed to cool and stored at 4^oC (for a maximum of 7 days).

D-glucose/Glycogen: The following standards were run: 5 mM, 2.5 mM, 1 mM and 0.5 mM (90 mg/100 ml and 81 mg / 100 ml = 5 mM for D-glucose and glycogen respectively).

1 ml of anthrone reagent and 50 ul of sample were added to a 5 ml test-tube, mixed well and incubated in a boiling water bath for 10 minutes. The tubes were then cooled on ice for 10 minutes and the absorbance measured at 620 nm (compared to a reagent blank).

Glycogen and oligo-/mono-saccharide fractions of haemolymph samples were separated as follows: 100 µl of haemolymph was added to 500 µl of absolute ethanol and stored on ice for 2 hours. The precipitated glycogen was then centrifuged down (10 minutes at 10,000g), the supernatant removed, and the pellet re-suspended in another 500 ul ethanol. After further centrifuging, the two supernatants were pooled and the pellet re-suspended in PCA. The pooled supernatant (oligo-/monosaccharides) and the re-suspended pellet (glycogen) were then assayed as above.

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