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THE CONTROL OF ESCAPE BEHAVIOUR IN, AND THE HISTOPATHOLOGY

OF, THE NORWAY LOBSTER, *Nephrops norvegicus* (L.).

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DECLARATION.

I declare that this thesis represents, except where note is made to the contrary, work carried out by myself. The text was composed by myself.

R. H. Field

24 February, 1992.

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Part 2. Initial Studies of the Histopathology and Epizootiology of a Novel Dinoflagellate Disease of *Nephrops norvegicus* (L.) on the West Coast of Scotland.

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ABSTRACT:

Nephrops norvegicus, like other lobsters and crayfish, react to threatening stimuli by producing of the tail-flip escape response. This response, which is important in both predator avoidance and capture by trawling, takes the form of repeated flexions and extensions of the abdomen, produced by the deep abdominal flexor and extensor muscles. Much research has been concentrated on both the metabolic and neuronal factors controlling and limiting the tail-flip swimming of decapod Crustacea, but little attention has been focused upon the interactions between these two areas.

This study has examined the tail-flip swimming of *N. norvegicus* in terms of both metabolic and neuronal limitations. Results have indicated that prolonged swimming and its recovery are limited neither by the availability of energy (from direct, stored sources - endogenous muscle ATP; or from indirect sources - by glycolytic production of ATP from D-glucose), nor by alteration of inter- or intra-cellular conditions by the build up of the glycolytic end product L-lactate. However, the part played by phospho-L-arginine (another short-term energy reserve in muscle) in the limitation of tail-flipping has not been unequivocally defined, and the possibility remains that there may be some metabolic influence in determining the endurance and recovery of swimming.

Despite the lack of definitive evidence against any metabolic limitation of escape swimming, the results of further experiments suggest that neuronal factors play a major role in the limitation of tail-flipping. These experiments were designed to assess the importance and site of action of neuronal factors, in particular habituation, in swimming. Furthermore, the waning of tail-flipping in response to repeated stimulation appears to represent true habituation.

By the use of both histochemical and morphological techniques, the roles of the muscles of the thoraco-abdominal joint in both tail-flipping and postural control have been partially elucidated. These results suggest both bracing and steering functions for the larger muscles of the two groups examined (thoraco-

abdominal extensors and abdominal abductors) and postural roles for the smaller muscles. These investigations also identify suitable areas for further research, not least an examination of the innervation and recruitment of these muscle groups during tail-flipping in order to determine unequivocally their bracing and steering functions.

N. norvegicus from fishing grounds on the west coast of Scotland have been found to harbour infection by a species of parasitic dinoflagellate. Chromosome morphology and ultrastructural features suggest that the parasite is a member of the botanical order Syndiniales, possibly related to *Hematodinium perezi* Chatton & Poisson 1931. The cells invading the haemal spaces, however, show no signs of flagella. The mode of transmission is not yet known, and a flagellate spore stage has not been identified. The infection appears to be fatal to its host, the main cause of death possibly being disruption of gas transport and tissue anoxia caused by the presence of large numbers of dinoflagellate cells in the haemolymph. Severe infection has an adverse effect on meat quality that has provoked comment from fisherman and processors.

Affected animals have been found at all west coast sites surveyed, with peak infection rates reaching 70% of trawled samples. The occurrence of infection shows marked seasonality coincident with the annual moult period of *N. norvegicus*. This coincidence suggests some link with the moult cycle, which is further supported by the consistently higher prevalence of infection in females than in males, and in intermoult than premoult animals. An increased prevalence of infection has been observed recently in some areas, suggesting a worsening of the problem, and that further research is necessary to assess the implications of this condition for west coast *N. norvegicus* and the fisheries they support.

PART 1. METABOLIC, NEURONAL AND ANATOMICAL FACTORS
IMPORTANT IN THE LIMITATION AND CONTROL OF ESCAPE
SWIMMING IN *Nephrops norvegicus* (L.).

CHAPTER 1. GENERAL INTRODUCTION

Norway lobsters, *Nephrops norvegicus*, are common members of the benthic fauna around the coasts of Scotland. They are generally found at depths of between 20 and 500m, on substrates ranging from fine, soft muds to muddy sands which are suitable for the construction of their burrows (Figueiredo & Thomas, 1967; Chapman *et al.*, 1975). The geographical range of this species extends from the African coastal waters off Morocco and the Mediterranean in the south, to Iceland in the north. Within this range, Scottish coastal waters support one of the biggest *N. norvegicus* fisheries, currently worth around £35 million per annum (C.J.Chapman pers. comm.). Commercial grounds occur off the east and west coasts of Scotland, at depths of 40m and below (Chapman *et al.*, 1975). The main method of capture of *N. norvegicus* in the Scottish fishery is by trawling, accounting for 93% of landings, whilst creeling accounts for the other 7% (Scottish Sea Fisheries Statistical Tables, 1987).

The efficiency of trawling is affected by many aspects of the behaviour and biology of *N. norvegicus*, as well as by the design of the fishing gear. Studies of the emergence rhythms of *N. norvegicus* have shown that they spend most of their time concealed in their burrows 200-300mm below the sediment surface, where they are unlikely to be caught by trawling, and only emerge for short periods to forage (Chapman *et al.*, 1975; Atkinson & Naylor, 1976). The exact timing and duration of these activity periods have been shown to vary with depth, and to be determined ultimately by the intensity of light on the sea bed. Using this knowledge it is possible for fishermen to trawl at times which coincide with periods of maximum emergence of *N. norvegicus*. Studies of *N. norvegicus* behaviour on the seabed, and particularly in response to fishing gear have also yielded valuable information to aid in the design of trawling gear (Main & Sangster, 1982, 1985; Newland & Chapman, 1989).

Of particular importance are the evasion reactions performed by *N. norvegicus* in response to the approach of trawl nets. When disturbed by predators, other *N. norvegicus*, noxious stimuli or fishing gear *N. norvegicus*

escape by means of the 'tail-flip' escape response, typical of macrurous decapod Crustacea. This response takes the form of repeated flexions and extensions of the abdomen, resulting in rapid backward movement of the animal. These rapid extensions and flexions are caused by sequential contractions of the large segmental deep flexor and extensor muscles of the abdomen. Clearly, such an escape reaction requires sophisticated neuronal control and coordination, efficient metabolic support and skeletal/muscular adaptation in order to be an effective evasion response. This study examines the limitations imposed on the tail-flip performance of *N. norvegicus* by these factors.

1.1. The neuronal basis of the tail-flip. The neuronal basis of this behaviour has been studied in detail in crayfish (reviews by Wine & Krasne, 1972, 1982) and more recent studies have shown it to be broadly similar in *N. norvegicus* (Newland & Neil, 1990a; Newland *et al.*, 1988, 1992b). Much research effort has been concentrated on the escape behaviour of crayfish, not only because it represents a relatively simple, stereotyped behavioural act based on neural circuitry of comparatively low complexity, but because of the involvement of large diameter giant axons which are amenable to experimentation. Thus, in 1947 Wiersma was able to show that stimulation of crayfish giant fibres elicited a full escape response. The escape responses of many animals are mediated by giant fibres, which have evolved independently in several groups: cnidarians, annelids, some insects, cephalopod molluscs, and fish, as well as crustaceans. Their evolutionary 'popularity' derives from the very fast reaction times they confer. This is in turn due to the increased conduction velocity achieved by their large diameter, and in some cases by their insulation. However, giant fibres are not essential in all escape responses, and many behaviours, including one form of tail-flip seen in both crayfish and *N. norvegicus*, involve other neural pathways.

In crayfish, two pairs of giant fibres in the ventral nerve cord, when

activated by stimuli of abrupt or unexpected onset, induce short latency tail-flips. These giant-mediated flips are of two forms, dependent on which pair of giant fibres is recruited (Wine & Krasne, 1972). An abrupt stimulus to the anterior of the animal (cephalothorax, chelae or other limbs) results in the activation of the medial giant fibres, producing medial giant (MG) tail-flips of a low trajectory. A similar stimulus applied to the posterior region of the body (the abdomen or telson) causes recruitment of the segmental series of lateral giant fibres, resulting in lateral giant (LG) tail-flips of more elevated trajectory. *N. norvegicus* and crayfish typically respond to such stimuli by producing a number of tail-flips or 'swimming bouts' (Newland *et al.*, 1988). A swimming bout consists of an initial, short latency giant fibre flip, followed by one or more longer latency 'non-giant' flips (NG's). This basic pattern is true both of crayfish and *N. norvegicus*, but Newland & Neil (1990a) described important differences in the LG mediated tail-flip trajectories of these species. In the crayfish, caudal stimuli result in LG flips which actually pitch the animal vertically and slightly forward of the starting point, subsequent NG flips resulting in a somersault manoeuvre which rotates the animal to land upright, in front of and facing the point of stimulation. In *N. norvegicus*, the LG flip is less elevated, being a backward movement at an angle of about 70°, followed by subsequent NG's which carry the animal backwards and away from the point of stimulation. Thus *N. norvegicus* move up and over the source of posterior stimuli, whilst the crayfish turns to face possible threat. Differences in the patterns of neuromuscular activation underlie these differences in trajectory of LG and MG tail-flips in crayfish and lobsters. In MG and NG flips, a caudo-rostral temporal gradient of segmental contraction has been noted in both crayfish and *N. norvegicus*. However, LG flips in crayfish involve only the activation and flexion of rostral segments, producing a power stroke of the telson in a vertical plane, whilst in *N. norvegicus* caudal segments are also activated in a rostro-caudal contraction sequence, causing a telson power stroke in a plane angled at

70° to the horizontal.

This division of labour between the three tail-flip initiating systems allows considerable flexibility of response. Giant mediated flips enable a very swift initial reaction to threat, but in a relatively stereotyped manner, whereas the non-giant flips (probably mediated by several systems - Wine & Krasne, 1982) make a more flexible response possible once escape is underway. The flexibility of this response lies in its ability both to scale and direct escape swimming. The production of swimming bouts consisting of both non-giant and giant tail-flips (elicited by stimuli of the same or different form, and having different latency) enables the animal to produce a variety of responses within a basically stereotyped motor behaviour, at a level appropriate to the particular situation. The level of response elicited is dependent on the flip-mediating system initiated, which in turn is determined by the site, nature and intensity of the stimulus applied.

1.2. Skeleto-muscular specialisations for the tail-flip. The directionality and steering of tail-flipping on a gross level are determined by the site of initial stimulus, due to the differing trajectories of the two giant mediated tail-flip forms. However, both crayfish and *N. norvegicus* are clearly capable of a much more accurately steered response, tailored to any particular threat situation, away from danger to known places of safety (for example, their burrows). Reichert & Wine (1983) reported that crayfish stimulated to swim by initiation of LG activity by means of laterally applied stimuli, showed initial giant mediated flips with little or no lateral vector directed along the caudo-rostral axis of the animal's original orientation, whilst subsequent non-giant flips had response trajectories preferentially directed away from the lateral stimulus. Similar reactions have been found in *N. norvegicus* (Newland & Neil, 1987) and in the spiny lobster *Jasus lalandii* (Newland *et al.*, 1992a). These authors examined the tail-flips produced in response to laterally placed tactile stimuli.

In both species steering is produced by the lateral direction of the force produced by the power stroke of the uropods. Laterality of force direction is achieved by a tilting of the abdomen towards the side of stimulation, accompanied by asymmetrical flexion of the uropods, those on the downward side of the abdomen being more flexed. Of particular interest is the fact that some steering component is actually present in the initial giant mediated flips of *N. norvegicus* (Newland & Neil, 1990b). Some steering effect has also been attributed to the use of streamlined thoracic appendages much like a rudder (Cooke & Macmillan, 1985) and to the swimmerets (Cattaert *et al.*, 1988).

Despite the obvious role of uropods and thoracic appendages in tail-flip steering, the thoraco-abdominal joint also plays an important role. It is not only involved in the tilting of the abdomen, as an integral part of righting reactions and tail-flipping in *N. norvegicus* and *Jasus lalandii* (Newland & Neil, 1990b; Newland *et al.*, 1992a), but slow, postural movements also occur about this joint, adjusting the attitude of the abdomen (Knox & Neil, 1991). These movements are brought about by two groups of muscles which span the joint (Pilgrim & Wiersma, 1963). However, in order to retain mobility at this joint, there is little skeletal strengthening, beyond the arthrodial membrane itself, and so the considerable force generated by tail-flipping must be transmitted through the control muscle systems to the heavily calcified thorax and chelae. Thus some component of these muscle systems would be expected to have a bracing function.

1.3. The metabolic support of muscle activity. In muscle, work is done by the conversion of the chemical energy contained in the phospho-adenylate adenosine triphosphate (ATP) into the mechanical energy of contraction. ATP is not only the sole source of energy for muscular contraction, but is also a prerequisite for life itself, being the primary fuel of all cell reactions. Consequently, much study has been concentrated on the mechanisms of its

generation and utilization. Until the middle of this century it was thought that all ATP generation was brought about in the presence of oxygen, through complete oxidation of carbohydrates, fatty acids and proteins to CO_2 and H_2O. It has become apparent, however, that under certain conditions, some animals cannot meet their energy requirements solely through this aerobic metabolism, and resort to anaerobic methods of ATP generation. Glycolysis releases the energy of carbohydrate fuels by anaerobically phosphorylating sugar residues and, although liberating less ATP per unit fuel, is faster and can proceed without the presence of oxygen.

Some animals (particularly aquatic animals) periodically experience very much reduced oxygen tensions. Under these hypoxic conditions, the animal's total energy needs exceed the available supply of oxygen, and the shortfall is made good by recourse to anaerobiosis - 'environmental anaerobiosis'. However, some animals may experience a 'functional' anoxia in a particular tissue, where oxygen supply to that tissue is insufficient to allow aerobic metabolism to meet energy demand. Such is the case in vigorously exercising muscle, particularly in large muscles used for powerful burst activity. It is the availability of ATP energy, and the capacity to liberate it, that determines the capability of a muscle to contract.

1.4. Muscle enzyme histochemistry. Within muscle cells, ATP specific enzymes - ATP-ases - facilitate the release of bond energy from ATP by separating off inorganic phosphate molecules from the remainder of the adenylate molecule. This energy is then utilised to power the movement of muscle filaments over one another, resulting in contraction. Muscles performing different functions have long been known to have differing strategies of energy metabolism, depending on the rate and duration of their activity. In vertebrates, the dichotomy between 'slow'/'red' and 'fast'/'white' skeletal muscle fibres has been known for some time. This basic division is based upon the link between

colour and function, the red fibres being so because of the presence of high levels of cytochromes and myoglobins, whilst these are absent in white fibres. The functional basis for this difference is that red fibres are in constant use, performing low level postural movements. Hence they require a constant but relatively low supply of energy, which is most efficiently supplied by oxidative phosphorylation. White fibres, however, are used less frequently and for high level rapid or powerful contractions. These fibres have only the limited oxidative capacity which is necessary to maintain themselves, and obtain the large amounts of ATP energy needed rapidly for burst activity from anaerobic glycolysis.

The crude red/white classification of muscle has recently been greatly elaborated. Vertebrate striated muscle is known to contain four fibre types; one slow, two fast and a transitional form (Brooke & Kaiser 1970). This classification is based not on the relative oxidative capacities of the fibres but on the relative density and activity of ATP-ase enzyme sites, which give a measure of relative abilities to liberate energy from the ATP molecule. The use of such histochemical techniques has led to the characterisation of muscle fibres in terms of the adaptation of their cellular machinery to a particular mode of energy production and rate of energy usage (Brooke & Kaiser 1970; Mabuchi & Sréter 1980; Green *et al.* 1982; Snow *et al.* 1982). These techniques have also been applied successfully to invertebrate muscle, particularly crustacean muscle, in studies which have attempted to relate biochemical structure to function and innervation (Jahromi & Atwood 1969; Ogonowski & Lang 1979; Silverman & Charlton 1980; Kent & Govind 1981; Govind *et al.* 1981; Govind & Kent 1982; Parsons & Mosse 1982; Maier *et al.* 1984).

1.5. Factors limiting tail-flip swimming. An important aspect of the tail-flip behaviour (and other startle or burst escape behaviours), and one that has received much attention (in crayfish) is the endurance of the response and

those factors which impose limits upon it. The swimming performance of *N. norvegicus* is of obvious importance in the efficiency of capture of this animal. Newland (1985) and Main & Sangster (1985) have shown that *N. norvegicus* cannot avoid capture by outswimming trawl nets moving at normal towing speeds. However, both studies also stated that animals may escape capture using the tail-flip reaction around the sides of the trawl and over the side warps. These observations not only show the importance of the persistence of the tail-flip itself, but also raise the questions of what factors limit its performance, and how these factors affect the subsequent swimming ability of animals that have already avoided capture.

Many studies on several species of crayfish, *N. norvegicus* and other decapods, have shown that with repeated tactile or electrical stimulation the production of tail-flip escape swimming wanes. The rate at which waning occurs varies with the type, frequency and position of the stimulus applied. Constraining factors on this kind of rapid, stereotyped, repetitive, and relatively 'simple' reflex reaction are of two main types - those which relate to the perception and transmission of threatening stimuli, and those which involve the mechanisms of reacting to them, i.e. the performance of the muscular systems.

Firstly, a burst escape reaction can only be as rapid as the perception and transmission of a threatening stimulus and therefore its production may be limited by the efficiency of the receptors, sensory afferents, interneurones, and efferent motor pathways involved. Such limitations may be due to sensory adaptation, infringement of the neuronal refractory period or habituation, as defined by Hinde (1970). Detailed knowledge of the pathways involved in the crayfish tail-flip has enabled the probable neuronal sites of failure of the tail-flip to be identified. Krasne & Woodsmall (1969), working on intact animals, showed that the cessation of swimming in response to a tactile stimulus applied to one area of the abdomen could be reversed by application of the same stimulus in other areas, or by delivering a more intense stimulation in the same

region. A similar phenomenon was observed in *N. norvegicus* by Newland *et al.* (1988), suggesting that habituation does occur. Using isolated abdominal nerve cord preparations of the crayfish *Procambarus clarkii*, Zucker (1972) confirmed that the response decrement in the LG tail-flip reaction was due to habituation, and that it occurred at the chemical synapse between the tactile afferent neurones and the sensory interneurones.

The second possible area of limitation of escape swimming is the constraint placed on the ability of an animal to swim by the endurance and fatigue resistance of the swimming muscles themselves. Ultimately, the capacity of a muscle to do work is determined by the availability of energy.

In view of the importance of the endurance of tail-flip swimming in the capture of *N. norvegicus*, an investigation has been undertaken into the energy metabolism within the abdominal muscles of this animal and the roles which this and neuronal factors play in limiting escape swimming. The results of this study are presented in Chapter 2.

Chapter 3 presents the results of an investigation of the adaptations of the muscle systems of the abdomen and thoraco-abdominal joint to production of the burst activity of tail-flipping, and its control and steering. The morphology and histochemical properties of the thoraco-abdominal muscles have been examined to elucidate their various roles in steering and bracing during escape swimming. These muscles and also the deep abdominal muscles have been analysed by histochemical fibre typing techniques, to characterise them in terms of enzyme system adaptations to functional anaerobiosis.

CHAPTER 2. THE RELATIVE IMPORTANCE OF METABOLIC AND NEURONAL FACTORS IN LIMITING TAIL-FLIP SWIMMING IN

Nephrops norvegicus (L.).

2.1 INTRODUCTION

For some years, the importance of the swimming ability of N. norvegicus has been recognised. The tail-flip escape reaction is elicited in response to disturbance and presumably its primary function is as a predator avoidance mechanism. N. norvegicus has been shown to be capable of many tail-flips in response to repeated stimulation. Newland et al. (1988) reported means of 67.1 ± 4.8 flips for males and 57.3 ± 7.1 flips for females in response to repeated single tactile stimuli. Studies of the behaviour of N. norvegicus in relation to fishing gear have shown that the tail-flip is an integral part of the capture process. It has also been shown that in many cases animals perform relatively few tail-flips before they are captured, and that these numbers by no means represent their full capability.

N. norvegicus encountering trawls produce tail-flips to escape from the threatening stimulus of the approaching ground gear. The type of tail-flipping performed is dependent upon their orientation on the sediment surface in relation to the trawl (Newland & Chapman, 1989). Animals facing away from an oncoming trawl will be stimulated abdominally, and capture will result as a consequence of the first bout of LG mediated escape swimming carrying the animal over the ground rope and back into the net. N. norvegicus oriented head-on to a trawl will be stimulated in the anterior regions of the body, eliciting MG mediated tail-flips, the animal swimming directly away from the approaching net, but elevated above the ground gear. Mean swimming speeds of N. norvegicus are less than commercial towing speeds and hence almost all MG swimming animals are overtaken and caught within the first few swimming bouts (Main & Sangster, 1985). Thus animals in the central region of the path of an oncoming trawl are highly likely to be captured rapidly, and their swimming ability is inconsequential beyond the fact that swimming is initiated. Moreover, Newland & Chapman (1989) also showed that swimming in response to ground gear tended to be oriented at 90° to the groundrope, parallel to the direction of

net travel, further reducing the possibility of escape, especially around the sides of an oncoming trawl. However, animals on the sediment surface in the more lateral areas of the trawl path were shown to have a greater chance of escape. This is either because the ground gear at the sides of the trawl, closer to the trawl doors, becomes elevated from the sediment surface and passes over foraging *N. norvegicus* without stimulating them to swim, or because animals swimming over the ground gear do not pass into the net. Newland (1985) suggested the use of 'tickler' chains to cause animals in the path of the lateral trawl warps to be stimulated to swim. This raises the possibility of 'herding' of swimming animals into the central regions of the net path, and ultimately into the net itself. In such cases the swimming endurance of *N. norvegicus* in response to the repeated stimulation of the 'herding' ground gear becomes important.

Of equal importance to endurance is recovery of the ability to swim after long-term tail-flipping. U.K. Fisheries legislation requires that *N. norvegicus* below a certain size (20mm carapace length in some areas, 25mm in others) may not be landed (Main & Sangster, 1985), and size selection is partly determined by the mesh size of the net used. However, most animals in the towing path of the net, of whatever size, may initially be stimulated to swim and many of these may be caught by the trawl. Some of the smaller animals may then escape through the mesh of the side panels or cod-end (Main & Sangster, 1985), but they will have been induced to perform prolonged swimming before escape. As prolonged tail-flipping is likely to be energetically very costly, the ability to recover full swimming potential after severe exercise may have an important bearing on the subsequent behaviour and survival of *N. norvegicus* following escape from a trawl. This will also be true for animals caught and then subsequently discarded from fishing boats, either because they are undersized, or for other reasons.

Thus factors limiting the continued production of escape tail-flips, and the

recovery of this ability, are important in assessing the likelihood of capture on subsequent occasions. Several possible factors may result in the waning (or 'behavioural habituation') of the escape swimming reaction of *N. norvegicus*. Hinde (1970) defined habituation as 'the decrement of a response as a result of repeated or continuous stimulation.' This definition has certain limitations, the most important of which is that it does not apply to cases that involve infringement of refractory period by stimulation frequency, sensory adaptation or effector (muscle) fatigue. Thus the observed 'behavioural habituation' in *N. norvegicus* may be due to true neuronal habituation, other associated neuronal factors, or to metabolic constraints.

Studies on crayfish (Wine *et al.*, 1975) have shown that abdominal flexions (tail-flips) always follow activity in the giant fibres, and that excitability of these pathways is reduced by up to 50% after as few as 10 tactile stimuli have been applied at 5 minute intervals. Furthermore, these researchers have established that both LG and NG tail-flips show habituation of several hours duration (see also Wine & Krasne, 1972). Up to 10 possible sites of neuronal lability have been demonstrated. Various afferent sites are sensitive to repeated stimulation, but loss of transmission has been shown to be adequately compensated for by other parallel routes, resulting in no apparent decrement of response. Indeed, Zucker (1972) has shown that the behavioural habituation observed in the waning of crayfish escape swimming is the result of a failure of synaptic transmission between tactile afferent and sensory interneurones. The behavioural observations of Krasne & Woodsmall (1969) support the contention of an afferent seat of habituation, since changing the stimulus site causes further response in previously habituated animals. Similar observations in *N. norvegicus* (Newland, 1985) suggest that this kind of habituation may also be responsible for the limitation of escape swimming in this species.

Metabolic factors may also result in response decrement, similar to habituation. Indeed, Hinde's (1970) definition of habituation excludes apparent

habituation which involves muscular fatigue. Metabolic fatigue can occur in two general areas, the depletion of available energy supply, and the build-up of waste or by-products of the processes replenishing that energy supply.

Energy for muscle contraction is supplied by the phosphate bond energy contained within molecules of ATP, muscle contraction resulting in the breakdown of ATP to ADP (adenosine diphosphate) and Pi (inorganic phosphate). Muscles have a short term store of ATP but, once this is utilised, it must by replaced from other sources. ATP is regenerated by the rephosphorylation of ADP; the method of replenishment depends upon the muscle involved, and the intensity of work being done. Short term replenishment is from phosphagen stores within the muscle cells. Phosphagens (creatine phosphate in vertebrates, arginine phosphate in most invertebrates) are labile compounds, which readily give up phosphate to ADP when it is present. These stores are adequate for low intensities of activity, but prolonged work requires additional ATP to be supplied from sources external to the muscle. The ultimate source of metabolic energy for rephosphorylation of ADP and also muscle phosphagens differs with muscle type. Muscles of vertebrates and invertebrates show both anaerobic (glycolytic) and aerobic respiratory activity, but the relative contributions of these pathways vary considerably. Muscles performing functions involving different activity regimes have different ultrastructural bias to aerobic or anaerobic functioning. Those muscles which effect rare but high power activity, such as those involved in escape and startle responses, tend to have low aerobic capacity (to supply resting needs) whilst having the capacity to work rapidly for short periods under functional anaerobiosis, deriving energy from glycolysis. The deep abdominal muscles of *N. norvegicus* effecting the escape response are such muscles (See Chapter 3) relying on glycolysis for provision of ATP energy by rephosphorylation of ADP during prolonged exercise.

Most animals require the availability of enough oxygen to provide a

continuous energy supply for survival. This presents few problems to air-breathing animals, but for those which live in water it can be a major limiting factor, as they may be subjected to frequent, or sometimes continuous periods of hypoxia or even anoxia. A number of strategies are available to animals encountering reduced oxygen availability. These range from behavioural avoidance responses in motile animals (e.g emmersion reactions in shore crabs (*Carcinus maenas*) in response to severe hypoxia (Hill, 1989)), increasing ventilation rates and the use of stored oxygen, to the development of high anaerobic metabolism capacities. As well as environmental oxygen limitations, some animals may experience hypoxia or anoxia within particular tissues, usually muscles, due to vigorous activity (so-called 'functional' hypoxia or anoxia).

For many years the production of lactate via the Embden-Meyerhof-Parnas (EMP) pathway associated with severe muscular work in mammals was thought to be the only anaerobic means of energy production. The anaerobic energy metabolism of invertebrates has been the subject of much interest in recent years, both with reference to environmental and functional anaerobiosis. This interest has revealed that aquatic invertebrates exploit a number of catabolic pathways and combinations of pathways for anaerobic energy production. Bivalve molluscs (particularly sessile species) subjected to hypoxia have developed a pathway involving the breakdown of glycogen to succinate and the fatty acids propionate and acetate. This pathway yields more ATP per mole of glucosyl units than the classical lactate pathway, but also has the advantage of producing volatile end-products (e.g. work on *Mytilus edulis* by Klutymans *et al.*, 1975). However, despite an increased energy yield, the rate at which this energy is produced is decreased.

In response to the functional anoxia of vigorous muscular activity, some bivalves resort to mechanisms of energy supply other than the succinate/propionate pathway. More active species are known to accumulate a

variety of compounds after vigorous swimming. These compounds have been identified as end products of glycolysis alternative to L-lactate, known as opines (strombine, octopine, alanopine, tauropine (Gäde & Meinardus-Hager, 1987)). In contrast, crustaceans rely almost solely upon the L-lactate glycolytic pathway for anaerobic energy production, during both functional and environmental anoxia. This is possibly because the majority of bivalve molluscs have only limited motility (even the more active swimming species) compared to Crustacea, and hence cannot resort to behavioural means to avoid anoxia, so must withstand it. Thus the succinate/propionate pathway holds more benefits for these animals than the EMP pathway, providing more energy, whilst a more modest supply rate is sufficient. In crustaceans, however, the emphasis tends to be on rate of energy supply needed during escape reactions. They are subjected to conditions of limited environmental oxygen perhaps less frequently and less severely, and the faster energy supply of glycolysis is favoured.

Studies of the energy metabolism of crustaceans during exercise have shown that L-lactate accumulates in both muscle tissue and haemolymph, indicating glycolytic supply of ATP energy (Phillips et al., 1977; England & Baldwin, 1983; Onnen & Zebe, 1983; Gäde, 1984; Booth & McMahon, 1985; Morris & Greenaway, 1989). L-lactate concentrations continue to increase after the cessation of exhaustive swimming or ambulatory exercise, implying the continued replenishment of the phosphate energy stores of ATP and phosphagens after immediate demand has ceased. Associated with L-lactate increases, intracellular (England & Baldwin, 1983; Raffin et al., 1988) and haemolymph (Phillips et al., 1977; Hamilton & Houlihan, 1992) pH decreases have been observed, and these have been postulated to have regulatory effects upon glycolysis (England & Baldwin, 1985). England & Baldwin (1985) report that the observed decline in pH has effects upon the functioning of L-lactate dehydrogenase, as well as other glycolytic enzymes (phosphofructokinase and pyruvate kinase), and also that altered adenylate concentrations may be

responsible for glycolysis regulation in abdominal muscle of the yabby (*Cherax destructor*) during swimming. However, changes in phospho-metabolites during burst swimming and recovery suggest that in many species such exhaustive or long term activity is limited by energy availability. Onnen and Zebe (1983) and Gäde (1984) reported first phosphagen depletion, and then ATP depletion through long term tail-flip swimming in *Crangon crangon* and *Orconectes limosus* respectively. This has also been noted in *Cherax destructor* (England & Baldwin, 1983).

[31]Phosphorous Nuclear Magnetic Resonance (^{31}P NMR) studies of intact crayfish (*Procambarus clarkii*) and shrimps (*Palaemon spp.*) have produced results that support the contention that failure of ATP supplies is implicated in the onset of exhaustion (Chiba *et al.*, 1989; Thébault *et al.*, 1987; Raffin *et al.*, 1988;). There is common agreement in these studies that ATP and phosphagen levels return very rapidly to normal after cessation of exercise, possibly accounting for the observed rapid recovery of some swimming ability.

The three experiments reported here were undertaken to examine the tail-flip escape swimming of *N. norvegicus*. Swimming ability was assessed in terms of tail-flip performance under repeated stimulation, the rate of its recovery, and the associated metabolic effects, in order to ascertain whether the limiting factors of this response are metabolic or neuronal.

Firstly, the measurement of metabolic changes due to 'exhaustive' swimming was undertaken in order to establish whether fatigue was occurring and to determine the absolute swimming ability of *N. norvegicus*.

Secondly, the metabolic and behavioural changes due to long term swimming and recovery were assessed, to determine whether habituation was occurring, and if so, whether the metabolic effects and performance were the same as those seen in 'exhaustive' swimming (if this is so it is likely that 'exhaustive' swimming is halted by multiple habituation, especially if there is no evidence of fatigue.) This also allowed an assessment to be made of any links

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between metabolic and behavioural recovery.

Lastly, investigations were made into the effects of shifting the stimulation site on tail-flip performance within one tail-flip mediating pathway, and between two pathways, to ascertain if habituation was occurring, and if so, at what point it acted within these pathways.

2.2 MATERIALS AND METHODS

2.2.1 Experimental animals. Adult *N. norvegicus* of both sexes were collected by trawling or creeling from the Firth of Clyde. Animals were returned to the Zoology Department, Glasgow University or the SOAFD Laboratory, Aberdeen, and maintained in well aerated, running seawater (salinity between 30 and 33%.) at temperatures of between 11 and 13°C for a maximum of 5 days before experimentation. They were fed every 2-3 days on white fish or squid flesh but food was withheld for approximately 24 hours before experimentation. Experimental animals were kept in large, communal aquaria, and provided with shelter and gravel substrate to reduce stress. Aquarium space restrictions required that animals were kept at unnaturally high densities and consequently all had their chelae immobilised with elastic bands to prevent injury and stress through fighting. Handling was kept to a minimum and animals were allowed to recover for at least 24 hours after handling and/or emmersion of any length of time before experimentation, to avoid hyperglycaemic or other stress effects (Telford, 1968). All animals used were in intermoult as determined by the degree of withdrawal of the epidermis from the cuticle of the pleopod (after Aiken, 1980), corresponding to Drach's stage C4 (Drach, 1939). Experiments were conducted on *N. norvegicus* of both sexes, ranging in carapace length from 31-65mm. Experiments were conducted at temperatures between 11 and 13°C.

2.2.2 Experimental procedures. Three experiments were undertaken in order to examine the relative importance of metabolic and neuronal factors in the waning and recovery of escape swimming in *N. norvegicus*.

1. Measurements of the changes in anaerobic metabolite concentrations in the abdominal deep muscle and haemolymph of *N. norvegicus* due to 'exhaustive' escape swimming.

Twenty-three animals of both sexes were stimulated to tail-flip via both giant- and non-giant-mediated pathways until no further swimming could be induced. This state was defined as 'functional exhaustion' - animals in this state

will have ceased to swim because of: fatigue in the effector muscles; habituation at separate points in all three tail-flip mediating pathways; or habituation at one point common to all three pathways.

Swimming was induced by repeated prods with a stick applied to any region of the body, and thus involved all three (LG, NG and MG) flip-mediating pathways. The total number of flips performed by each individual was recorded. Although Newland *et al.* (1988) found that there were sex differences in the swimming ability of *N. norvegicus*, these differences were found to be in the relative sizes and numbers of swimming bouts, and they concluded that overall swimming ability did not differ significantly between the sexes. Therefore animals of both sexes were used in this experiment, although berried females were not used.

Immediately following cessation of tail-flipping, animals were removed from the water, a pleopod was removed for moult staging, the abdomen was cut off and placed immediately into liquid nitrogen ($N_{2(l)}$), and a haemolymph sample was taken via a syringe from the arthrodial membrane of a 5th pereiopod. The duration of sampling was kept below 30 seconds to prevent changes in metabolite levels due to handling stress (Telford, 1968). Haemolymph and muscle samples were taken in the same way from ten unexercised animals, which had performed no tail-flips. As a control against handling stress effects, haemolymph samples were also taken from nine unexercised animals from which no muscle samples were taken. Nine unexercised animals which performed tail-flips during capture and sampling were treated separately to allow assessment of the metabolic effects of low level exercise.

Sequential sampling of haemolymph from individuals was found to be impractical. It caused great handling stress to the animals, and involved the removal of significant quantities of haemolymph, rendering metabolite concentration results meaningless due to dilution effects. Sequential sampling

of muscle tissue by biopsy was also thought to be impossible in practice. Sacrificial sampling methods were therefore adopted. Comparison of immediately pre- and post-exercise metabolite concentrations was made by pooling data from the sub-populations of experimental animals either subjected to 'exhaustive' exercise or not. In this way the metabolic consequences of 'exhaustive' swimming were determined.

2. Changes in anaerobic metabolite concentrations in *N. norvegicus* abdominal muscle and haemolymph due to escape swimming and subsequent recovery, and their relation to swimming performance.

Only male *N. norvegicus* were used in this experiment as the composition of swimming sequences was being examined in terms of bout size, and this has been shown to differ between the sexes in *N. norvegicus* (Newland *et al.*, 1988). *N. norvegicus* were stimulated to swim via the LG pathway alone, since this allowed an unambiguous distinction between giant-mediated flips (having an upward trajectory) and NG-mediated flips (with a flat trajectory). This stimulus, in the form of a standardised tap, was applied to a localised region of the telson of each animal. In initial tests, a mechanical trigger system was used in order to standardise stimulus strength. However, the very localised and abrupt nature of this stimulus made it rather ineffective in inducing swimming bouts. Taps delivered manually proved to be more effective, probably because they stimulated a greater number of receptors over a more extended period of contact. Manual taps were therefore adopted as the standard stimulus in this experiment, and care was taken to maintain the same intensity and positioning. Taps were delivered repeatedly (frequency = 1/20Hz) until no further response was elicited. Failure to respond to three consecutive taps was taken to represent the waning of tail-flipping in response to this stimulus. The total number of tail-flips performed by, and the total number of stimuli delivered to each animal were recorded.

The tail-flips of each swimming bout were divided into LG and NG flips,

according to the angle of their trajectory and temporal occurrence within a bout (i.e. flips subsequent to initial flips were assumed to be NG-mediated). The initial swimming sequence of each animal tested was divided into quarters to allow the changes in responses to the repeated application of the standard stimulus with time to be detected, whilst acting as a control for the variation between individuals in total swimming performance. For each quarter, mean bout size (expressed as the number of NG flips in each bout only, to allow statistical analysis), number of LG flips expressed as a percentage of the total number of flips performed, and percentage of stimuli applied which provoked a response were calculated.

Following bouts of escape swimming, groups of six to eight animals were then either sampled immediately for haemolymph and muscle tissue (as above), or allowed to recover for periods of between 5 minutes and 10 hours before sampling. In order to assess any decrement of arginine phosphate during escape swimming a further group of six animals was sampled after performing around 30 tail-flips, which represented approximately 60% of the mean total number performed in response to this stimulus. In a parallel series of experiments, further groups of six to eight *N. norvegicus* were stimulated to swim in response to the same stimulus, but after similar recovery periods were stimulated to swim again. Recording the number of tail-flips performed in this second swimming period yielded a measurement of recovery of swimming ability (expressed as the percentage of initial swimming ability for each individual). This allowed a comparison to be made of metabolic and behavioural recovery at discrete time periods after the cessation of swimming, and the identification of any metabolic factors linked to behavioural recovery over time.

3. Changes in recovery of swimming ability due to alteration of the site of stimulation.

Animals were stimulated to swim via the LG pathway in response to a standard stimulus applied to a localised region of the telson, or via the MG

pathway in response to the stimulus applied to the rostrum (in this case, the standardised stimulus elicited only single giant-mediated tail-flips, and virtually no swimming bouts were observed). Stimulation was continued until no response could be obtained to three consecutive stimuli. Six stimulation protocols were followed using different groups of between nine and sixteen animals. These were as follows:

I) Stimulation on one region of the telson (eliciting LG flips) to the point of tail-flip failure, recovery for 30 minutes, then restimulation at the same point with the same stimulus (n = 16).

II) Stimulation on the left uropod (LG flips), recovery for 30 minutes, then restimulation on the right uropod (n = 16).

III) Stimulation on the left uropod (LG flips), followed immediately by stimulation on the right uropod (i.e. no recovery period) (n = 16).

IV) Stimulation on the rostrum (MG flips), recovery for 30 minutes, then restimulation at the same point (n = 13).

V) Stimulation on the rostrum (MG flips), followed immediately by stimulation on the telson (LG flips) (i.e. no recovery period) (n = 9).

VI) Stimulation on the telson (LG flips), followed immediately by stimulation on the rostrum (MG flips) (i.e. no recovery period) (n = 10).

In this way it was possible to determine the influence of afferent neuronal factors upon production of tail-flips via the LG pathway, and to determine whether swimming in response to the standard stimulus ceased due to habituation to that stimulus.

2.2.3 Preparation of muscle tissue and haemolymph samples for analysis of metabolite concentrations.

Haemolymph. Immediately after collection, haemolymph samples were mixed with an equal volume of chilled 0.6M perchloric acid (PCA), to precipitate out proteins and to denature enzymes. After mixing, samples were centrifuged for 20 minutes at 6240g at 0-4°C in an Heraeus Minifuge RF

refrigerated centrifuge. The supernatant was removed and then neutralised by dropwise addition of $1/20^{th}$ volume of 2M potassium bicarbonate ($KHCO_3$). The precipitate of potassium perchlorate was removed by a further 10 minutes centrifugation (6240g at 0-4°C). The samples were then stored at -20°C until required.

Abdominal Muscle. After freezing in liquid nitrogen ($N_{2(l)}$), a small portion of the deep flexor muscle was dissected out of each abdomen and ground to a powder under $N_{2(l)}$ in a mortar. From each muscle sample, three subsamples of approximately 50mg were transferred to chilled Eppendorf centrifuge tubes (exact weights were recorded for use in concentration calculations). To each subsample, $500\mu l$ of chilled 0.3M PCA was added, the mixture was thoroughly agitated, and centrifuged for 10 minutes at 6240g and 0-4°C. The resulting supernatant was removed and stored on ice. An additional $500\mu l$ PCA was added to the remaining pellet, mixed and centrifuged for a further 20 minutes. The supernatant was then removed, combined with the first and neutralised with 2M $KHCO_3$. The samples were centrifuged a final time to remove the potassium perchlorate precipitate, and then stored at -70°C until required.

2.2.4 Analysis of metabolite concentrations.

Haemolymph. PCA extracts from haemolymph samples were assayed for the following metabolites;

L-lactate. Concentrations of L-lactate were determined using the method of Gutmann & Wahlefeld (1974) incorporating the modifications of Engel & Jones (1978) and Hill (1989). L-lactate is oxidised to pyruvate, catalysed by lactate dehydrogenase (EC.1.1.1.28), reducing NAD+ to NADH. It is assumed that the formation of NADH is proportional to the amount of L-lactate present.

D-glucose. D-glucose concentrations were determined using the method of Slein (1965) as modified by Hill (1989). This is a two step reaction involving the phosphorylation of D-glucose to glucose-6-phosphate (G-6-P) by hexokinase

(EC.2.7.1.1) followed by oxidation of G-6-P to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (EC.1.1.149). NADP+ is reduced to NADPH, the concentration of which is assumed proportional to the concentrations of G-6-P and D-glucose.

Abdominal muscle. In addition to those specified above, PCA extracts from abdominal muscle tissue were assayed for the following;

ATP and phospho-L-arginine. Concentrations of phospho-L-arginine were determined using the method of Lamprecht & Trautschold (1974) as modified by Hill (1989). Arginine kinase catalyses the dephosphorylation of phospho-L-arginine, which phosphorylates ADP to ATP. ATP concentrations are then determined by the method specified above for D-glucose, except that ATP is the limiting factor.

Glycogen. Concentrations of glycogen were determined by the method of Keppler & Decker (1974) as modified by Hill (1989). This involves hydrolysis of 1-4 and 1-6 glycosidic bonds of glycogen by 1-4,1-6 amylglucosidase (EC.3.2.1.20). The resulting glucosyl units are assayed by the reaction specified for D-glucose determination above.

All concentrations in muscle tissues are expressed as μmol g^{-1} wet weight, except those of glycogen which are in μmol g^{-1} dry weight, all concentrations in haemolymph samples are in μmol ml^{1-}. All determinations were made using a Shimadzu RF 5000 spectrofluorophotometer. Internal standards using known metabolite concentrations were run for all assays, and all except the phospho-L-arginine assay showed consistent recoveries of over 90%.

2.2.5 Statistical analysis of data. In all statistical tests differences were considered to be significant when $P \leq 0.05$. Metabolite concentrations during exercise and subsequent recovery were compared using oneway analysis of variance, followed by Tukey tests. Analysis of swimming sequence composition was done by arcsine transformation followed by oneway analysis of variance and Tukey tests. All other comparisons were made using two-sample t-tests.

2.3 RESULTS

2.3.1 Swimming performance and its recovery. When induced to perform 'exhaustive' exercise by stimulation of all tail-flip mediating pathways (Experiment 1), the mean number of tail-flips performed by *N. norvegicus* was 103.9 ± 33.4 tail-flips. This figure is significantly higher than the 52.0 ± 30.6 flips performed in response to stimulation of LG mediated swimming alone (Experiment 2)($P < 0.05$, t-test, Figure 2.1).

During repeated application of standard stimuli to the abdomen, eliciting both LG mediated tail-flips and subsequent non-giant swimming flips (swimming bouts) (Experiment 2), the two types of flip showed different rates of waning. Figure 2.2 shows that through the time course of initial swimming sequences there was a significant reduction in bout size in response to single stimuli (from 1.4 ± 1.0 NG flips to 0.9 ± 1.2 NG flips per bout, $P < 0.05$, ANOVA), as the number of NG flips accompanying each LG response declined, and LG responses thus became a significantly higher proportion of the total number of flips produced (increasing from 53.6 ± 8.7% to 68.9 ± 11.6%, $P < 0.005$, ANOVA, Figure 2.3). This reflects the more rapid waning of the NG flip, compared to the LG response. A steady waning was observed in the production of LG flips; the percentage response to stimulations decreased significantly from 98.5 ± 5.5% in the first quarter to 78.6 ± 7.9% in the last quarter of swimming sequences ($P < 0.005$, ANOVA, Figure 2.4). The waning of the tail-flipping response in these experiments appeared artificially abrupt, due to the stimulation regime used. This was because waning was assumed to have occurred after three consecutive failures to respond, and stimulation was therefore discontinued.

Figure 2.5 shows the course of recovery of total LG and NG swimming ability over time, expressed as the percentage of initial total swimming performance of each individual regained when restimulated. This controlled for the large variation seen in individual swimming ability in response to the

FIGURE 2.1. Histogram showing the variation in the mean number of tail-flips performed by *N. norvegicus* in response to stimulation of all tail-flip mediating pathways (exhaustive stimulation) and the LG pathway (standard stimulation). Error bars represent standard deviation about means. (n = 23 for Exhaustive stimulation; n = 85 for Standard stimulation.)
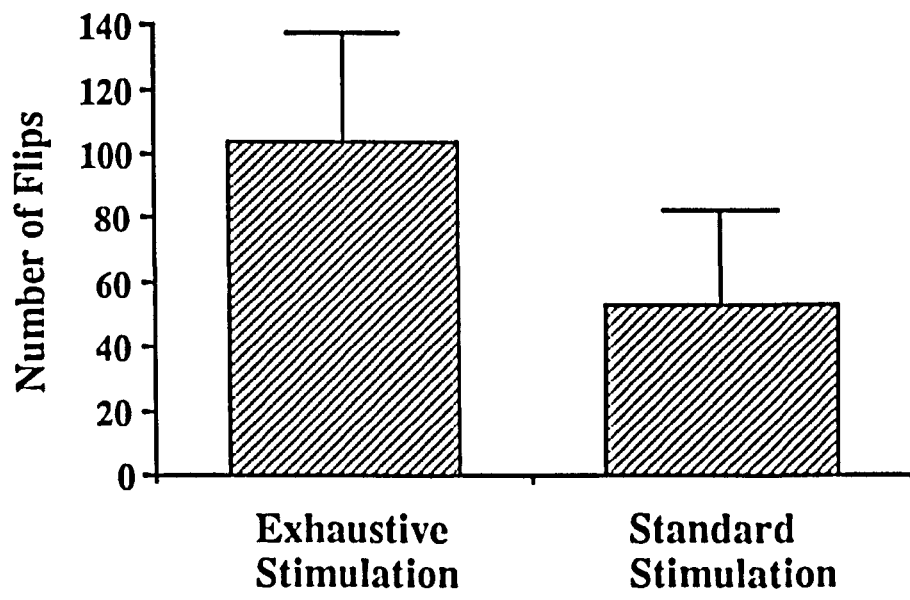
FIGURE 2.2. Histogram showing the reduction in mean bout size (in numbers of NG tail-flips) produced in response to single stimuli over the duration of swimming sequences of *N. norvegicus*. Error bars represent standard deviations about means. (n = 85).

FIGURE 2.3. Histogram showing the increase in LG tail-flips as a percentage of the total number of tail-flips produced by *N. norvegicus* in each quarter of swimming sequences, over the duration of those swimming sequences. Error bars represent standard deviations about means. (n = 85).
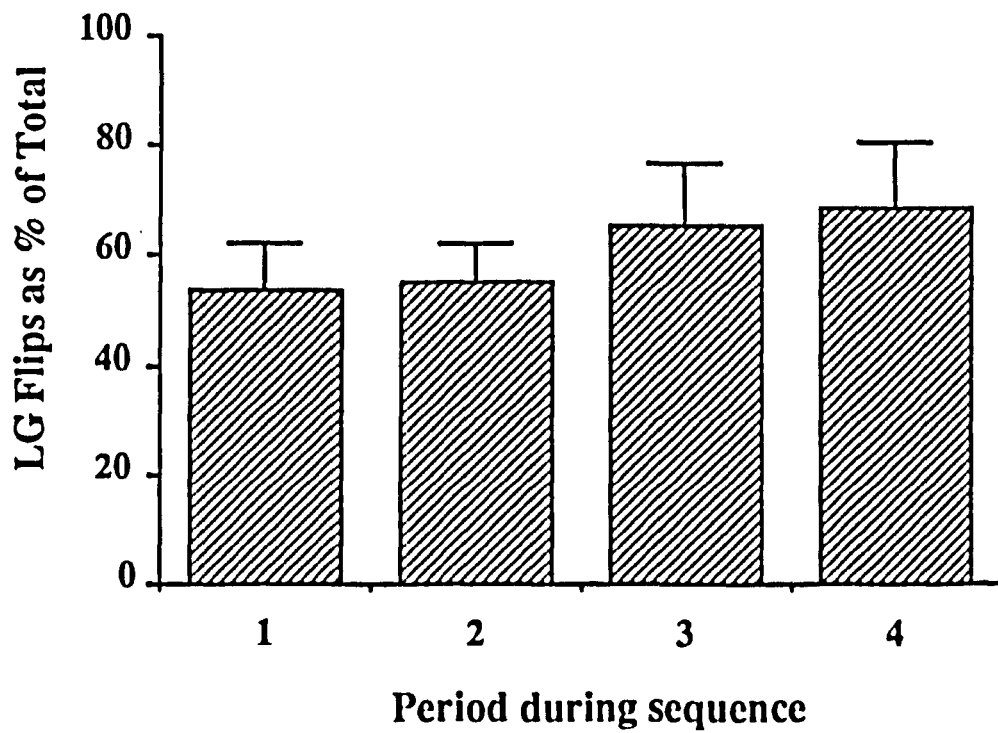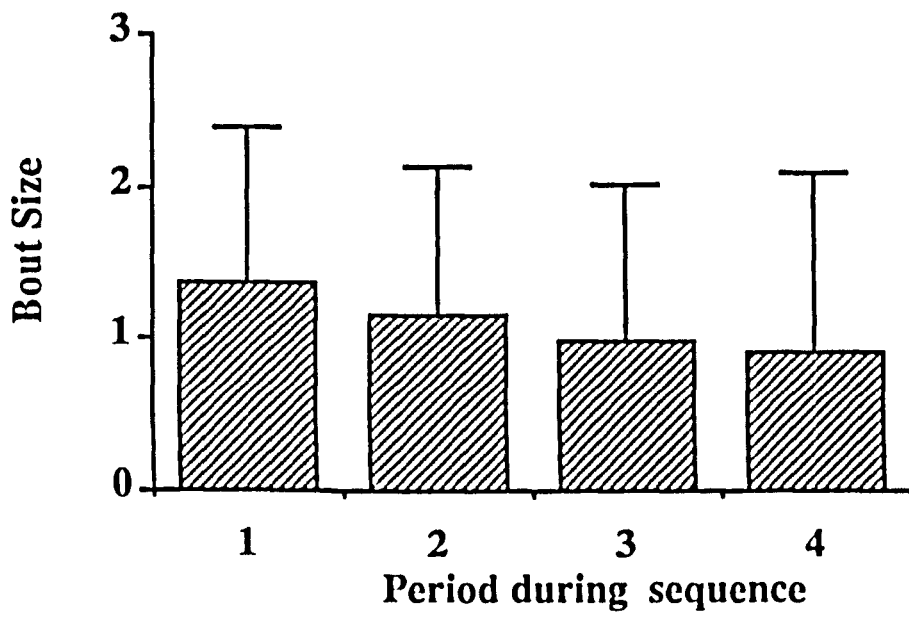
FIGURE 2.4. Histogram showing the decrease in the percentage response to stimuli by *N. norvegicus* over the course swimming of sequences. Error bars represent standard deviations about means. (n = 85).

FIGURE 2.5. Graph showing the recovery of total LG and NG swimming ability of *N. norvegicus* over time after stimulation of swimming to cessation with a standard stimulus, expressed as mean percentages of initial total swimming ability of each individual. Error bars represent standard deviations about means. (n = 7 for each group, except those tested at 5 and 180 minutes, where n = 6).
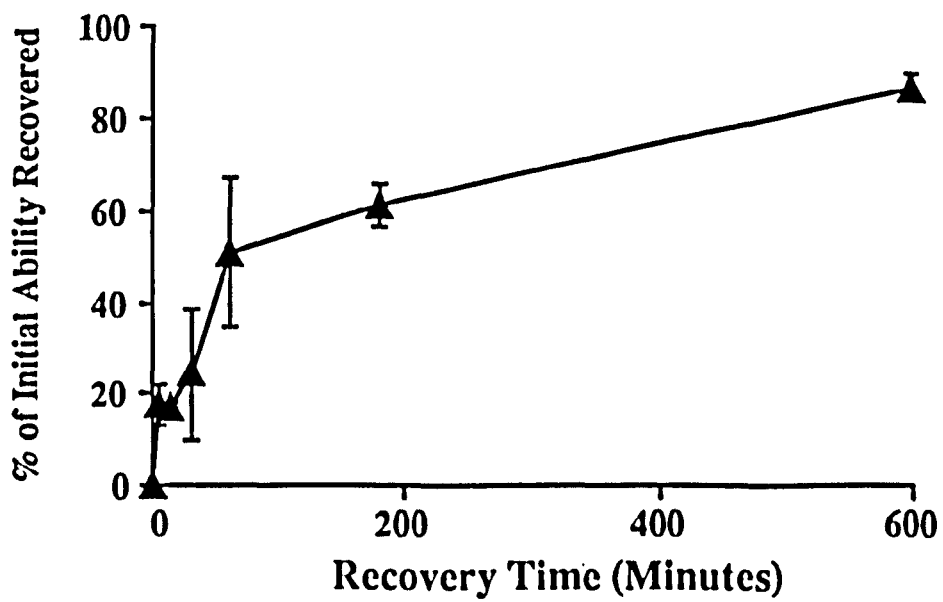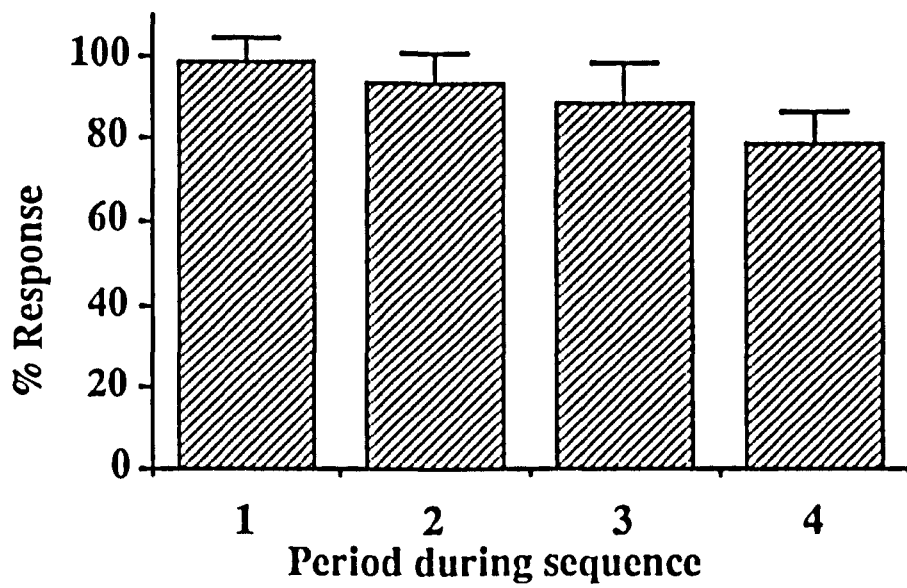
standard stimulus. Over 80% of full swimming ability was regained within a recovery period of 10 hours. This recovery was initially rapid, beginning within 5 minutes of initial failure to respond, and reached over 50% of initial ability within the first hour and 60% after 3 hours. Therefore, although the greater part of swimming ability is lost for only a short period after prolonged swimming, full tail-flipping potential takes longer to recover.

A change in the site of uropod stimulation from one side to the other resulted in the restoration of a greater proportion of tail-flipping ability than was shown in response to restimulation at the same site (Experiment 3 , parts I & II). However, this difference was not significant (P>0.05, t-test). After a recovery period of 30 minutes following failure of tail-flipping in response to stimulation through the telson, restimulation at the same site resulted in animals performing a mean of 27.4 ± 5.0% of their initial tail-flip performances. In response to a shifting of stimulation site from the left to right uropod after 30 minutes recovery, restimulation resulted in the production of slightly more tail-flips, expressed as a percentage of initial flip numbers (43.2 ± 7.6%; Figure 2.6). Nevertheless, transferring the site of stimulation immediately upon cessation of tail-flipping, allowing no recovery time, resulted in the production of an additional 21.8 ± 6.1% of tail-flips (expressed as a percentage of initial number performed) (Figure 2.7). There was, however, no difference in the number of LG flips elicited by stimulation at the two different sites on the abdomen (a mean of 53.8 ± 32.0 LG flips produced by stimulation of the telson compared to a mean of 58.9 ± 23.9 LG flips produced as a result of stimulation of the left uropod, P>0.05, t-test), which excludes the possibility that any measured differences in swimming performance could be due to differing responsiveness of sensory fields at the two sites.

The switching of stimulation site from the sensory field of MG mediation to that of LG mediation, after the cessation of MG tail-flipping did not result in any decrement of LG tail-flipping ability. There was no significant difference

FIGURE 2.6. Histogram showing the effect of a change in stimulation site on recovery of LG swimming ability of *N. norvegicus* 30 minutes after stimulation of swimming to cessation with a standard stimulus, expressed as mean percentages of initial swimming ability of each individual. 1) = initial and second stimulation sequences applied to the telson, n = 16; 2) = initial stimulation sequence applied to left uropod, second sequence applied to right uropod, n = 16. Error bars represent standard deviations about means.

FIGURE 2.7. Histogram showing the effect of a change in stimulation site on recovery of LG swimming ability of *N. norvegicus* immediately after stimulation to cessation with a standard stimulus, expressed as mean percentages of initial swimming ability of each individual. Stimulation regimes as for Figure 2.6, n = 16. Error bars represent standard deviations about means.
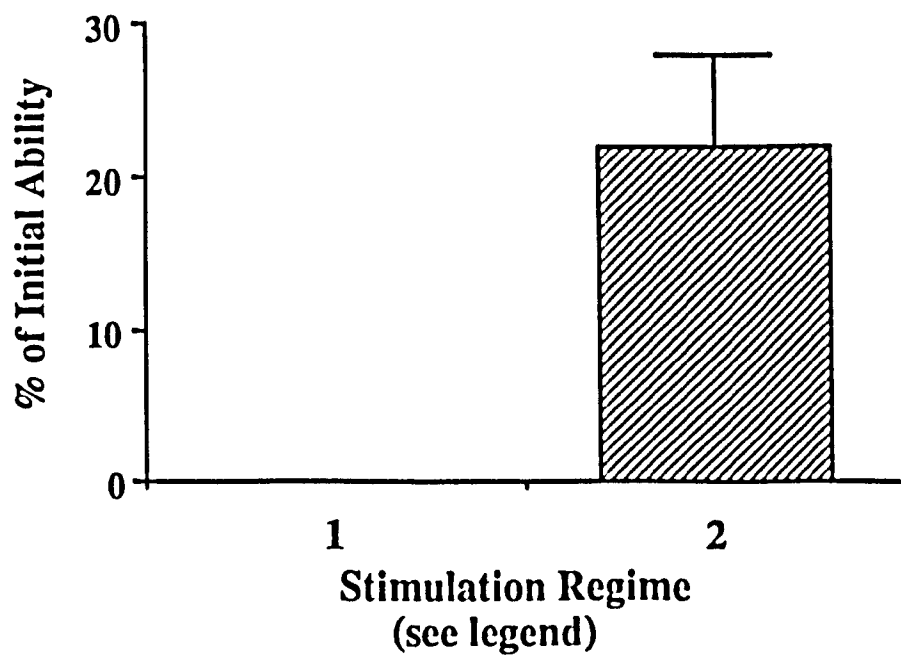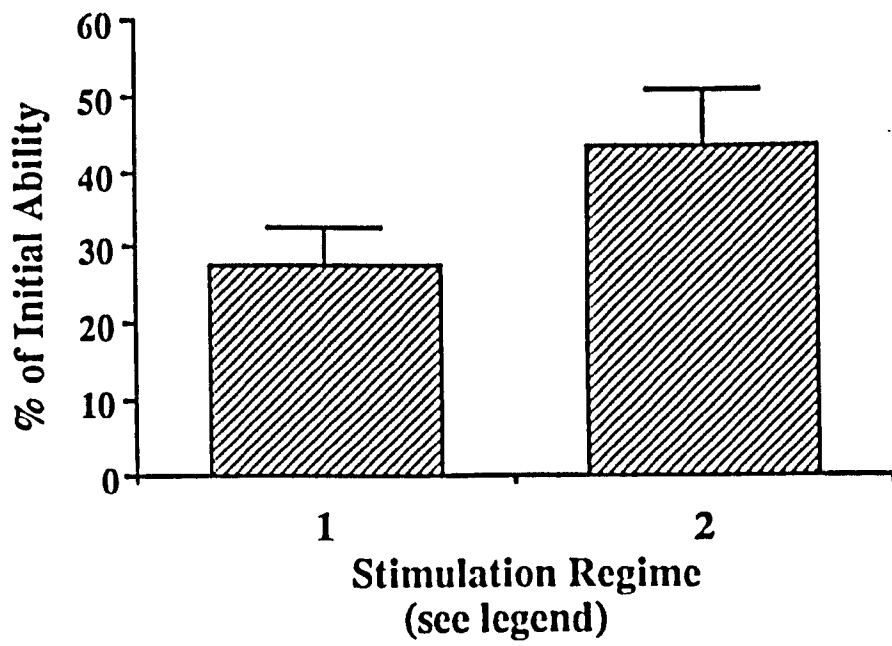
between the number of LG flips performed by experimentally naive animals and the number performed by animals previously stimulated via the MG pathway (56.6 ± 27.7 compared with 60.4 ± 31.2; P>0.05, t-test; Figure 2.8). Interestingly, there was an alteration of MG flipping performance due to the performance of LG tail-flips. The number of MG mediated flips performed by animals which had already been stimulated to swim to cessation via the LG pathway was significantly lower than the capacity of animals stimulated only by rostral taps (4.2 ± 4.6 compared with 15.8 ± 6.9; P<0.05, t-test; Figure 2.9).

2.3.2 Metabolite concentration changes due to exercise and recovery.

'Exhaustive swimming' (Experiment 1) resulted in large, significant changes in both muscle and haemolymph L-lactate concentrations (14.3 ± 8.3 to 26.2 ± 7.4 μmol g^{-1} P<0.001, ANOVA, and 0.51 ± 0.06 to 0.86 ± 0.38 μmol ml^{-1} P<0.01, ANOVA respectively) (Figures 2.10a & b respectively). The increases in both tissues did not occur in the initial stages of exercise and indicate that no significant change was brought about by low level exercise. The build-up of L-lactate in muscle and its release into the haemolymph indicated that energy for swimming was being derived from anaerobic glycolysis in the latter period of swimming sequences.

A similar situation was observed for muscle D-glucose (increasing from 0.62 ± 0.56 to 1.29 ± 0.61 μmol g^{-1} P<0.005, ANOVA), and again no change occurred during the initial period of swimming (Figure 2.10c), but a significant increase was evident immediately after exercise. This increase in D-glucose also suggests the onset of replenishment of ATP supplies, possibly representing the mobilisation of resources for aerobic or anaerobic respiration. Aerobic contribution to the ATP pool cannot be discounted, but high L-lactate production does imply a large anaerobic contribution to ATP production in the latter period of severe escape swimming. In contrast, haemolymph D-glucose concentrations exhibited a small but statistically insignificant increase as a result of exercise and showed much variation within groups (Figure 2.10d). This would

FIGURE 2.8. Histogram showing the maintenance of LG swimming ability of *N. norvegicus* after stimulation of MG swimming to cessation, expressed as mean numbers of tail-flips. Error bars represent standard deviations about means. (n = 16 for LG stimulation alone; n = 9 for LG after MG stimulation).

FIGURE 2.9. Histogram showing the reduction of MG swimming ability of *N. norvegicus* after stimulation of LG swimming to cessation, expressed as mean numbers of tail-flips. Error bars represent standard deviations about means. (n = 9 for MG stimulation alone; n = 10 for MG after LG stimulation).
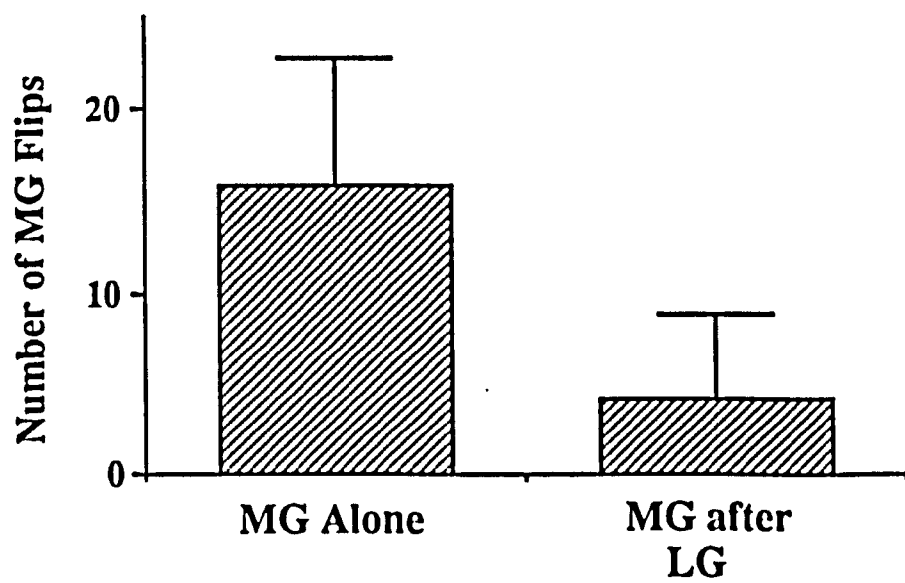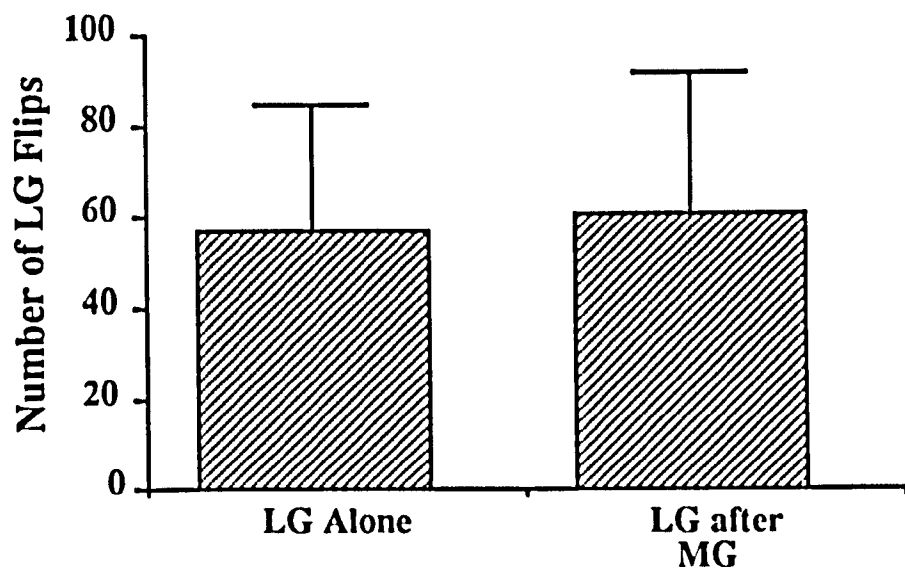
FIGURE 2.10. Histograms showing the mean concentrations of metabolites in the tail muscles of groups of *N. norvegicus* before, during and after 'exhaustive swimming' in response to repeated stimulation via all tail-flip mediating pathways. R = rested animals, n = 10; Lt = animals performing a low number of tail-flips, n = 8; E = animals apparently exhausted, n = 23. a) muscle L-lactate; b) haemolymph L-lactate; c) muscle D-glucose; d) haemolymph D-glucose; e) muscle ATP. Error bars represent standard deviations about means.
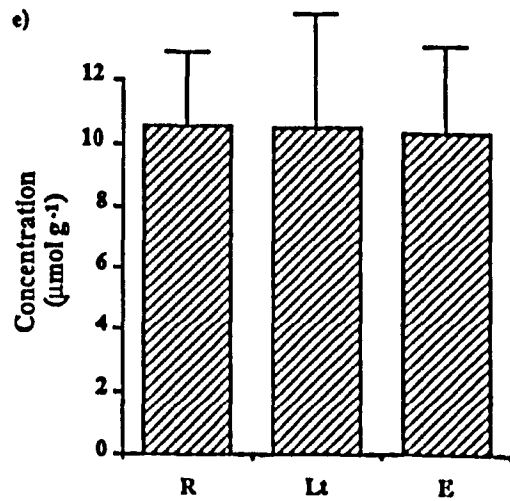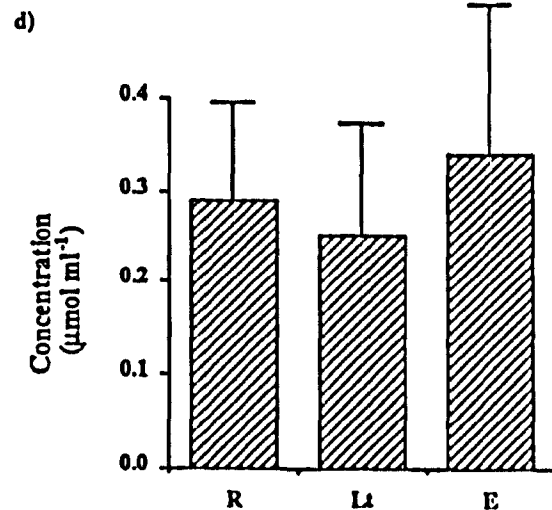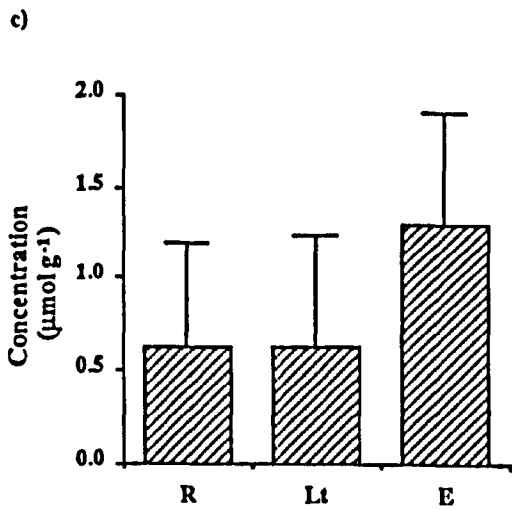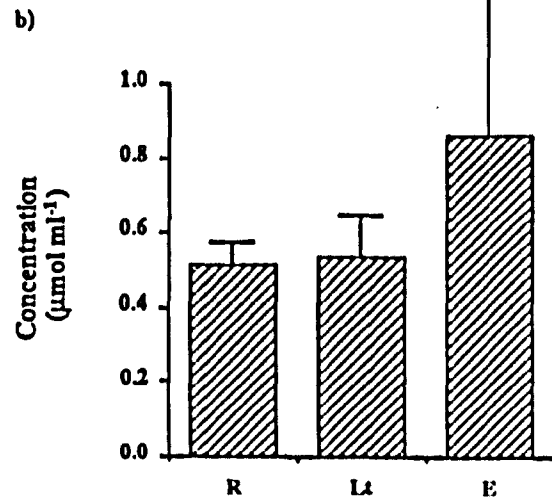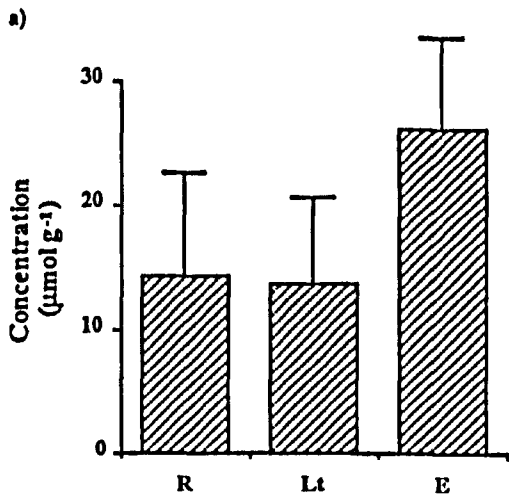
suggest that if resource mobilisation is occurring to meet the demands of escape swimming, then it is the mobilisation of endogenous muscle reserves (of glycogen/glucose) rather than the delivery of fuels from exogenous sources via the haemolymph. In addition, haemolymph D-glucose concentrations in control animals subjected to handling but not muscle tissue sampling showed no significant deviation from those observed in animals which were sampled for muscle tissue. This indicates that the act of abdomen removal itself, although massively traumatic, did not result in the short-term elevation of D-glucose concentration symptomatic of handling stress effects (Telford, 1968). This, however, does not rule-out the possibility that rested and control haemolymph D-glucose concentrations are themselves elevated above the true values present in un-handled animals. Nevertheless such elevation seems unlikely in the light of Telford's finding that, in *Homarus americanus*, the increased concentration of D-glucose in the haemolymph due to handling was not manifested until at least 30 seconds after handling. Handling times in the present experiments were kept below 30 seconds.

At the point of cessation of swimming after severe exercise, ATP concentrations ($10.27 \pm 2.81 \, \mu$mol g^{-1}) showed no significant difference from those of animals at rest ($10.50 \pm 2.34 \, \mu$mol g^{-1}, P$>$0.1; Figure 2.10e). Furthermore, after the initial period of exercise (a few tail-flips) ATP concentrations were unchanged ($10.49 \pm 3.80 \, \mu$mol g^{-1}). This suggests either that ATP re-supply to the muscles is very efficient, or that ATP production by glycolysis was being channelled to other areas, such as the replenishment of phosphagen stores depleted during the initial stages of exercise.

Lower levels of swimming in response to a standard stimulus (Experiment 2) resulted in broadly similar catabolic changes. Muscle tissue L-lactate concentrations (Figure 2.11) were again increased and, although not significantly elevated immediately post-exercise, this L-lactate production continued during the initial hour of the recovery period, after the immediate

FIGURE 2.11. Graph showing the variation of mean concentrations of L-lactate in the tail muscles and haemolymph of groups of *N. norvegicus* before, and during recovery from, LG mediated swimming to cessation in response to repeated standard stimulation. Error bars represent standard deviations about means. (n = 7 animals for each group, except for that tested at 600 minutes, where n = 6; the concentration for each animal is based on the mean of the concentrations in three replicate PCA extracts).

FIGURE 2.12. Graph showing the variation of mean concentrations of D-glucose in the tail muscles and haemolymph of groups of *N. norvegicus* before, and during recovery from, LG mediated swimming to cessation in response to repeated standard stimulation. Error bars represent standard deviations about means. (Sample sizes as in Figure 2.11).
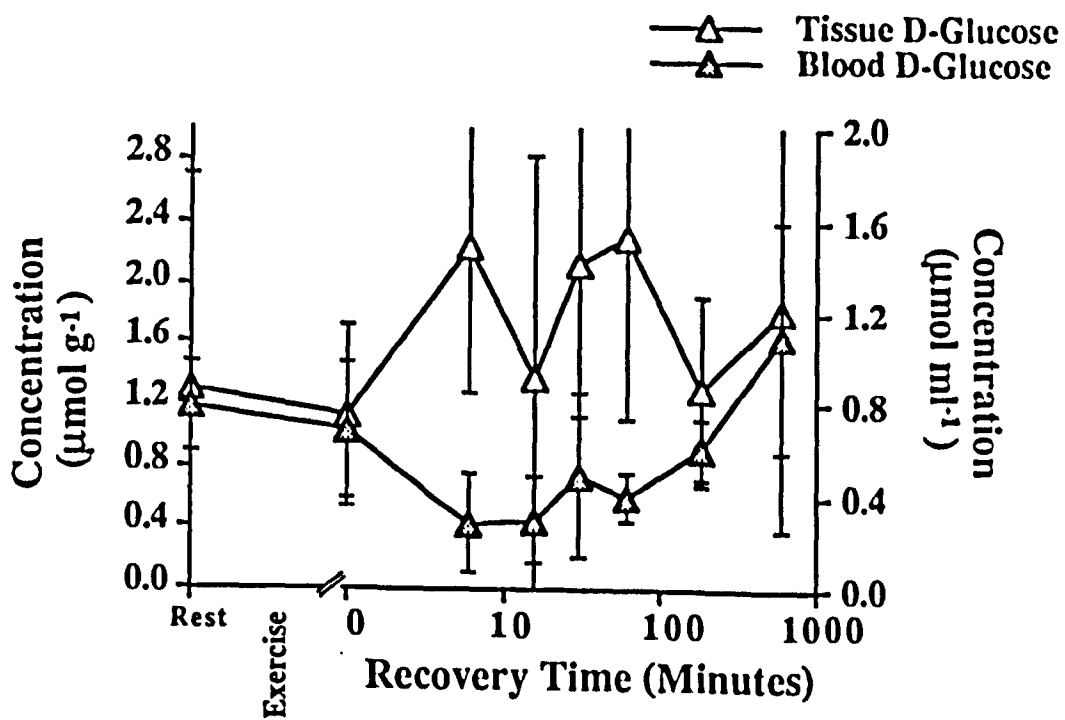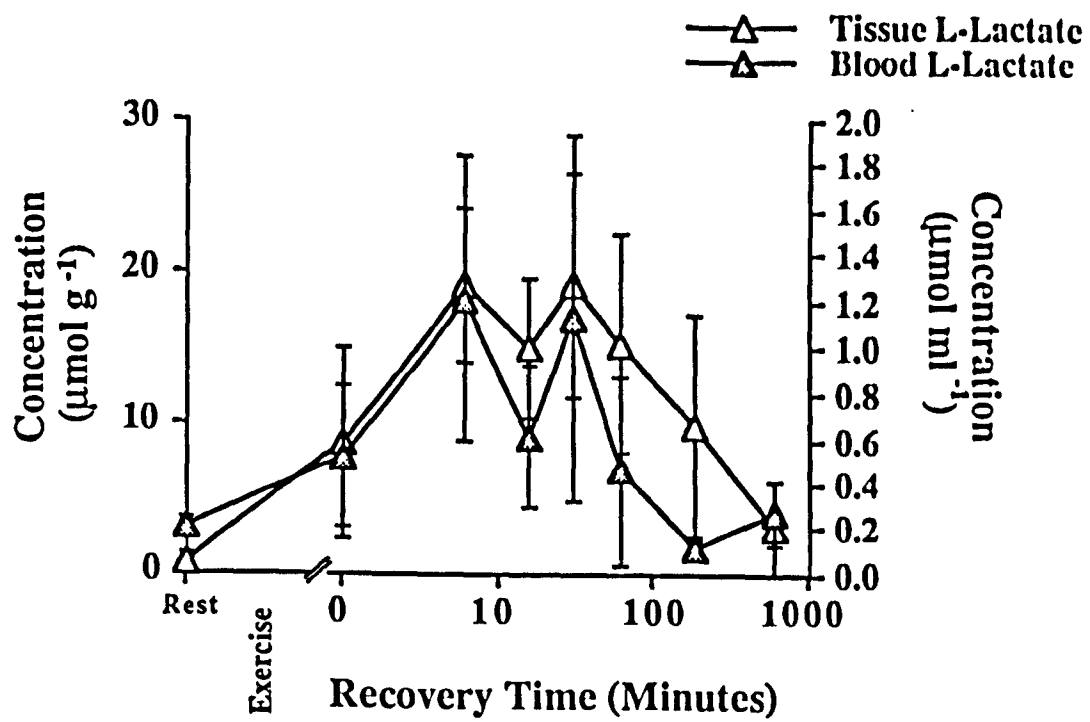
energy requirements of exercise had ceased. This further suggests a replenishment of the muscle pool of phosphagens and/or phosphates. Peak L-lactate concentrations were reached very quickly after the cessation of escape swimming (by 5 minutes, at a mean concentration of $19.12 \pm 5.01 \ \mu\text{mol g}^{-1}$) and remained elevated through recovery, gradually decreasing until after approximately 10 hours the mean L-lactate concentration (3.12 ± 3.16 $\mu\text{mol g}^{-1}$) was not significantly different from pre-exercise levels (0.69 ± 0.68 $\mu\text{mol g}^{-1}$). This maintenance of elevated L-lactate concentrations during most of the measured recovery period is suggestive of some delay in lactate clearance mechanisms (either some form of gluconeogenesis, or redirection of L-lactate back into the latter stages of aerobic respiration and the Tricarboxylic acid (TCA) cycle), or of continued L-lactate production.

A very rapid initial release of L-lactate from muscle to haemolymph was indicated by the marked synchrony with which concentrations of this metabolite rose in these two tissues. The pattern of L-lactate concentration changes in the haemolymph due to exercise was similar to that seen in muscle tissue (Figure 2.11). Immediately after exercise L-lactate concentrations in the haemolymph had risen from their resting value of $0.21 \pm 0.04 \ \mu\text{mol ml}^{-1}$ to $0.52 \pm 0.32 \ \mu\text{mol}$ ml^{-1}, and within 5 minutes of recovery had significantly increased to the peak value of $1.22 \pm 0.62 \ \mu\text{mol ml}^{-1}$ ($P<0.001$, ANOVA). Despite the similarity in patterns of initial change, elevated L-lactate concentrations within the haemolymph persisted for a shorter period, and after 1 hour were indistinguishable from resting values.

However, the L-lactate concentrations seen in this second experiment were significantly lower than in the first (compare Figures 2.10a & b with Figure 2.11). This was true of both the immediately post-exercise levels ($P<0.001$, t-test) and the rested concentrations ($P<0.05$, t-test). This suggests that the discrepancy was due either to calibration error during assaying, or to a general population difference between the two groups of animals involved, rather than

a difference in response to differing exercise regimes. This argument is supported by the fact that the concentration changes in each experiment are of similar magnitudes, indicating a similar response.

D-Glucose concentrations within abdominal deep flexor muscle tissues showed much variation through exercise and recovery, with no significant differences being detected ($P > 0.1$, ANOVA; Figure 2.12). These concentrations show some parallels with the glucose-6-phosphate results (Figure 2.13), and their large variations may be due to compounding factors, such as their common involvement in aerobic as well as anaerobic respiration, and also possible handling stress effects through recovery. This second point seems unlikely, however, as initial large elevations in D-glucose concentrations were not seen.

Haemolymph D-glucose concentrations did not change as an immediate consequence of exercise, but showed a significant decrease after 5 minutes ($P < 0.001$, ANOVA; Figure 2.12). Concentrations remained below resting levels for around 1 hour, before returning to a level no longer significantly different to those observed pre-exercise.

These D-glucose results are in direct contrast to those seen as a result of 'exhaustive' tail-flipping, and suggest that in this case glucose resources were being imported from exogenous stores via the haemolymph to the muscles of the abdomen. The use of endogenous sources of muscle fuel is further called into question by the results obtained for assays of muscle glycogen.

Muscle tissue ATP concentrations showed no significant deviations from resting values due to exercise or during recovery, and indeed were remarkably constant at between 8.27 ± 1.72 and 9.73 ± 1.53 μmol g^{-1} (the resting figure being 8.67 ± 0.85 μmol g^{-1}; Figure 2.14), complementing the results obtained in response to 'exhaustive' swimming. These results suggest that at no point during swimming or recovery does the muscle ATP pool drop to a level likely to limit muscular activity.

FIGURE 2.13. Graph showing the variation of mean concentrations of glucose-6-phosphate in the tail muscles of groups of *N. norvegicus* before, and during recovery from, LG mediated swimming to cessation in response to repeated standard stimulation. Error bars represent standard deviations about means. (Sample sizes as in Figure 2.11).

FIGURE 2.14. Graph showing the variation of mean concentrations of ATP in the tail muscles of groups of *N. norvegicus* before, and during recovery from, LG mediated swimming to cessation in response to repeated standard stimulation. Error bars represent standard deviations about means. (Sample sizes as in Figure 2.11).
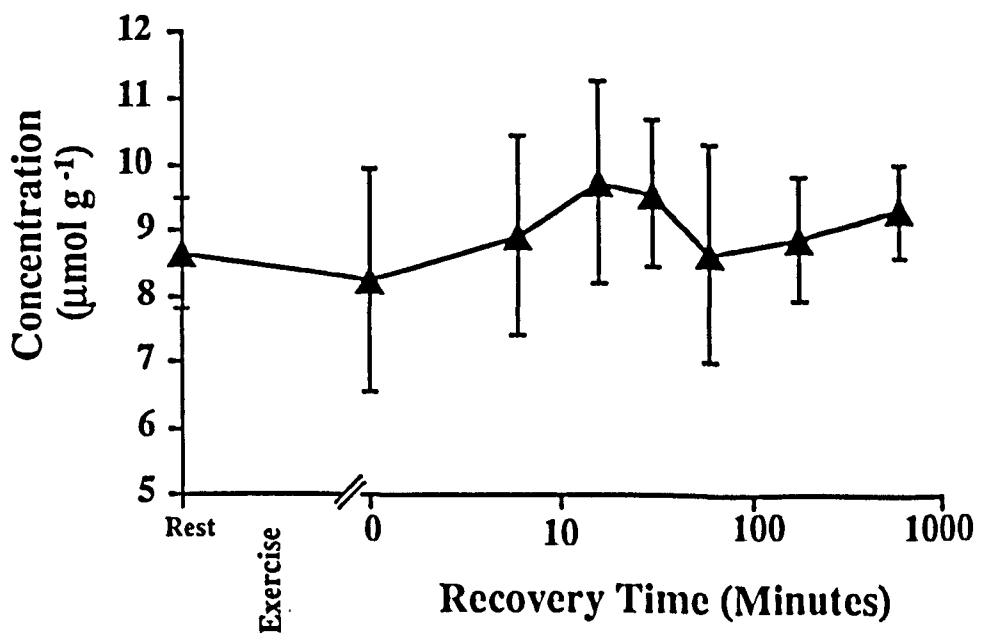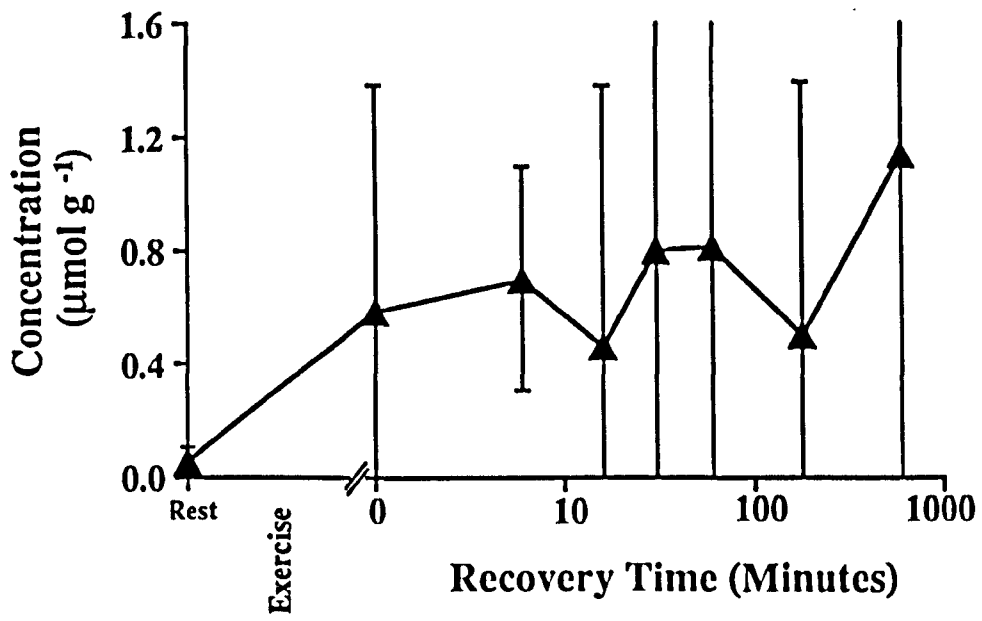
There was, however, a significant change in the concentration of the other important phosphometabolite in abdominal muscle, phospho-L-arginine (Figure 2.15; P<0.05, ANOVA). Anomalously, this significance was the result of a slight increase above rested concentration during muscular activity, compared with a slight fall during the early period of recovery.

Interestingly, glycogen concentrations within abdominal deep flexors also showed no significant variations from those seen in resting animals, either directly due to exercise, or during recovery (Figure 2.16; P>0.05, ANOVA). These figures, expressed as μmol g^{-1} dry weight, also appear to be very low, possibly as a result of assaying error, but are supported by the results presented in Chapter 3.

2.3.3 Relationship between metabolic and behavioural recovery. In Figures 2.17 & 2.18, the changes in L-lactate concentrations of both muscle and haemolymph tissue have been plotted against recovery of swimming ability. This allows an assessment to be made of the correlation between L-lactate concentrations and cessation and recovery of tail-flipping. Figure 2.5 shows that the regaining of swimming ability begins almost immediately after swimming cessation, and Figure 2.11 that L-lactate concentrations continue to rise after failure to swim. Figures 2.17 & 2.18 confirm that in the early stages of behavioural recovery, L-lactate concentrations remain considerably elevated, and that approximately 70% of behavioural recovery was achieved in the presence of muscle and haemolymph concentrations above those present immediately post-exercise.

FIGURE 2.15. Graph showing the variation of mean concentrations of phospho-L-arginine in the tail muscles of groups of *N. norvegicus* before, and during recovery from, LG mediated swimming to cessation in response to repeated standard stimulation. Error bars represent standard deviations about means. (Sample sizes as in Figure 2.11).

FIGURE 2.16. Graph showing the variation of mean concentrations of glycogen in the tail muscles of groups of *N. norvegicus* before, and during recovery from, LG mediated swimming to cessation in response to repeated standard stimulation. Error bars represent standard deviations about means. (Sample sizes as in Figure 2.11).
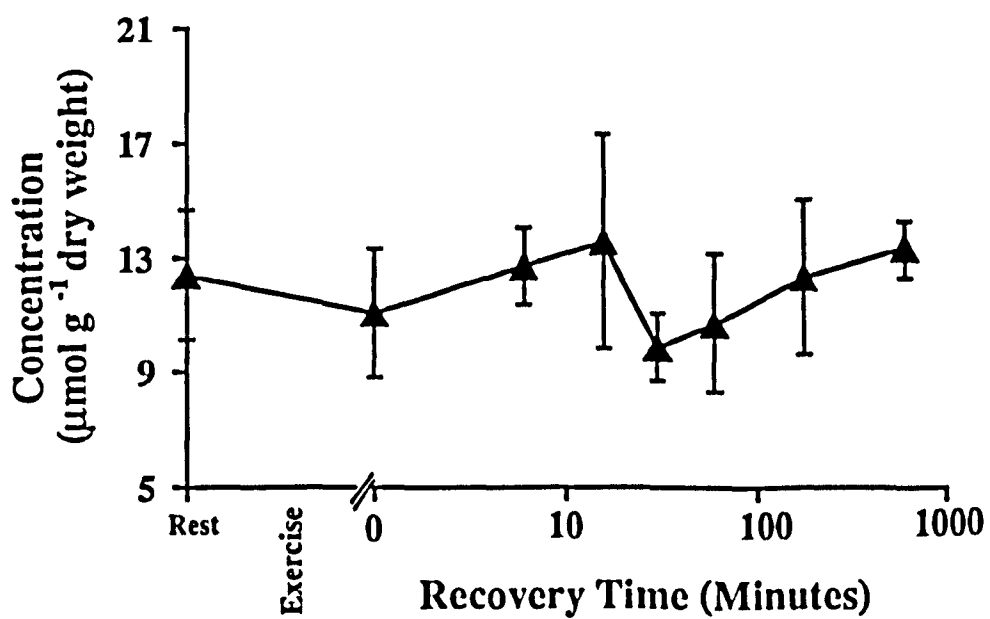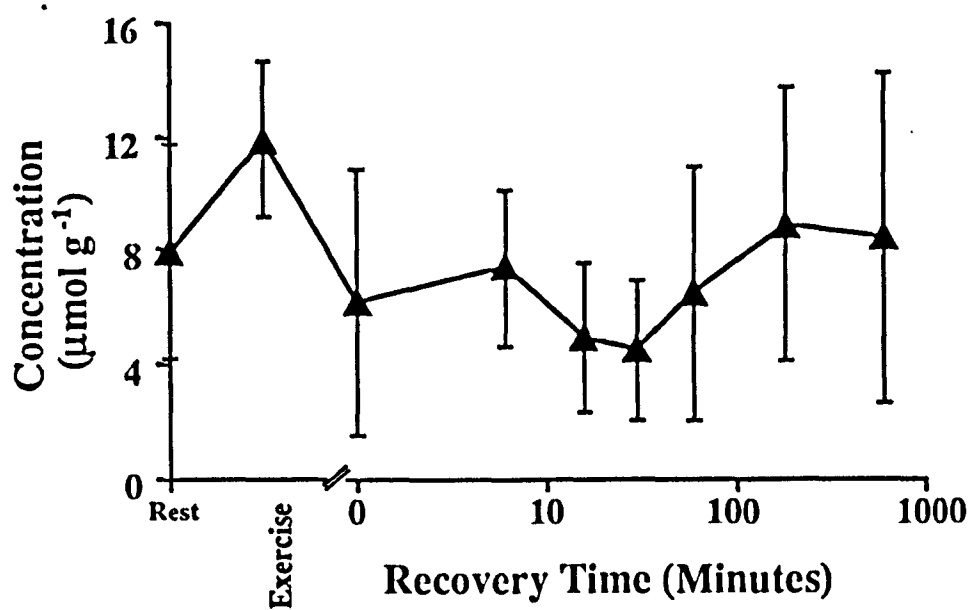
FIGURE 2.17. Graph showing the variation of L-lactate in the tail muscles of groups of *N. norvegicus* in relation to the recovery of total LG and NG swimming ability after stimulation of swimming to cessation with a standard stimulus, expressed as mean percentages of initial total swimming ability of each individual. Error bars represent standard deviations about mean concentrations. (Sample sizes as in Figure 2.11).

FIGURE 2.18. Graph showing the variation of L-lactate in the haemolymph of groups of *N. norvegicus* in relation to the recovery of total LG and NG swimming ability after stimulation of swimming to cessation with a standard stimulus, expressed as mean percentages of initial total swimming ability of each individual. Error bars represent standard deviations about mean concentrations. (Sample sizes as in Figure 2.11).
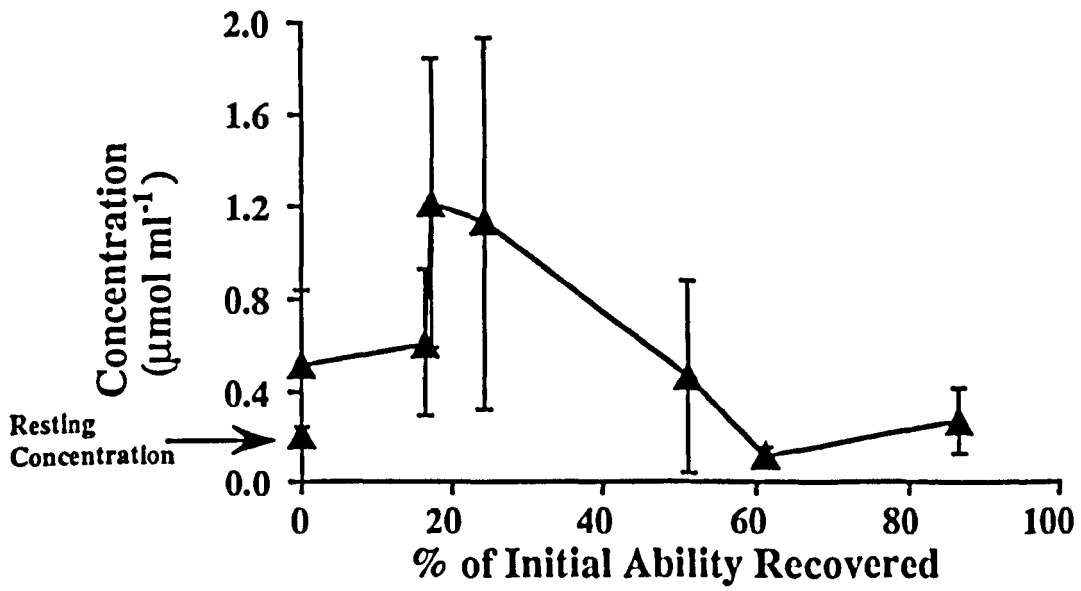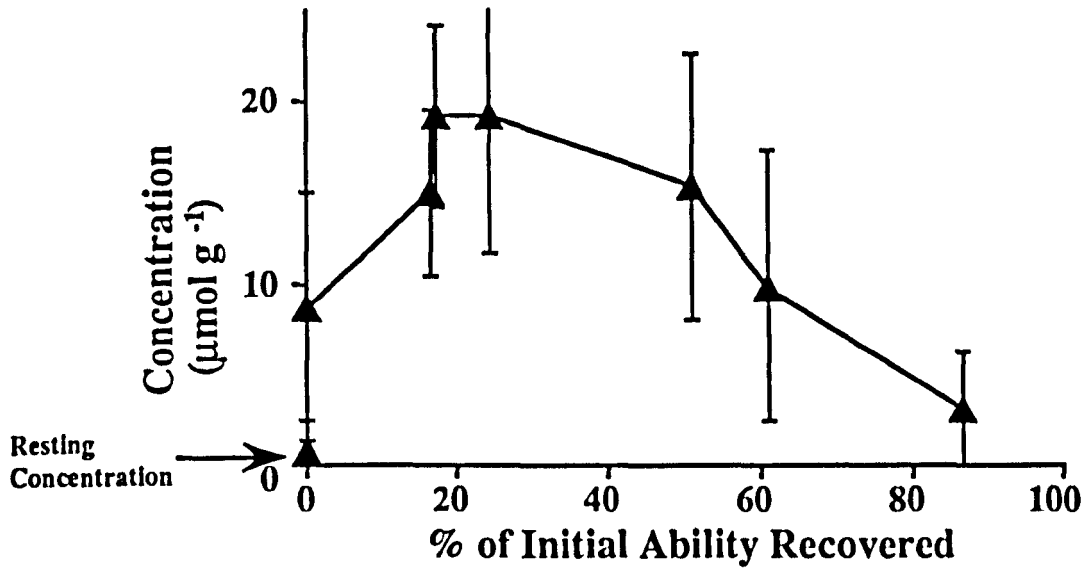
2.4 DISCUSSION

As in many other decapods, the intense muscular work of 'exhaustive' escape swimming in *N. norvegicus* results in an increase in muscle tissue and haemolymph L-lactate concentrations, signifying anaerobic supply of ATP to facilitate further muscular exercise under functional anaerobiosis and to replenish muscle phosphometabolite reserves. Similar occurrences have been noted in a variety of other decapod species e.g. *Cherax destructor* (England & Baldwin, 1983; Phillips *et al.*, 1977), *Orconectes limosus* (Gäde, 1984), *Callinectes sapidus* (Booth & McMahon, 1985), *Birgus latro* (Morris & Greenaway, 1989), *Crangon crangon* (Onnen & Zebe, 1983), *Homarus gammarus* (Phillips *et al.*, 1977). This implies the breakdown of initial stores of both ATP and muscle phosphagens, resulting in the need for rephosphorylation of ADP by glycolytic means.

In metabolic studies of long term escape activity in Crustacea, cessation of swimming has been attributed to muscular fatigue, i.e. the depletion of available energy to the point where contractions can no longer occur (Chiba *et al.*, 1989; Thébault *et al.*, 1987; Raffin *et al.*, 1988). However, at the point of cessation, *N. norvegicus* showed no apparent lack of available contractile energy. Indeed, ATP levels remained remarkably constant after 'exhaustive' exercise. Thus the waning of *N. norvegicus* escape swimming under repeated multiple stimulation may be due to some factor other than fatigue. It is possible that the large accumulations of L-lactate in the haemolymph and muscle may have an inhibitory effect upon further muscular activity, as the pH decrease associated with such occurrences has been shown to have regulatory effects upon Lactate dehydrogenase (LDH), and thus on glycolytic production of ATP (England & Baldwin, 1985). It is also possible that this pH decrease may influence neuronal functioning directly, and thus affect swimming performance.

In the LG mediated swimming sequences induced by repeated standard stimulation, it is unlikely that fatigue was responsible for the response

decrement observed, since ATP concentrations remained unchanged after exercise and there was no apparent correlation between recovery of swimming ability and recovery of resting L-lactate concentrations. It is more likely that some neural factor caused the waning of response to this standard stimulus alone. This is confirmed by casual observations made during experimentation, that animals were able to swim in response to different stimuli even after waning of the response to the standardised stimulus. Furthermore, total swimming performance in response to this standardised stimulus was significantly less than that achieved at 'functional exhaustion', indicating that the potential existed for further exercise. The estimate of swimming endurance of *N. norvegicus* made by Newland *et al.* (1988) was of a similar magnitude to that obtained under standardised stimulation here. Since it was obtained using a similar stimulation regime, it must therefore represent an underestimate of the potential endurance.

However, the metabolic effects of such LG mediated swimming induced by repeated standardised stimulation showed marked similarities to those of 'exhaustive' swimming. ATP concentrations remained unchanged by exercise, and indeed did not alter throughout recovery, and the same synchronous rise in the concentration of L-lactate in the haemolymph and in muscle was seen. Although these L-lactate concentrations were lower than seen in the 'exhaustive' exercise experiment, this appeared to be an artefact of a shift in the base-line level of calibration of the assay, as the absolute changes were of the same magnitude. The observed increase in L-lactate concentrations continued into the recovery period, and recovery of normal levels showed no link with the behavioural recovery of swimming ability. The similarity of the patterns of L-lactate changes in the two cases implies that anaerobic glycolytic metabolism occurs at similar levels during and after both maximal and non-maximal work, and therefore that direct metabolic factors are not significant in halting swimming at 'functional exhaustion'. Furthermore, the lack of a link between

behavioural and L-lactate recovery refutes the suggestion that L-lactate has significant inhibitory or regulatory effects on tail-flipping. Any inhibition of anaerobic glycolysis appears to be relevant only in the later stages of recovery, when L-lactate concentrations were higher, whilst any inhibitory effects upon ATP usage or neuronal mechanisms can also be discounted by the lack of any behavioural/metabolic link.

The presence of elevated L-lactate concentrations undoubtedly indicates glycolytic ATP production, but the reason for this production appears unclear. ATP levels do not appear to be altered by long term tail-flipping as has been reported by other authors working with various species of decapods (Gäde, 1984; Onnen & Zebe, 1983; Raffin et al., 1988). This could be the result of the different assaying procedures used to measure ATP concentrations, but the repeatability of this assay and the accuracy with which it could be calibrated tend to argue against this. The results seem to suggest that glycolytic replenishment of ATP is very efficient, so that ATP concentrations do not become limiting before other factors (possibly neuronal) halt tail-flipping. However, this does not account for the situation during the initial period of tail-flipping, where L-lactate concentrations were not seen to change in abdominal muscle. The bulk of L-lactate production occurred after the cessation of tail-flipping and its direct demand for ATP.

It seems likely, on the basis of the results presented here, that N. norvegicus is much like other decapods in its energy supply for exercise. If this is the case, the energy for initial rephosphorylation of ADP to ATP would be obtained from the breakdown of phospho-L-arginine. This was not actually observed for N. norvegicus but doubts must be expressed about the reliability of the assay method used. Much difficulty was experienced in calibrating the assay reliably, and a great deal of background fluorescence was encountered, possibly from the enzyme preparation used. Furthermore, the phospho-L-arginine concentrations obtained were markedly lower than those reported for other

species. This may have resulted from the spontaneous breakdown of this labile compound during storage and sample preparation, since although care was taken to keep samples frozen at -70°C, and the number of times samples were thawed kept to a minimum, freezing itself is likely to degrade phospho-L-arginine. In the light of this, it is possible that the surge in L-lactate production after the cessation of swimming is a result of the glycolytic replenishment of muscle phosphagen stores utilised to power the initial stages of escape swimming. The later stages of escape swimming appear to be fuelled relatively efficiently by glycolytic ATP production, and cessation of swimming seems to be due to habituation to inducing stimuli, rather than to metabolic limitation.

The source of fuel for the anaerobic L-lactate production observed is also unclear from the results obtained. The values for glycogen concentrations within *N. norvegicus* abdominal fast flexor and extensor muscles are markedly low when compared with those found in other decapod species, and also display little change due to glycolytic ATP and L-lactate production. Again this could be construed as an artefact of assaying, but further evidence that these results are representative is found in the histochemical staining reactions of these muscles (Chapter 3). When whole abdomen sections were stained with the Periodic Acid Schiff stain, the abdominal fast muscles showed a very low level of reaction. The stain was, however, considered to be effective as both swimmeret and slow postural muscles exhibited dense staining in the same sections. The D-glucose concentration changes seen also showed no consistent pattern, but may indicate some import of D-glucose from other tissues to the muscles.

The continued rise in L-lactate concentrations of both haemolymph and muscles well into the period of recovery from exercise implies continued glycolytic activity. Since tail-flipping has ceased and there is no more demand for ATP for muscular work, it is possible that continued anaerobic glycolysis is not only for the replenishment of muscle phosphagen stores, but also related to the restoration of other factors after exercise. Onnen & Zebe (1983) postulated

that continued anaerobic glycolysis during the recovery from intensive exercise in *Crangon crangon* was to enable the restoration of muscle function as rapidly as possible, but they attributed the cessation of swimming to metabolic exhaustion. Hill *et al.* (1991) working on *Carcinus maenas* (pooled tissues) and Head & Baldwin (1986) working on *Cherax destructor* (abdominal muscle) recorded significant increases in L-lactate concentrations during the recovery from environmental anoxia and intense activity respectively. These authors have suggested that this may indicate that anaerobic glycolysis is important in supplying energy to aid the recovery process. The situation in *N. norvegicus* appears to be similar, although there is probably a significant role for aerobic energy production during recovery also. This has yet to be evaluated.

L-lactate appears to be removed to a certain extent from muscle to haemolymph, as witnessed by the synchronous nature of the rise and fall in concentrations in both tissues. The fate of L-lactate once in the haemolymph has been the subject of much debate. It seems that little is excreted by crustaceans, and the possibility of a Cori cycle similar to that of mammals has been raised. However, there has been considerable argument over the site of such a cycle, the hepatopancreas (= midgut gland) has been nominated (Munday & Poat, 1971; Giles *et al.*, 1975), as have the haemocytes (Johnston *et al.*, 1973). Some of these ideas, however, have been disputed by Phillips *et al.* (1977), who found no evidence of gluconeogenesis in the midgut gland of *Cherax destructor.*

England and Baldwin (1983) report a transitional 'switch-over' period in the tail-flip sequence of the yabby (*Cherax destructor*), representing the point at which direct energy supplies are depleted and anaerobic re-phosphorylation becomes necessary. This is manifested behaviourally; animals respond to stimulation with defensive behaviour rather than further muscular work, which would involve anaerobic respiration. This 'switch-over' period is not evident in *N. norvegicus*, either behaviourally (no period of reduced

responsiveness to stimulation within swimming sequences being apparent) or metabolically (though this may be a result of sampling frequency).

Furthermore, in experiment 3 of this study, application of a standard stimulus to the rostrum elicited a much smaller number of MG flips (15.8 ± 6.9) than the number of LG flips elicited by abdominal stimulation (60.4 ± 31.2), suggesting either that animals were less sensitive to this stimulus to the rostrum, or that they habituated to it more rapidly. This clearly illustrates the importance of stimulus form and intensity in determining the tail-flip capability of *N. norvegicus*. This is further illustrated by the fact that the MG flipping performance reported by Newland *et al.* (1988), was elicited by single tactile stimuli which, although relatively standardised, were applied over a large and inconsistent area of the rostrum and chelae (D.M.Neil. pers comm.). In contrast, the taps used to elicit MG flips in this study were applied over a very small area of the rostrum between the eyes, and this may account for the differences in tail-flip performance between the earlier study (a mean of 67.1 ± 4.8) and those reported here (a mean of 15.8 ± 6.9).

The differential waning rates of LG and NG tail-flips apparent in Experiment 2 could be indicative of either fatigue or habituation. NG flips waned more rapidly than LG flips, in a manner very similar to that observed in crayfish by Reichert & Wine (1983). They attributed this differential waning to different sensitivities to habituation in the two mediating systems. A similar explanation could account for the fact that in *N. norvegicus* LG flips are still possible after NG flips have waned, implying that energy is still available to produce muscular contractions.

Hinde (1970) included conditions within his definition of habituation to exclude cases of decrement of response due to effector fatigue, sensory adaptation and infringement of refractory period. Clearly, neuronal refractory period has not been infringed, as stimulation frequency was 1/20 Hz or less.

According to Hinde, 'if, after the waning of one response, the same

effectors can be used in another, waning cannot be due solely to muscular fatigue.' This is true in the case of LG tail-flip swimming in *N. norvegicus* in response to standardised stimulation. MG and NG tail-flips can still be elicited via other stimuli after the cessation of LG mediated swimming. This is further supported by the fact that upon the cessation of LG mediated swimming, shifting the site of stimulation, albeit within the LG system, allows some decrement of flipping to be reversed, even though exactly the same musculature is involved (Figure 2.7). Also, shifting of the stimulus between two essentially separate mediating systems (LG to MG) can allow almost complete recovery of swimming ability in an apparently exhausted animal (Figure 2.8), although this is much reduced in the reciprocal experiment (MG to LG, Figure 2.9). However, the case of cessation of swimming in response to 'exhaustive' stimulation is not so obvious and because no more swimming could be induced by any means, cessation could be due to failure of the effector muscles. The results of the examination of tail muscle metabolism, however, would tend to refute this.

The differentiation between sensory adaptation and habituation as causes of response decrement is less certain, however. The recovery of only partial LG swimming ability after the switching of stimulation to a previously unstimulated receptor field implicates habituation at some point central to receptors, rather than receptor adaptation. Receptor adaptation would have led to a complete recovery of the response. The result obtained here may indicate habituation at multiple locations, so that partial habituation is observed upon stimulus switching. This fulfils a second of Hinde's conditions: 'If, after waning there is also a reduction in the strength of the response elicited through a previously unstimulated sense cell, sensory adaptation can be ruled out'.

Fulfilment of a third condition adds further weight to the argument for habituation. That is: 'If, after the waning of one response to a stimulus, the same stimulus elicits a different response, sensory adaptation can be ruled out..'

(Hinde, 1970). Casual observations made during experiments involving the repeated application of standard stimuli to a localised point showed that even though tail-flipping had waned, the stimulus could still elicit defensive and aggressive posturing oriented towards the direction of stimulation. This indicates that although the escape behaviour had failed, receptors were still allowing the perception of threat. This is seen in the habituation of many other escape and withdrawal responses (e.g. fleeing in ducklings (Melzack, 1961), 'tail flip' in goldfish (Rogers *et al.*, 1963)), in which the orientation to threat wanes much more slowly than does the escape/withdrawal response itself.

CHAPTER 3. SKELETO-MUSCULAR ADAPTATIONS OF THE THORACO-ABDOMINAL JOINT OF *Nephrops norvegicus* (L.) TO TAIL-FLIP SWIMMING.

3.1 INTRODUCTION

In addition to the metabolic and neuronal factors important in the production and control of *N. norvegicus* tail-flipping, skeleto-muscular factors may also play a significant role. Clearly, the muscles of the abdomen must be capable of producing not only the slow contractions necessary for maintenance and adjustment of posture during normal activity, but also the rapid, energy consuming and often prolonged, contractions of the tail-flip. The muscles responsible for posture maintenance are the superficial flexor and extensor muscles which form thin sheets along the ventral and dorsal borders of the abdomen respectively. The much larger deep flexors and extensors produce the effective and recovery strokes of the tail-flip respectively. The morphology of these muscles in *N. norvegicus* has been described by Newland (1985).

The tail-flip power stroke produced by the rapid contraction of the deep flexor muscles of the abdomen propels the animal backwards through the water with considerable force. This force must act through the thoraco-abdominal joint to pull the heavily calcified thorax and chelae. However, this joint is composed almost entirely of thin arthrodial membrane with little skeletal strengthening. Therefore all the force produced by a tail-flip must be transmitted to the anterior of the individual via the muscle groups connecting the two regions, acting as braces.

However, tail-flipping is not merely an uncontrolled explosive action, but is a steered response to directional stimuli (see Chapter 1). It is during the initial flips of swimming bouts that coarse steering occurs, dependent on the direction of the flight initiating stimulus. A strong tactile stimulus to the rostrum or chelae will initiate a medial giant (MG) mediated flip, which is directed almost exclusively horizontally, whilst a stimulus to the abdomen will initiate a Lateral Giant (LG) mediated flip which produces a downward thrust of the abdomen, lifting the animal away from the substrate. This steering is dictated by the order of contraction of the segmented deep flexor muscles (Newland &

Neil, 1990a), but also involves an angling of the abdomen in the vertical plane, particularly the first abdominal segment, due to the contraction of the muscles of the thoraco-abdominal joint (Newland *et al.*, 1992b). In addition, fine steering, and steering in the horizontal plane, are achieved both by angling of the trailing thoracic appendages (Cooke & MacMillan, 1985) and by directional flexing of the tailfan (Newland & Neil, 1987; Newland *et al.*, 1992a). There may also be some component of horizontal steering at the thoraco-abdominal joint, involving lateral movement of the abdomen via the first abdominal segment.

In view of the gross and subtle ways in which the thoraco-abdominal joint contributes to swimming locomotion and postural adjustments, an examination of the morphology of the muscles of this joint, and their histochemical properties has been undertaken. From these results, a picture emerges of a complex skeleto-muscular system in which a heterogeneous group of muscles have adaptations appropriate for bracing the joint and for slow, postural movements.

3.2 MATERIALS AND METHODS

The lateral abdominal abductor and the dorsal thoraco-abdominal extensor muscle systems of the thoraco-abdominal joint of *N. norvegicus* were investigated in terms of their structure and function. This was achieved by examination of their morphology, by dissection, and by histochemical examination of their enzyme ultrastructure. For comparison, the enzyme histochemistry of the muscles of an abdominal segment was also investigated.

3.2.1 Tissue sample preparation. The morphology of the two thoraco-abdominal muscle groups was investigated by dissection of the posterior thoracic region under *N. norvegicus* saline (Miyan, 1984). During dissections, muscles of these groups were removed and separated from their cuticular attachments.

For histochemistry, muscles were mounted in blocks of two or three, separated by thin strips of tissue paper to provide internal controls for staining density. Blocks were mounted on strips of cork using OCT mounting medium, and frozen in liquid nitrogen ($N_{2(l)}$). In a similar manner, whole abdomens removed from *N. norvegicus* were glued to a rigid support in an extended position, and frozen rapidly in $N_{2(l)}$. Care was taken to ensure that immersion of whole abdomens in liquid nitrogen was neither too slow, causing disruptive formation of ice crystals, nor too fast, leading to splitting of the cuticle. Frozen tissue blocks were then mounted on cryostat chucks with OCT medium.

3.2.2 Tissue section staining. Transverse sections were cut using a Bright Starlet 2212 Cryostat, at a thickness of $20\mu m$ and a temperature of $-25^{\circ}C$. Sections were lifted onto coverslips and air-dried at room temperature. Sections of thoraco-abdominal muscles were stained for succinic dehydrogenase (SDH), and total myofibrillar ATP-ases. Sections of whole abdomen were stained for SDH, NADH dehydrogenase (NADH = nicotinamide adenine dinucleotide, reduced form), total myofibrillar ATP-ases, myosin ATP-ases stable at pH 5.4 and polysaccharides (including glycogen) using the Periodic Acid Schiff reaction

(PAS).

SDH. The oxidative capacity of muscle fibres, indicative of their fatigue resistance, was determined from their succinic dehydrogenase activity (Lojda et al., 1976). The incubation medium contained 1M sodium succinate, 0.1M sodium phosphate and 1.0mg ml^{-1} nitroblue tetrazolium (Sigma N6876). The cryosections were placed in a petri dish on moist filter paper, and a drop of incubation medium was applied to each coverslip. Incubation was for up to 2 hours at 40°C. The sections were then dehydrated in an alcohol series, cleared in Histoclear (National Diagnostics, New Jersey, USA) and mounted in Histomount.

NADH.Dehydrogenase. NADH.Dehydrogenase activity, another indicator of oxidative capacity, was determined using the method reported by Ogonowski & Lang (1979). The incubation medium was made up from 2ml of buffer containing 0.1M sodium phosphate and 1.0mg ml^{-1} nitroblue tetrazolium (pH 7.5) added to a tube containing 2mg NADH (disodium salt) (Sigma 340-102). Cryosections on coverslips were incubated in this solution as described for SDH. Sections were dehydrated and mounted as above.

Myofibrillar ATP-ases. The total myofibrillar ATP-ase activity of muscles was determined using a method derived from those used by Mabuchi & Sréter (1980) and Snow et al. (1982). From a stock incubation buffer containing 20mM sodium barbital, 10mM calcium chloride and 10mM mangenese chloride, an incubation medium was prepared by adding ATP (disodium salt) at 1.5mg ml^{-1}, and adjusting the pH to 9.4. Sections were dehydrated and mounted as above.

Myosin ATP-ases stable at pH 5.4. The stability of myosin ATP-ase activity at low pH was determined using a method derived from Maier et al. (1984), as modified by Fowler (1990). The medium for acid preincubation contained 50mM calcium chloride, 35mM sodium acetate and 38mM sodium barbital, adjusted to pH 5.4. Sections were preincubated for 5 minutes, washed in the incubation buffer and incubated as described for myofibrillar ATP-ases.

Sections were dehydrated and mounted as above.

PAS reaction for glycogen and other polysaccharides. The concentration of polysaccharides (including glycogen) in muscles of the abdomen was assessed by the Periodic Acid-Schiff reaction modified from Drury & Wallington (1967). Sections were oxidized in an aqueous 1% periodic acid solution for 5 minutes, washed and incubated in Schiff's reagent at room temperature for 20 minutes. Sections were then rinsed in 3 changes of 0.5% sodium metabisulphite, washed, dehydrated and mounted.

3.3 RESULTS

3.3.1 Muscles of the abdomen: Histochemical profiles. The staining pattern resulting from treatment of sections of whole abdomen with the PAS stain for glycogen and other polysaccharides is shown in Figure 3.1. Both groups of large deep muscles showed very low staining densities, whereas the small superficial muscles (responsible for the production of the small, slow and sustained contractions of postural abdomen movements) and the lateral swimmeret muscles exhibited greater, though varying degrees, of reaction. This indicates low levels of polysaccharide (and therefore glycogen) storage within the deep muscle fibres, whilst the postural and swimmeret muscles have greater amounts of endogenous glycogen. Furthermore, very dark staining was visible in the extra-cellular spaces (the haemal spaces) between the deep muscle fibres. This suggests that although there may be no glycogen storage within the deep muscle fibres themselves, the haemolymph, or more specifically the haemocytes, may play a role in storing glycogen.

The paired deep extensor and flexor muscles showed high staining density for total myofibrillar ATP-ases (Figure 3.2), indicating a high concentration of these enzymes, and therefore the ability to perform rapid contractions. In contrast, the superficial muscles showed much lower densities with this stain, suggesting a lower concentration of myofibrillar ATP-ases and hence smaller capacity for rapid contraction.

The test for myosin ATP-ase stability conducted after an acid preincubation (Figure 3.3) showed intermediate staining densities (indicative of acid lability) in the fibres of the deep, fast acting muscles, whilst the fibres of the slow acting superficial muscles, exhibited a range of reactions. Most fibres were acid stable, but some showed the lighter staining indicative of acid-lability. This suggests considerable heterogeneity of fibre properties within the superficial muscles.

The correlation of enzyme concentrations of muscles with their functions

FIGURE 3.1. Transverse section of *N. norvegicus* abdomen stained for glycogen with Periodic Acid Schiff reaction. MSE = median superficial extensor; LSE = lateral superficial extensor; MSF = median superficial flexor; LSF = lateral superficial flexor; DE = deep extensor; DF = deep flexor; Sw = swimmeret muscles. Bar = 1mm.

FIGURE 3.2. Transverse section of *N. norvegicus* abdomen stained for total myofibrillar ATP-ases. MSE = median superficial extensor; LSE = lateral superficial extensor; LSF = lateral superficial flexor; DE = deep extensor; DF = deep flexor; V = ventral nerve cord; Sw = swimmeret muscles. Bar = 2.5mm.

FIGURE 3.3. Transverse section of *N. norvegicus* abdomen stained for myosin ATP-ases stable at pH 5.4. MSE = median superficial extensor; LSE = lateral superficial extensor; MSF = median superficial flexor; LSF = lateral superficial flexor; DE = deep extensor; DF = deep flexor; V = ventral nerve cord; Sw = swimmeret muscles. Bar = 2.5mm.

FIGURE 3.4. Transverse section of *N. norvegicus* abdomen stained for NADH.Dehydrogenase. MSE = median superficial extensor; LSE = lateral superficial extensor; LSF = lateral superficial flexor; DE = deep extensor; DF = deep flexor; V = ventral nerve cord; Sw = swimmeret muscles. Bar = 2.5mm.
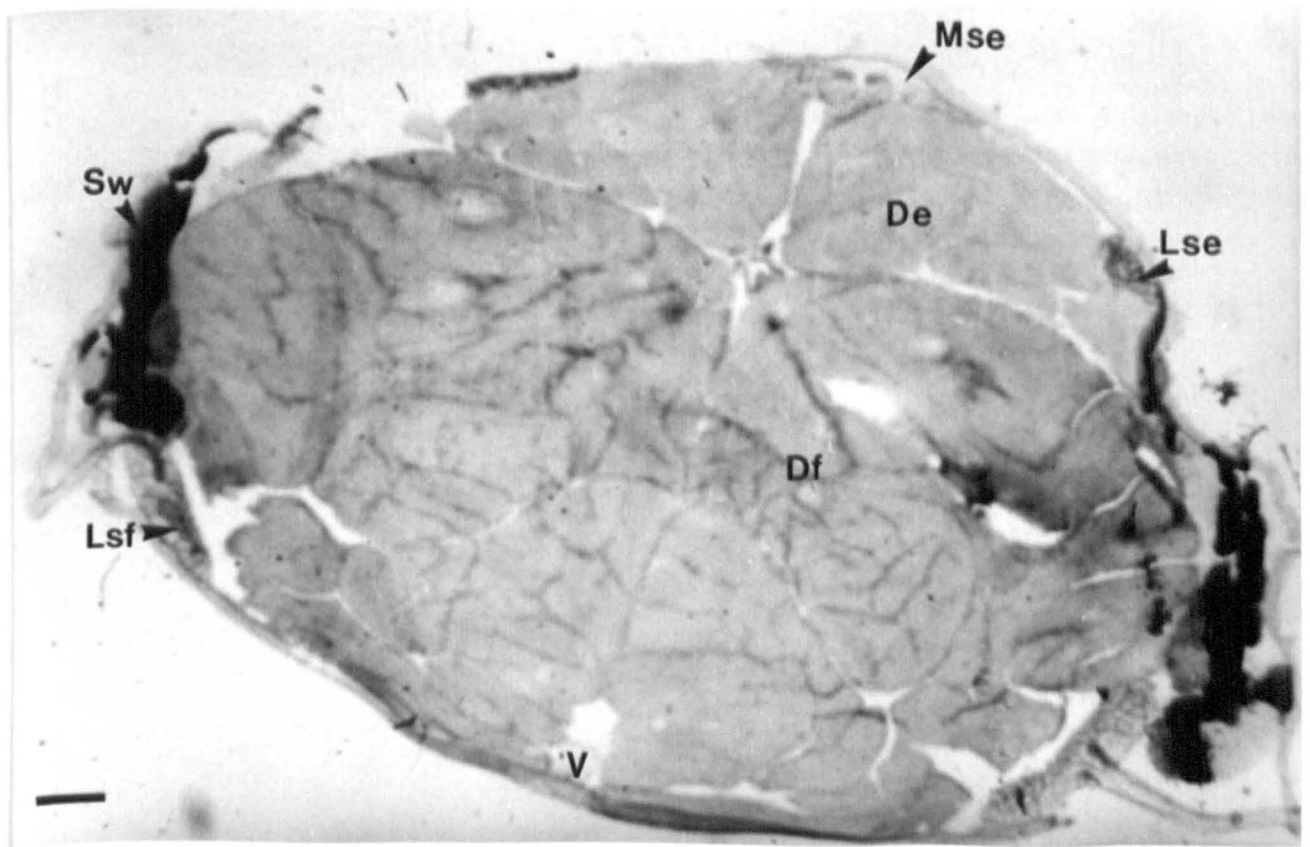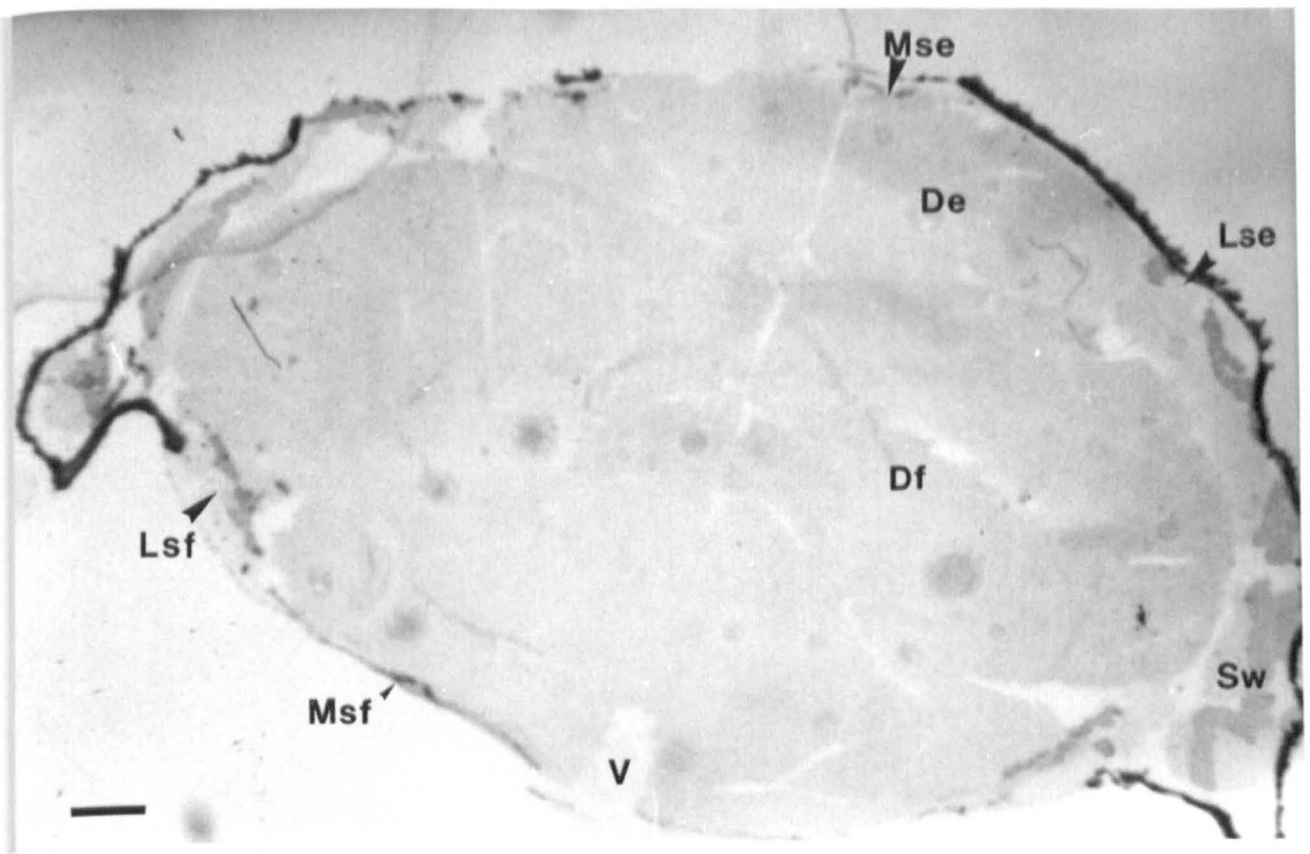
is reinforced by the histochemical results for NADH.Dehydrogenase and SDH (Figures 3.4 & 3.5). In both tests the deep, fast acting muscles stained very lightly and uniformly, and thus have low levels of oxidative mitochondrial enzymes, indicative of low oxidative capacity. In contrast, the slow functioning superficial muscles showed much darker staining, especially around the fibre peripheries. This reflects a higher oxidative capacity, typical of slow-acting muscles. In fact, the distribution of stain corresponds to large numbers of mitochondria in the 'peripheral fringes' (Silverman & Charlton, 1980). There was also some fibre heterogeneity in reaction to these stains.

This combination of histochemical tests suggests that although the overall functioning of the superficial muscles is slow and sustained in nature, a spectrum of properties exists which confers a range of contractile abilities to control posture.

3.3.2 Muscles of the thoraco-abdominal joint.

In this work, the nomenclature used by Pilgrim & Wiersma (1963) in their study of the abdomen and thorax of *Procambarus, Panulirus and Astacus* has been used to aid comparison, and avoid confusion (see their paper for a review of previously used nomenclature).

I) **Abdominal abductors: Histochemical profile.** The relative sizes of the three muscles in this group suggest different functions. The dorsal abductor is by far the largest, and its line of action, being almost parallel with the longitudinal axis of the solea, appears to have greatest mechanical advantage of the three. These factors suggest that this muscle is involved in rapid powerful activity, possibly raising of the abdomen during tail-flipping (particularly LG flips) and bracing against the force produced by flexion of the abdomen. This is generally borne out by the results of enzyme histochemistry on this muscle. The dorsal abductor showed a total myofibrillar ATP-ase staining pattern typical of a 'fast', burst activity muscle: high concentrations in all fibres of this muscle (Figure 3.6), and uniform levels of SDH (Figure 3.7) indicating a relatively low

FIGURE 3.5. Transverse section of *N. norvegicus* abdomen stained for SDH. MSE = median superficial extensor; LSE = lateral superficial extensor; MSF = median superficial flexor; LSF = lateral superficial flexor; DE = deep extensor; DF = deep flexor; V = ventral nerve cord; Sw = swimmeret muscles. Bar = 2.5mm.
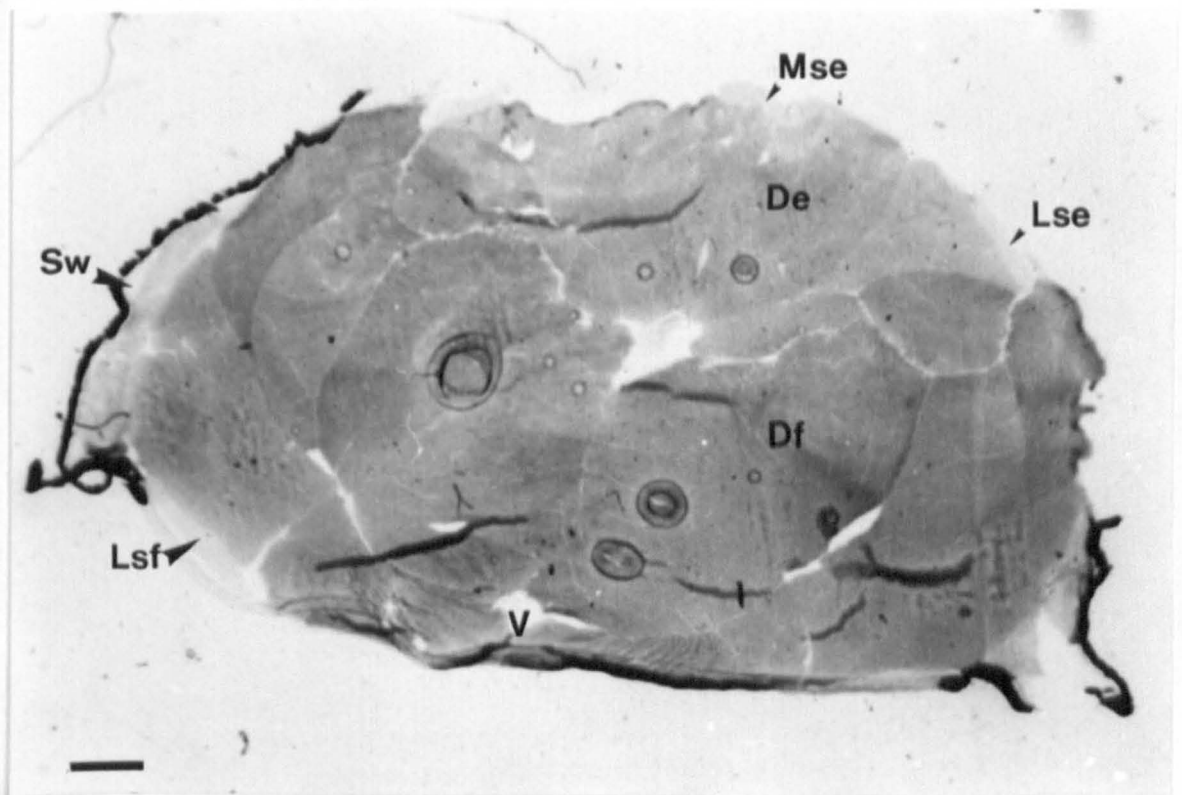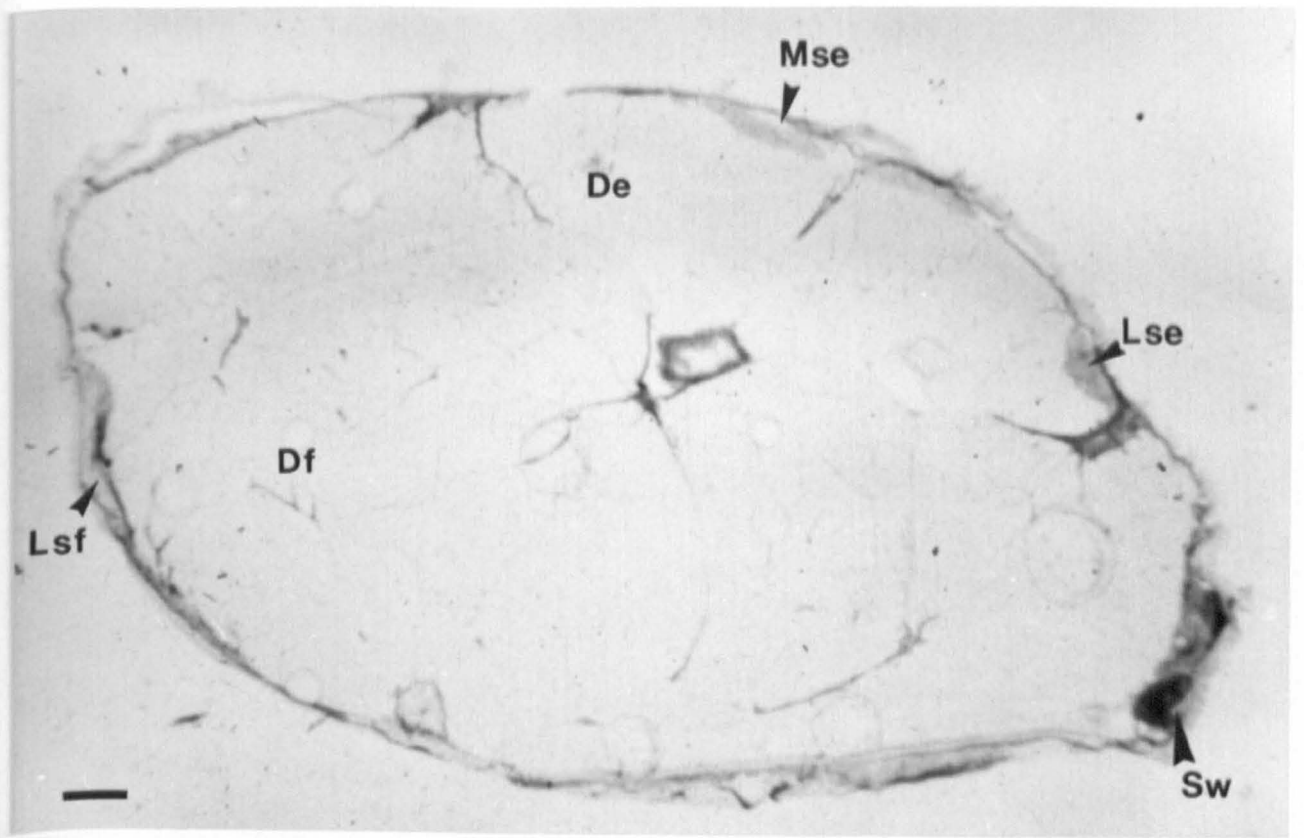
FIGURE 3.6. Transverse section of isolated abdominal abductor muscles of *N. norvegicus* stained for total myofibrillar ATP-ases. D= dorsal abdominal abductor; M = medial abdominal abductor; V = ventral abdominal abductor. Bar = 0.5mm.
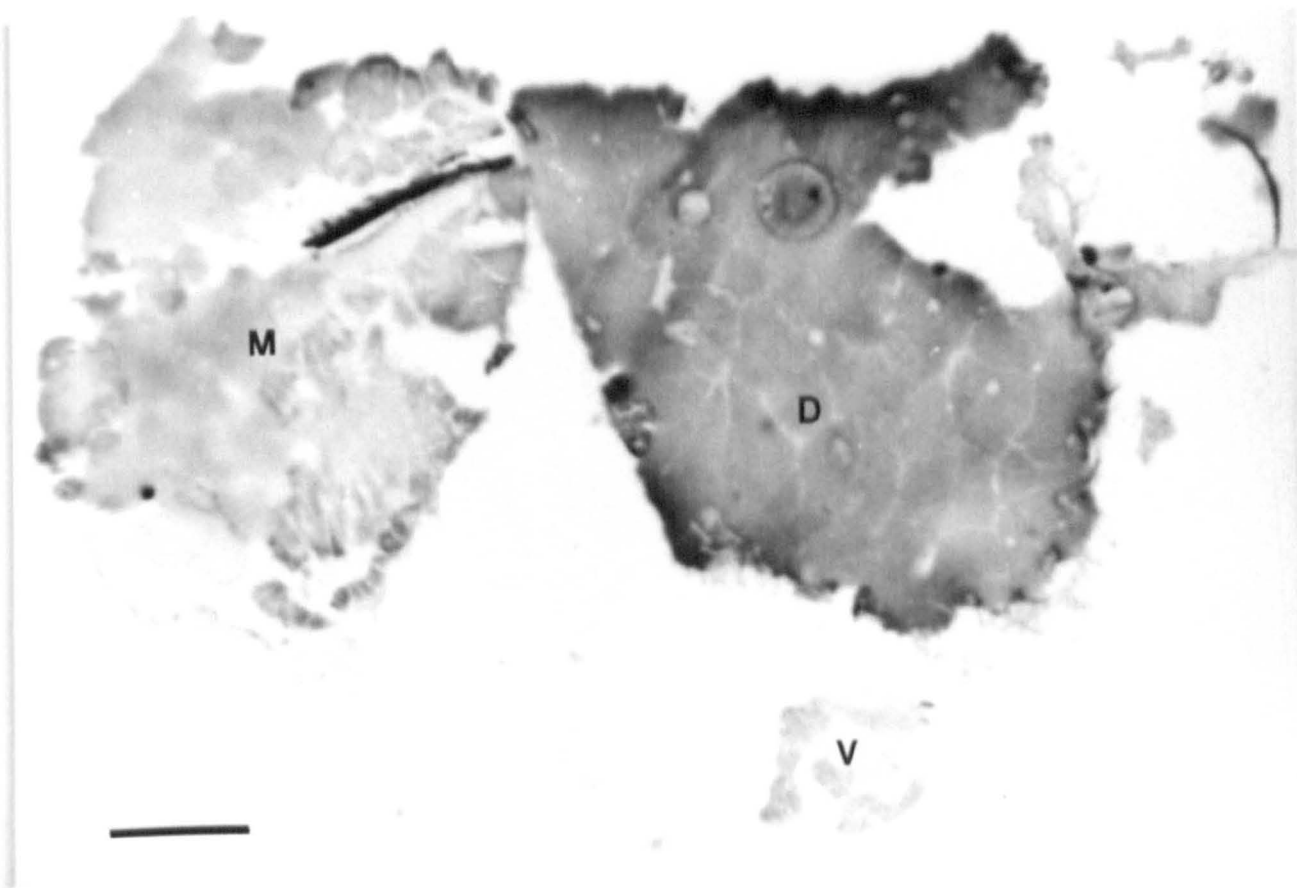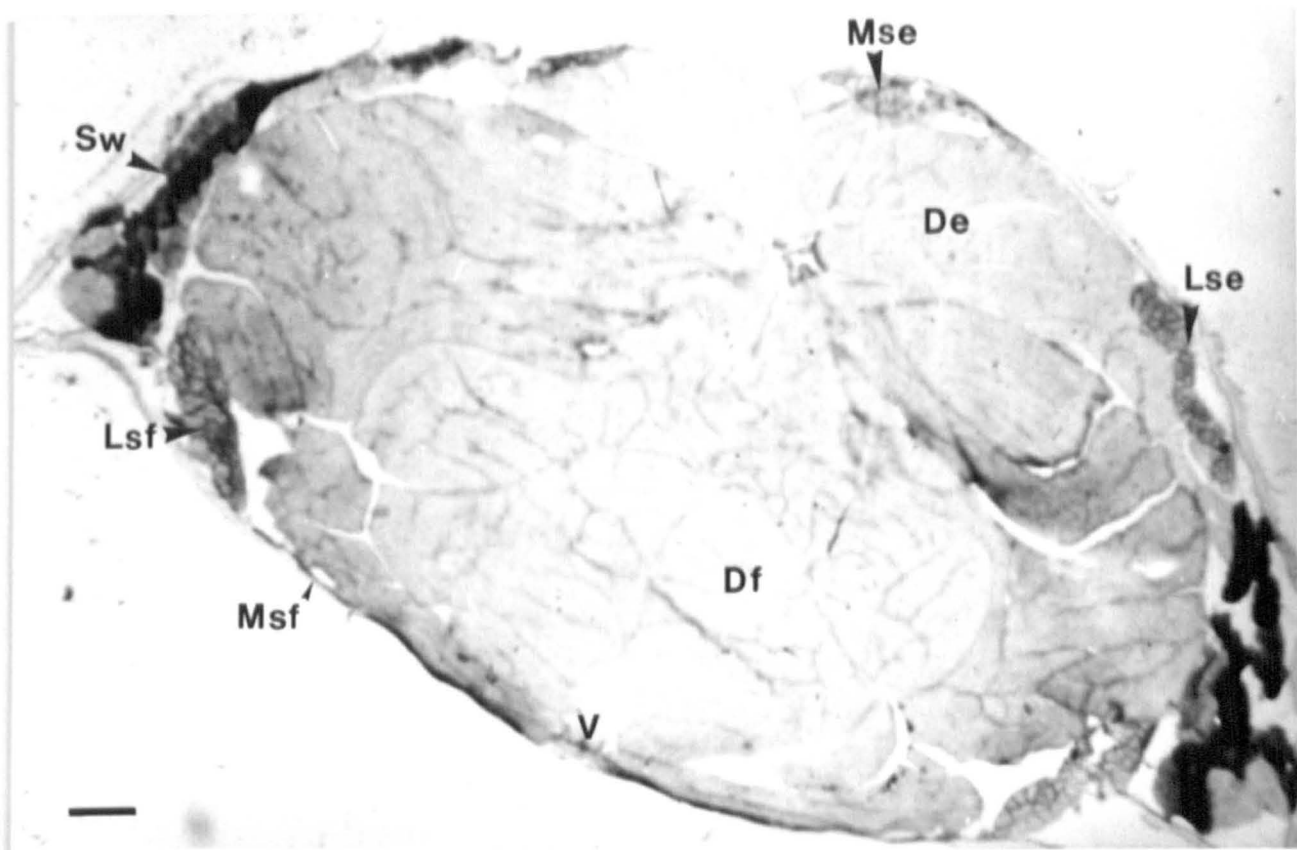
FIGURE 3.7. Transvers section of isolated abdominal abductor muscles of *N. norvegicus* satined for SDH. D = dorsal abdominal abductor; M = medial abdominal abductor; V = ventral abdominal abductor. Bar = 0.5mm.
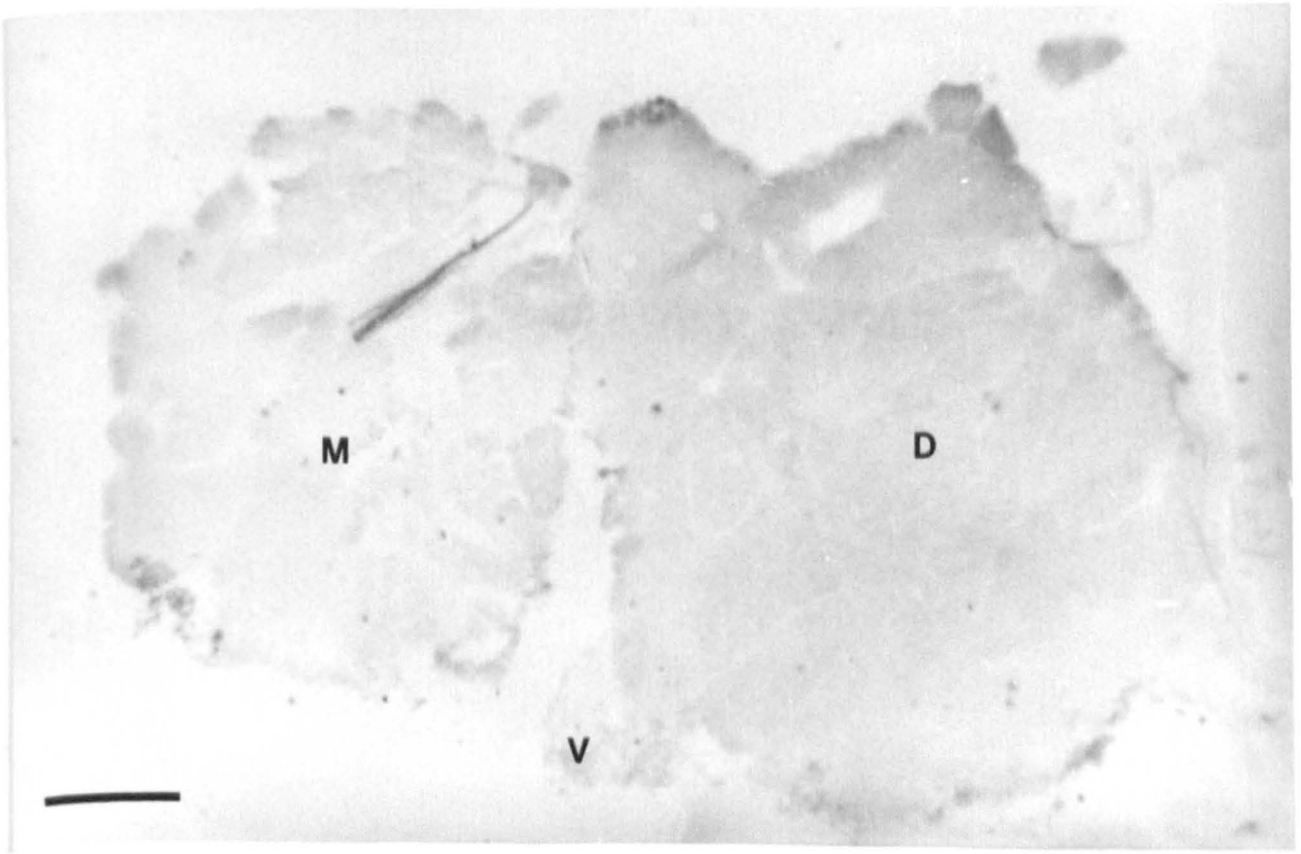
oxidative capacity. Conversely, the smaller medial abductor, which is oriented at a greater angle to its insertion on the solea, showed the heterogeneity of fibres more typical of 'slow' postural muscle. In this muscle the overall concentrations of SDH varied between fibres (Figure 3.7) and total myofibrillar ATP-ases were lower (Figure 3.6) than in the dorsal abductor. The SDH staining of the dorsal abductor, although uniform, was apparently of greater density than that of the medial abdominal abductor. This may suggest some fatigue resistance in the dorsal muscle, despite its essentially 'fast' contractile properties. The ventral abdominal abductor, which is very small and difficult to dissect out and mount, showed the heterogeneity of SDH fibre staining densities, and low ATP-ase activity of a typical 'slow' postural muscle, and it seems likely that this is how it functions.

II) Abdominal abductors: Morphology. The abdominal abductor system comprises three muscles (Figure 3.8); the dorsal, medial and ventral abdominal abductors. The whole group is situated laterally within the thorax, spanning the thoraco-abdominal joint and probably originated from thoracic muscles. All three muscles of the group have their insertions on a group of small moveable sclerites within the joint's arthrodial membrane (Figures 3.8 & 3.9). These sclerites articulate between the lateral posterior margin of the epimeral plate (the side wall of the thorax, which also constitutes the inner wall of the branchial cavity) and the anterior margin of the cuticle of the first abdominal segment. This group of calcified thickenings of the arthrodial membrane, collectively named the secula, comprises two separate but articulating plates. More dorsal is the solea, more ventral the uncus (Figure 3.9). The dorsal and medial abductors insert onto the dorsal edge of the solea, whilst the ventral abductor inserts onto the anterior margin of the uncus. Insertion of the dorsal abductor on the solea is inside and more dorsal than that of the medial abductor. Contraction of the dorsal and medial muscles appears to draw the solea upwards and forwards, acting as a 'hinge' and lifting the first abdominal segment. The anterior head of

FIGURE 3.8. Semi-diagramatic representation of the abdominal abductor muscles of the thoraco-abdominal joint of *N. norvegicus*, drawn as viewed from inside the thorax and slightly flattened. Bar = 2.5mm.

FIGURE 3.9. Enlarged portion of Figure 3.8 to show thje sclerites of the secula of the thoraco-abdominal joint of *N. norvegicus*. Bar = 1mm.

Medial abdominal abductor

Dorsal abdominal abductor

Head/thorax suture

1st abdominal tergite

Arthrodial membrane

Ventral abdominal abductor

Secula

5th pereiopod

Epimeral plate with calcified thickenings

Chela

Solea

Arthrodial membrane

Uncus

Epimeral plate

Post-coxal arm of 8th thoracic sternite
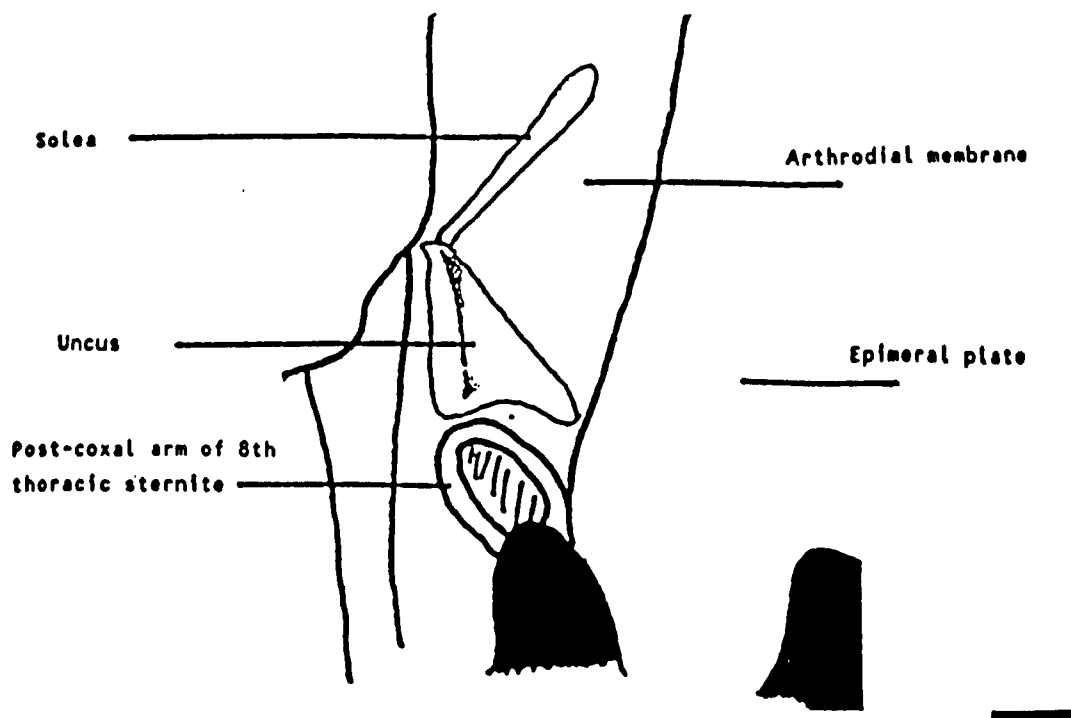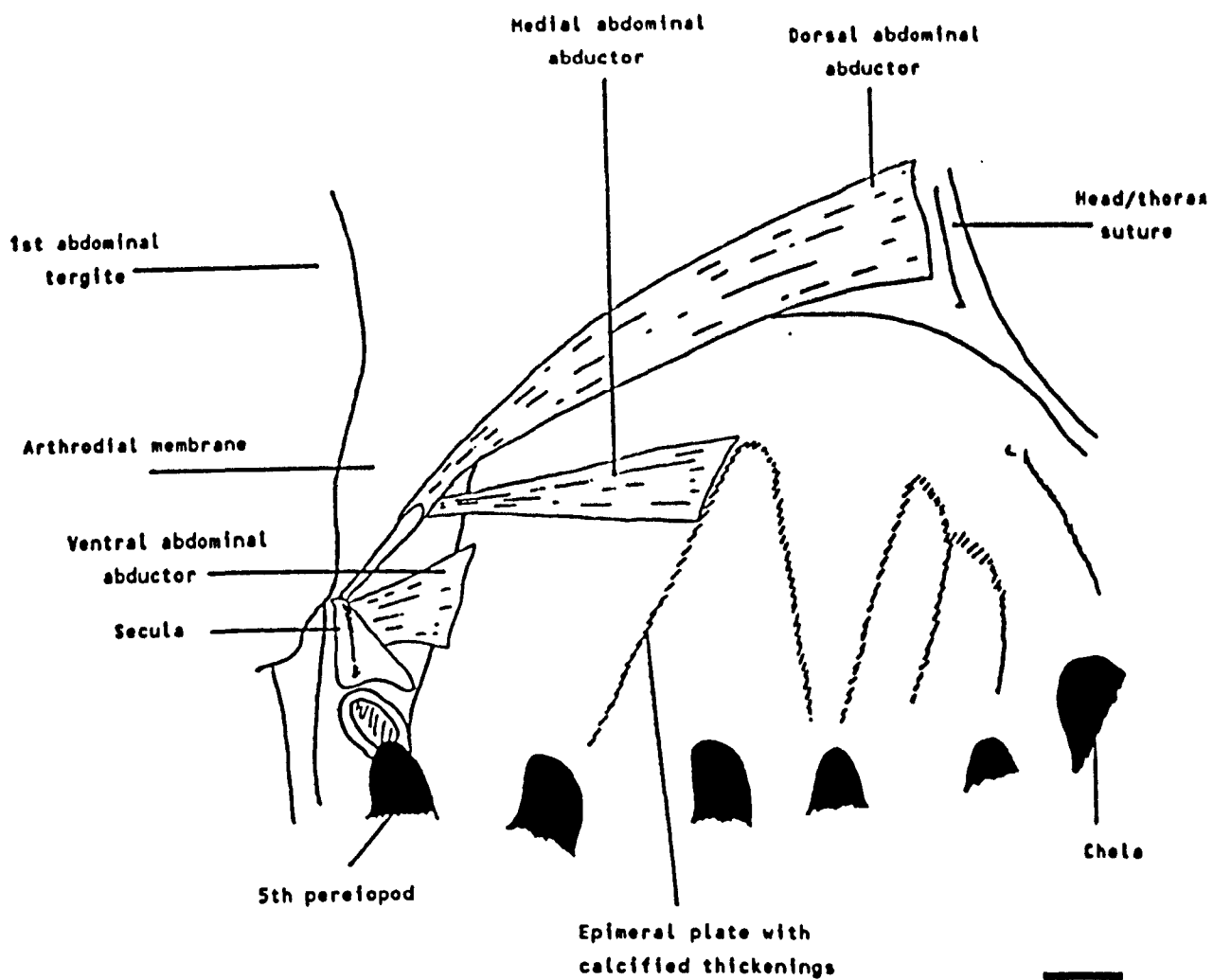
the dorsal abductor has its origin on the ventral surface of the dorsal carapace, just posterior to the suture between the head and thorax, on one side of the pericardium. The medial muscle originates on a heavily calcified thickening of the interior surface of the epimeral plate above the third leg. The ventral muscle also originates on the epimeral plate, posterior and ventral to the medial muscle. The arrangement of this group of muscles and the apparent functioning of the secula are very similar to those reported for *Procambarus clarkii* (Pilgrim & Wiersma 1963) and *Panulirus edwardsii* (Parker & Rich (1893) reported in Pilgrim & Wiersma (1963)). However the shape and number of the sclerites of the secula of *Nephrops* differs somewhat to those of *Procambarus*. In *Procambarus* and *Astacus* a third, more anterior sclerite is present (the manubrium), but (as in *Panulirus interruptus* (Pilgrim & Wiersma 1963)) this is not present in *Nephrops*. In addition, the solea is relatively longer and oriented more horizontally, whilst the uncus has become considerably more massive, being wider in the anterior-posterior plane and oriented more vertically. These differences suggest a more robust articulating system as *Nephrops* is a larger animal and has a relatively longer abdomen than crayfish.

III) Thoraco-abdominal extensors: Histochemical profile. As with the abdominal abductors, the relative sizes of the muscles which comprise the thoraco-abdominal extensor group imply differing functions. The large size of the dorsal, medial and ventral extensors suggest that these muscles are involved in powerful, short-term contractions, in a similar manner to the deep muscles of the abdomen. This is again supported by the results of histochemical analysis. Both the inner and outer slips of the dorsal extensor showed staining reactions typical of fast, anaerobic muscles. With total ATP-ase staining (Figure 3.10), these muscles were densely and uniformly stained, indicating high concentrations of myosin ATP-ases, and thus implying the ability to contract rapidly. The characterisation of these two muscles as anaerobic is supported by the results of SDH staining (Figure 3.12). Both muscles showed very low

FIGURE 3.10. Transverse section of isolated muscles of the thoraco-abdominal extensor system of *N. norvegicus* stained for total myofibrillar ATP-ases. OD = outer slip of dorsal thoraco-abdominal extensor; ID = inner slip of dorsal thoraco-abdominal extensor. Bar = 0.5mm.

FIGURE 3.11. Transverse section of isolated muscles of the thoraco-abdominal extensor system of *N. norvegicus* stained for total myofibrillar ATP-ases. M = medial thoraco-abdominal extensor; V = ventral thoraco-abdominal extensor; S = superficial thoraco-abdominal extensor. Bar = 0.5mm.
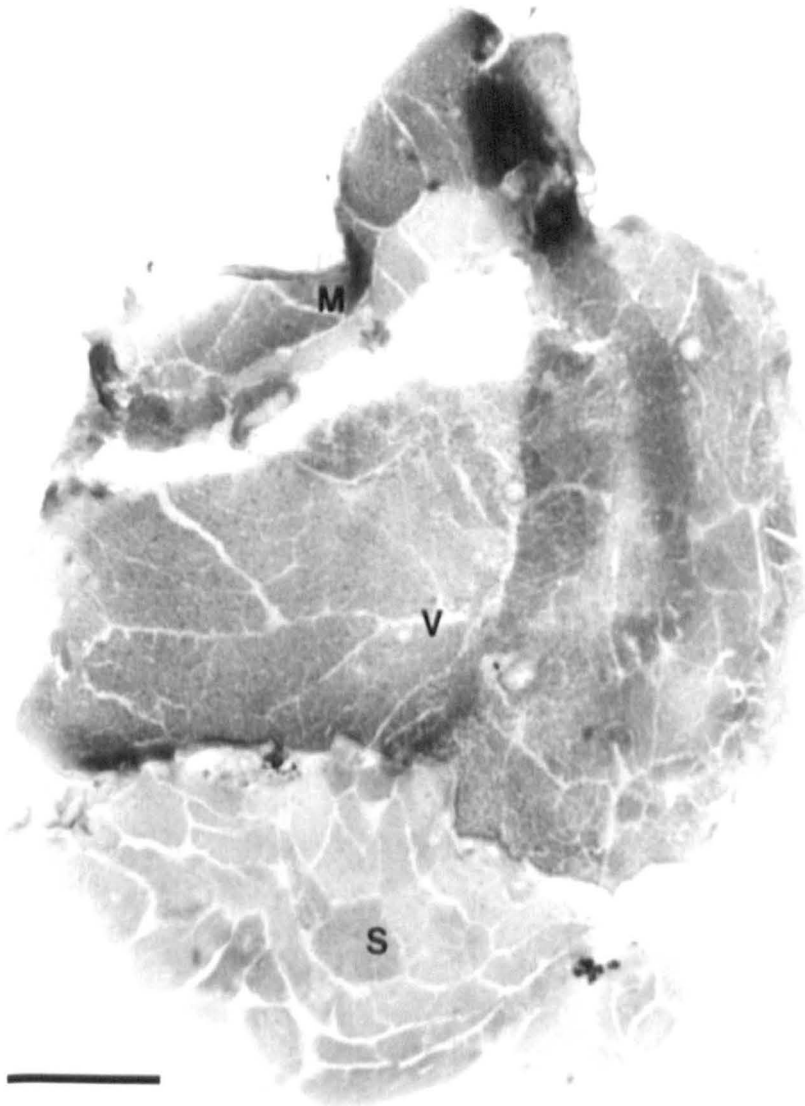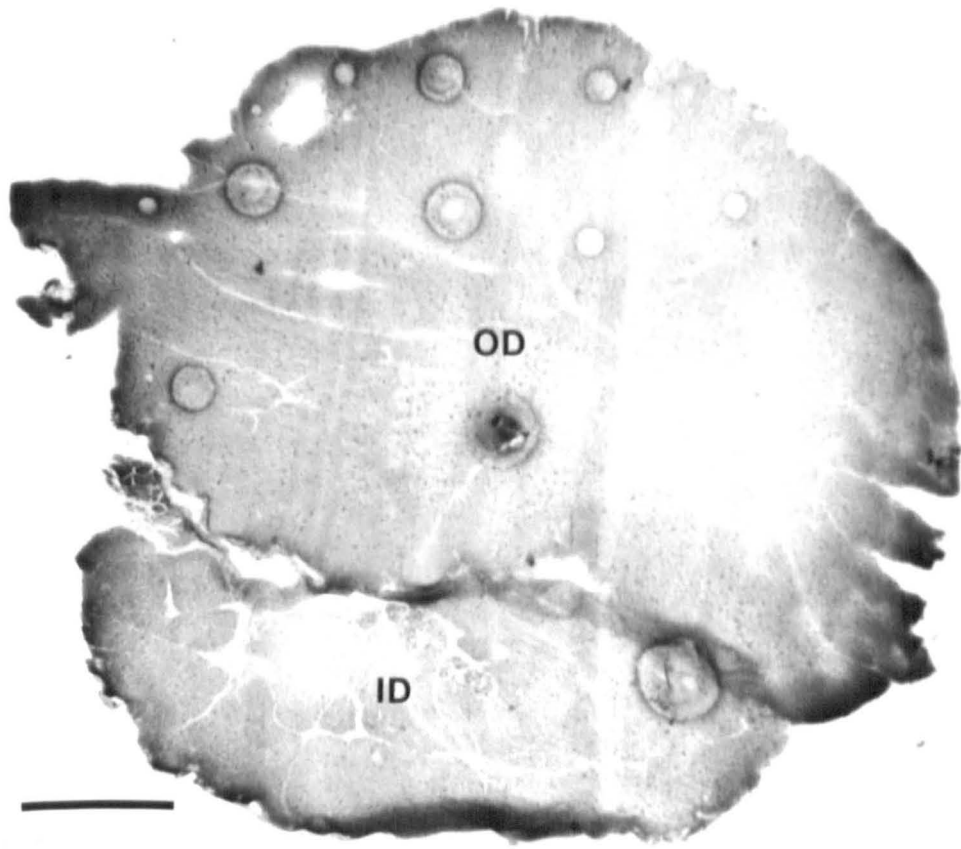
FIGURE 3.12. Transverse section of isolated muscles of the thoraco-abdominal joint of *N. norvegicus* stained for SDH. OD = outer slip of dorsal thoraco-abdominal extensor; ID = inner slip of dorsal thoraco-abdominal extensor; Hs = haemal space between fibres. Bar = 0.5mm.

FIGURE 3.13. Transverse section of isolated muscles of the thoraco-abdominal extensor system of *N. norvegicus* stained for SDH. M = medial thoraco-abdominal extensor; V = ventral thoraco-abdominal extensor; S = superficial thoraco-abdominal extensor. Bar = 0.5mm.
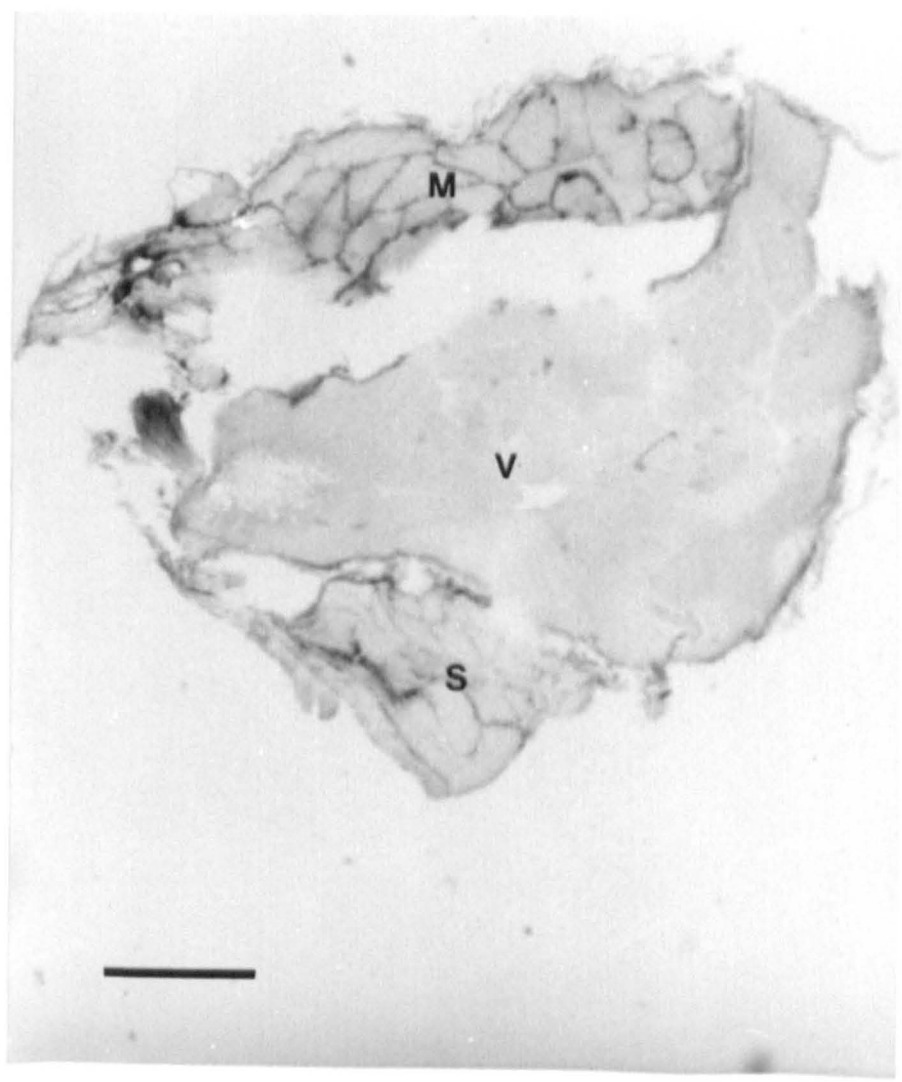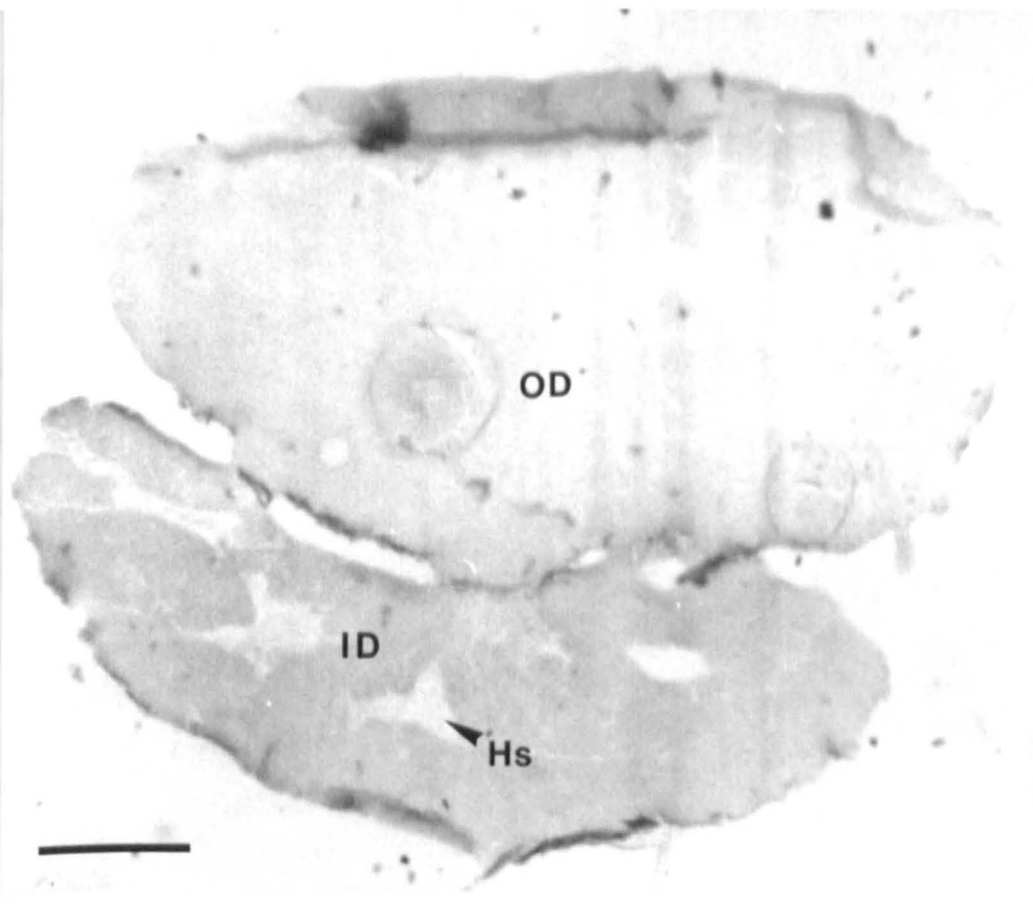
uniform intensities of staining for this enzyme, with no indication of a peripheral fringe.

In contrast to the dorsal extensors, the fibres of the medial extensor displayed a distinct heterogeneity of staining response to the total ATP-ase stain (Figure 3.11). The fibres with higher and lower ATP-ase activity were distributed in groups which appear as a characteristic banding pattern in the muscle, similar to that seen in slow fibres involved in longer term postural movement. Furthermore, the SDH response of the medial extensor was also characteristic of slow muscle (Figure 3.13), with fibres showing varying degrees of peripheral staining, indicative of mitochondrial fringes.

The ventral extensor displayed staining characteristics similar to those shown by the dorsal extensors, typifying it as a classic fast muscle. The superficial extensor was essentially similar to the medial extensor, being slow in character, displaying relatively high SDH activity (high aerobic capacity)(Figure 3.13) and low ATP-ase activity (Figure 3.11). However, in comparison to the medial extensor, this heterogeneity was less marked, most fibres having relatively low ATP-ase activity.

IV) Thoraco-abdominal extensors: Morphology. There are four main muscles in the dorsal thoraco-abdominal extensor system in *Nephrops* (Figure 3.14); dorsal, medial, ventral, superficial; as in *Procambarus*. The four most medial muscles are the deep extensors, whilst the most lateral of the group is the superficial extensor. The paired systems are situated either side of the mid-line extending anteriorly from the anterior dorsal margin of the first abdominal segment to the interior surface of the epimeral plate. They are situated medial to the abdominal abductor muscles.

The thoraco-abdominal superficial extensor appears to be sub-divided in *Nephrops* into two heads (unlike *Procambarus* but similar to *Astacus*), both of which insert on the anterior ridge of the first abdominal tergite, dorsal to the deep extensors (overlying them). The origin of this muscle is as yet uncertain

FIGURE 3.14. Semi-diagrammatic representation of the thoraco-abdominal extensor muscles of *N. norvegicus*, drawn as viewed from inside the thorax and slightly flattened. Bar = 2.5mm.

Inner slip of
dorsal extensor

Outer slip of
dorsal extensor

Medial extensor

Superficial extensor
(with probable origin
on secula)
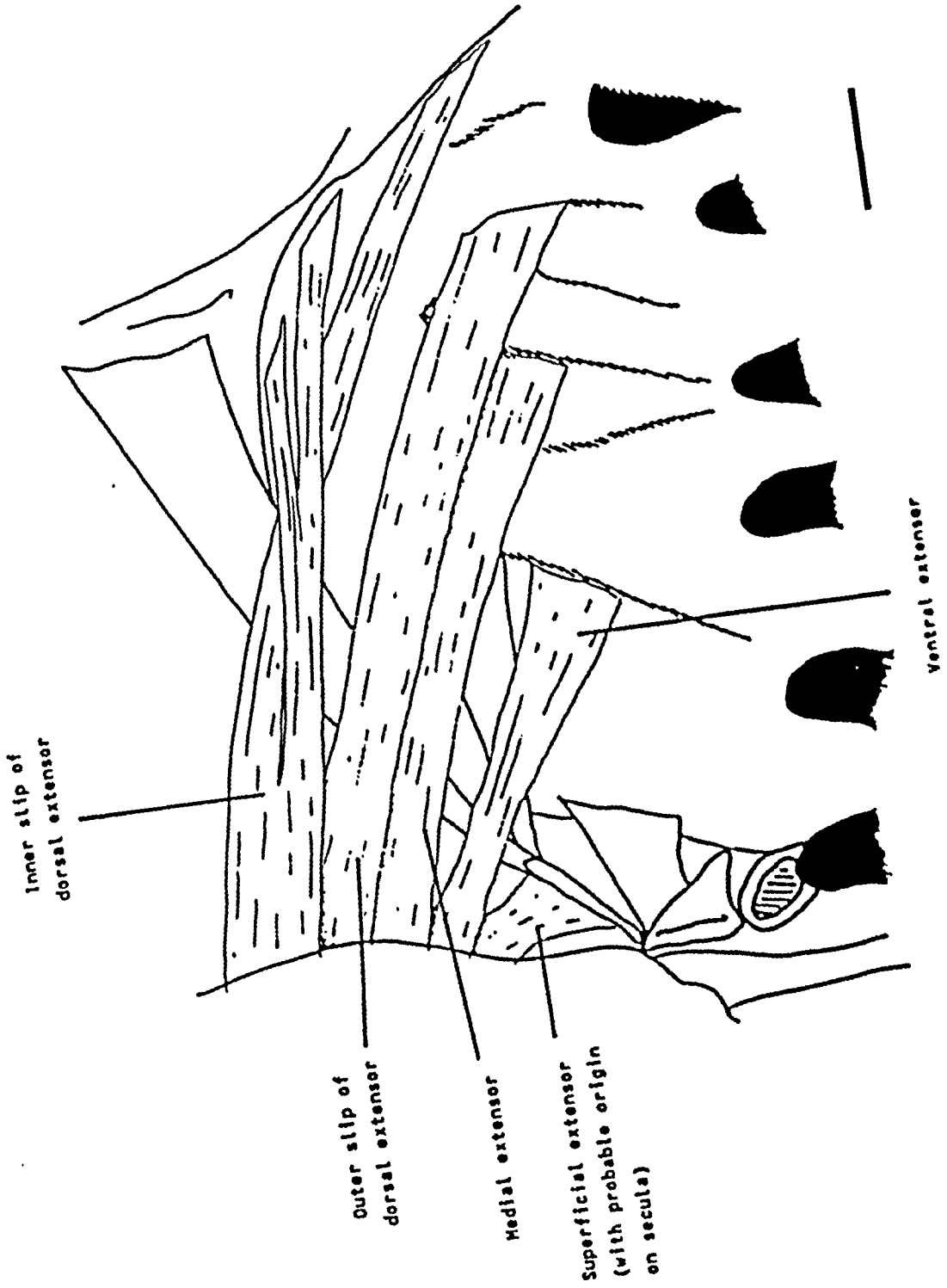
Ventral extensor

but is probably associated with the secula, as in *Procambarus*. The most lateral of the deep muscles is the ventral thoraco-abdominal extensor, which inserts on the first abdominal tergite medial and ventral to the superficial extensor, but partially overlain by it. This originates on the epimeral plate above the third walking leg, dorsal to the origin of the medial abdominal abductor, on the triangular calcification (this is opposite to the arrangement in *Procambarus* where the medial abdominal abductor originates more dorsally). The medial thoraco-abdominal deep extensor originates from another, more anterior, triangular calcification of the epimeral plate, on its posterior ridge, above the first and second legs. This muscle, again, inserts on the first abdominal tergite medial and ventral to (and partially overlain by) the ventral thoraco-abdominal extensor. The dorsal thoraco-abdominal extensor has two distinct slips, which fuse just before their insertion on the first abdominal tergite. The outer (more lateral) slip has a single origin, on the epimeral plate on the anterior ridge of the anterior triangular calcification, above the first and second legs. The inner slip is comprised of three sub-units. The outer two originate on the epimeral plate at its anterior margin dorsal to the chela, with the outermost of these two having the more dorsal, posterior origin. These sub-units fuse close to their origins. The inner subunit also originates on the epimeral plate, dorsal and posterior to the outer sub-units, and crosses them ventrally before joining them close to insertion. This arrangement is similar to that found in *Procambarus*, except that in *Nephrops* the outer slip of the dorsal thoraco-abdominal extensor originates posterior and not dorsal to the multiple origin of the inner slip of the same muscle.

3.4 DISCUSSION

The staining patterns of the deep abdominal extensors and flexors of *N. norvegicus* are typical of fast anaerobic muscle, adapted for powerful contractions over a limited time period. They display a high myofibrillar ATP-ase activity combined with very low aerobic capacity. Ogonowski & Lang (1979), showed similar results in the abdominal muscles of the decapods *Homarus americanus* and *Procambrus clarkii*, but reported no heterogeneity of fibres in the superficial muscles under the NADH.Dehydrogenase stain. They did report a large degree of heterogeneity of fibres within the swimmeret muscles in reaction to both total myofibrillar ATP-ase and NADH.Dehydrogenase stains, supporting results reported here.

However, the reaction of these muscles to the PAS stain for glycogen and polysaccharides show that they contain a very low level of these compounds. It seems anomalous that muscles which evidently rely heavily on rapid anaerobic metabolism to fuel contraction should contain very little endogenously stored glycolytic fuel (glycogen). This may suggest that the stain did not reveal the full complement of this compound within the sections. However, other muscles, particularly the swimmeret muscles in the same sections displayed very high concentrations of polysaccharides (probably glycogen), indicating that the stain was revealing the true concentrations present. Further evidence that the deep muscles do indeed contain very little stored glycogen is presented in Chapter 2 (Figure 2.16), where actual glycogen concentrations were measured within the fast flexors, and these were very low compared with other decapod species.

Moreover, some dark staining is also apparent in the haemal sinuses between the deep muscle blocks in these sections, and this may indicate the presence of glycogen within the haemolymph. Johnston *et al.* (1973) suggested a carbohydrate storage role for haemocytes of *Carcinus maenas*, demonstrating that certain cell types contained significant amounts of glycogen. In their paper they verified the positive PAS reaction to be due to glycogen by diastase

extraction. The results reported here did not involve diastase extraction of glycogen, and therefore the positive result presented by the haemal sinuses can only be construed as showing the presence of high concentrations of polysaccharides or neutral mucopolysaccharides. Despite the fact that Phillips *et al.* (1977) dispute glycogen storage by haemocytes, the positive PAS result exhibited by *N. norvegicus* haemolymph, coupled with the surprising lack of any mucopolysaccharides (and therefore glycogen) within the deep flexor and extensor muscles, suggest that the haemocytes may be acting as a glycogen pool for the fuelling of anaerobic metabolism within these muscles.

Both groups of thoraco-abdominal muscles examined in *N. norvegicus* in this study display a range of histochemical staining properties similar to those of the muscles of the abdomen itself. This similarity implies a similarity in the range of functions also.

The position, size and staining properties of both the dorsal abdominal abductor and the dorsal (inner and outer slips) and ventral thoraco-abdominal extensors suggest that these muscles may act both in bracing and directing the abdomen during tail-flipping. The staining properties of these muscles are typical of fast phasic muscles (high myofibrillar ATP-ase concentrations, low SDH concentrations), and suggest rapid, powerful but short-term contractions similar to those performed by the similarly stained abdominal muscles which are responsible for tail-flipping. This would suggest that these muscles act only briefly during each tail-flip, to transmit the forces generated by the abdomen to the thorax. The fact that the SDH staining reaction of the dorsal abdominal abductor appeared to be greater than that of the medial abductor, to which more typically slow functioning has been ascribed, may indicate some fatigue resistance in the dorsal muscle. This in turn may implicate the dorsal abductor in more long term contractions within the burst act of tail-flipping, possibly some involvement in steering.

The lateral independence of the paired groups allows for some

differences in contraction timing and force which could facilitate a degree of steering at the thoraco-abdominal joint. The fact that the abdominal abductor system is bilaterally organised, with the muscles on each side being morphologically independent of the other, allows the possibility that the dorsal and medial muscles may have a function in lateral movements of the abdomen. These are known to occur both during postural movements and in escape swimming, and it may be suggested that the slow medial muscles of each group contribute to the former, and the fast dorsal muscles to the latter. The opposing arrangement of the two muscle groups (abdominal abductors and thoraco-abdominal extensors) may also indicate a role for these muscles in vertical steering of the tail-flip. The different angles of the abdomen evident in LG and MG tail-flips may be the result of differential contractions in these two muscle groups causing differences in the angle of the first abdominal segment.

Abdominal rotation, a prominent feature of righting reactions of *N. norvegicus* (Newland & Neil, 1990b) also occurs about the thoraco-abdominal joint, and may be facilitated by these two muscle groups. As part of righting tail-flips this rotation is not merely a transitory angling of the abdomen for the duration of a tail-flip, as occurs in giant-mediated flips, but is a repetitive response during a swimming bout. This continued angling allows a cumulative directional effect to achieve large directional changes. This may also occur in lateral direction changes during steered escape swimming. This need for more prolonged contraction to effect steering over a period of several tail-flips suggests a possible function for the medial extensor. This muscle, by virtue of its size and position, was expected to have similar function and staining properties to the other large muscles of the group. However, it exhibited staining properties best described as intermediate, both having relatively high ATP-ase concentrations and some SDH activity, implying both some rapid contraction ability and some fatigue resistance. This endows the medial extensor with both the power and fatigue resistance necessary to produce bout-long steering.

The antagonistic relationships implied between these two muscle groups by their morphology and histochemical properties, suggest various functions within the control and coordination of the complex behaviour of tail-flipping. However, these functions cannot be fully elucidated without an examination of the innervation of the muscles with respect to each other and the other effector muscles of the tail-flip. Moreover, definition of the temporal relationships between recruitment of the various muscles within a tail-flip will further illuminate this point.

The results of this investigation have gone some way to assigning functions to muscles of two of the three groups which span the thoraco-abdominal joint, by use of two separate but complementary techniques. These functions do, however, remain putative, until the involvement of all three muscle groups have been better defined, and the neuronal factors detailed above have been investigated.

CHAPTER 4. CONCLUSIONS AND PROSPECTS.

The tail-flip escape reaction of decapod Crustacea, and particularly that of crayfish, has been the subject of a great deal of research. In consequence, extensive information is available on many aspects of this behaviour. Best known are the neuronal control mechanisms and energy metabolism, whilst the functional anatomy and behavioural significance have received less attention. While a considerable amount is known about both the metabolic and neuronal factors limiting to escape swimming, the vast majority of work has concentrated on a particular aspect of one system, and there has been very little overlap between areas of investigation.

In intact, living animals, all these different systems operate in an integrated manner to produce what we call the 'tail-flip'. As a consequence, factors limiting escape swimming may lie within several systems or result from antagonistic or synergistic interactions between systems and this needs to be considered in the design of experiments.

In Chapter 2 of this study, an attempt has been made to overcome the limitations of traditional studies by adopting an inter-disciplinary approach to the assessment of the factors important in determining swimming ability in a decapod crustacean, *N. norvegicus*. By cross-relating the results of biochemical and neuronal tests performed on parallel groups of animals, it was hoped to provide data on the relative importance of metabolic and neuronal factors in swimming performance, which would not be readily forthcoming from conventional analyses of either system performed on individual animals.

This approach is valid and possible for the analysis of tail-flip sequences because, although not completely stereotyped, they represent a standardised behavioural act whose constituent parts are readily distinguishable. As such they can be quantified in a repeatable and meaningful manner during different types of experiment.

Using this broad approach, it is possible to draw at least limited conclusions about some of the factors important in limiting and controlling

escape swimming in *N. norvegicus*. Examination of the anaerobic metabolism of *N. norvegicus* during exhaustive exercise (Chapter 2, Experiment 1) revealed that even at the point of apparent (= functional) exhaustion metabolic factors do not appear to be limiting. Further evidence that cessation of swimming is not due to metabolic factors was gained from the results of experiments correlating behavioural and metabolic recovery after escape swimming (Chapter 2, Experiment 2). The results of this experiment were, however, somewhat equivocal. These results tend to indicate that the general pattern of anaerobic glycolysis during and after exercise in *N. norvegicus* is similar to those reported in other decapods, with increased L-lactate concentrations beginning during anoxia (functional or environmental) and continuing into the recovery period. However, ATP concentrations within abdominal muscles suggest that swimming is not limited by this source of phosphate energy, supporting the results obtained in Experiment 1. Notwithstanding this, the situation with regard to the more immediately available energy source of phospho-L-arginine is less certain. The possibility that swimming may be at least partially limited by a deficit of this compound still remains, as the assay used to determine the concentrations of phospho-L-arginine cannot be entirely relied upon, casting doubts upon the accuracy of these data. Before direct energy deficits can be unequivocally ruled out as limiting factors, a more reliable method of estimation of phosphate metabolism should be applied to the tail muscles of *N. norvegicus*. The use of ^{31}P NMR is such a method, as used by Thébault *et al.* (1987), Raffin *et al.* (1988) and Thébault & Raffin (1989, 1991) in the examination of exercise energy metabolism of other decapods, although its use in this context presents several serious practical problems, not least the size of the animals being investigated, and the integrated nature of the experiments.

Despite the lack of definitive evidence against metabolic limitation, the results of tests undertaken in Experiments 2 & 3 of Chapter 2 which were designed to assess the importance of neuronal factors, in particular habituation,

suggest that these play a major role in defining escape swimming ability of *N. norvegicus*. With the fulfilment of several of Hinde's (1970) conditions for the identification of true habituation, the waning of the escape reaction of *N. norvegicus* under repeated stimulation seems to fall into the category of habituation. However, these conditions do not represent a strict definition of habituation, and it is necessary to apply more stringent parameters to confirm the role of habituation in this case. The nine 'parametric characteristics of habituation' defined by Thompson & Spencer (1966) provide a more accurate method for the identification of habituation within a behavioural act, but require very specific experimental conditions. Within the bounds of the multi-disciplinary experimental strategy adopted in this study, fulfilment of these conditions has not been possible. However, the results presented here have indicated the predominant role of neuronal factors over metabolic factors in the limitation of escape swimming, and further experiments could now be designed to test whether Thompson & Spencer's (1966) more stringent conditions for the identification of habituation are met. This may be achieved both by behavioural experiments involving intact animals, and by more detailed neurobiological investigations, such as those used by Zucker (1972) and Wine & Krasne (1972, 1982) in their work on the control and limitations of crayfish tail-flipping.

The use of 'overlapping' techniques has also yielded much useful information in the examination of the functional morphology of the muscles of the thoraco-abdominal joint of *N. norvegicus* (Chapter 3). Previous studies of the musculature of decapod Crustacea have dealt only briefly with the muscles which span the joint between abdomen and thorax. The most notable exception to this is the work of Pilgrim & Wiersma (1963) which, although very thorough, deals only with the anatomy of the muscles of the joint. Despite the usefulness of such detailed information, it can tell us very little about the functioning of these muscles, and particularly their role in tail-flipping. Casual observation alone reveals that the joint between the abdomen and thorax is important in

postural movement and positioning of the abdomen, and the work of several authors has implied that this is true also of steering movements, righting reactions and the tail-flip.

The use of enzyme histochemical techniques in conjunction with an investigation into the morphology of the muscles of the thoraco-abdominal joint of *N. norvegicus* has allowed some inferences to be made of the functions of two of the three groups of muscles spanning this joint, which would not have been possible from a purely morphological study. The results presented in Chapter 3 have shown differences in the enzyme composition of muscles of both the abdominal abductors and the thoraco-abdominal extensors which, in conjunction with their size and positions, suggest differing functions. The larger muscles of both these systems have histochemical profiles and positioning suggestive of short-term fast phasic activity, implying that they play a role in the bracing of the thoraco-abdominal joint during tail-flipping. This is further supported by the opposition of these groups of larger muscles, and their paired nature, allowing bracing in all planes. The smaller muscles of both groups have typical 'slow' type enzyme profiles, implying postural functions. However, the results obtained for one muscle of the thoraco-abdominal extensor group, the medial extensor, present a more complex picture. There is an apparent anomaly between the histochemical profile and the size and position of this muscle, suggesting that it has an 'intermediate' character. This in turn suggests that it may have a long-term bracing function, and possibly involvement in tail-flip steering.

The implication that the muscles within these groups have different functions raises the question of how they are innervated. An investigation into the innervation and activation during tail-flipping of the various muscles of the thoraco-abdominal joint would not only reveal much about the functions of these muscles in the bracing and steering of the abdomen during swimming sequences, but may also shed light on the origins of these muscle groups. The

thoraco-abdominal extensors appear to be homologous to the similarly organised deep and superficial abdominal extensors, and their histochemical properties are consistent with such an affiliation. Examination of the innervation of the extensor muscles, and of the recruitment of these nerves in relation to those of the abdomen which are responsible for tail-flipping may help to reveal if this group is related to its abdominal counterparts.

The investigations presented in Chapters 2 & 3 have studied a number of intrinsic factors which affect the production of tail-flips in *N. norvegicus*, and have identified appropriate lines of future research. However, the escape reaction is also governed by many other intrinsic and extrinsic factors, not least of which is the overall health and general physiological state of the animal. One such intrinsic factor related to the health of *N. norvegicus* became apparent during the course of these studies. Trawl samples taken in the Clyde Sea Area in the mid-1980's showed increasing prevalences of apparently moribund animals. Initially this condition was thought to be related to the moult cycle and was investigated from the viewpoint of its possible influence on the swimming ability of *N. norvegicus* (see for example Cromarty *et al.*, 1991), but it soon became clear that it represented a serious problem, relevant to the survival of west coast populations of *N. norvegicus* and the fisheries they support, and worthy of investigation in its own right. For these reasons the research presented in the first half of this thesis was not pursued further along the lines identified above, but attention was turned more to an investigation of the moribund condition and its causes. Part 2 of this thesis presents the results obtained from this study.

PART 2. INITIAL STUDIES OF THE HISTOPATHOLOGY AND EPIZOOTIOLOGY OF A NOVEL DINOFLAGELLATE DISEASE OF

Nephrops norvegicus (L.) ON THE WEST COAST OF SCOTLAND.

CHAPTER 5. GENERAL INTRODUCTION.

During the course of routine investigations by the Scottish Office Agriculture and Fisheries Department (SOAFD) into the biology of *Nephrops norvegicus* in the Firth of Clyde region during the early 1980's the occurrence was noted of a low incidence of animals with an abnormal dull orange colouration (Figure 5.1). Such individuals were observed to be in a moribund state, with 'watery' opaque muscles, low haemolymph pressure and milky white body fluids, apparently associated with increased numbers of cells. Initially, this condition was thought to be associated with the moult cycle of *N. norvegicus*, as it coincided with the annual moult period of Clyde populations, between February and May. The condition was therefore designated 'Post moult trauma'. By 1987, the poor quality of the tissues and their 'watery' appearance had begun to provoke comment from fishermen and processors. Consequently, a regular sampling programme was initiated by SOAFD to define more precisely the seasonality of occurrence and geographical incidence of this condition around the west coast of Scotland. An investigation was launched concurrently into the physiology and pathology of affected animals.

The symptoms presented by *N. norvegicus* affected by this condition are broadly typical of those seen in many Crustacea in response to a range of intrinsic and extrinsic factors. Such factors include large scale migrations, environmental perturbations (particularly starvation), but most importantly, infection by pathogenic agents.

The Class Crustacea contains over 26,000 species (Barnes, 1974), the majority of which occupy a wide range of habitats within the marine environment. They display great variation in body form, and have become hosts to disease-causing organisms from most major phyla. The importance of some crustacean species in commercial fisheries and aquaculture operations has further exposed them to pathogenic disease. Intensive rearing and culturing techniques can alter agent/host interrelations in favour of pathogens. This has, however, led to a considerable amount of research effort being concentrated on

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FIGURE 5.1. Photograph showing the appearance of healthy (H) and abnormal (A) adult male *N. norvegicus* from trawl samples taken in the Firth of Clyde. Bar = 10mm.

H   A

such disease syndromes, vastly increasing the available knowledge of crustacean disease pathology.

The majority of diseases caused by microorganisms (bacteria, rickettsiae, viruses, fungi), are known mainly from outbreaks in intensive rearing situations. Of these microbial agents, viruses are the most abundant, affecting mainly the culturing of penaeid shrimps, and some crab species. Some 30 species of virus have been identified and classified (though most only tentatively) (Brock & Lightner, 1990). Some viral diseases are caused by individual agents, but it is common for more than one agent to infect a host (Johnson, 1983). It is thought that stress may be an important factor in inducing or exacerbating viral disease, accounting for large holding tank mortalities. The importance of viral disease in natural populations is poorly understood (Brock & Lightner, 1990), and although most viruses are known from wild populations as well as hatchery stocks (indeed the source of most significant infections is introduction to rearing facilities via infected broodstock or larvae), their effects in natural systems have not been investigated. It has been suggested, however, that pollution, in particular that by chlorinated hydrocarbons and heavy metals, has synergistic effects with *Baculovirus penaei* virus in *Penaeus* spp.(Couch & Courtney, 1977). Moreover, some viruses have been observed in large numbers, apparently with no detrimental effects on their hosts (Brock & Lightner, 1990).

Rickettsial and chlamydial infections in crustaceans are rare, with only 4 incidences known in penaeid shrimps and crabs. Of these, a chlamydia-like organism infecting dungeness crabs (*Cancer magister*) shows significant pathogenicity, and may have an impact on natural populations (Sparks *et al.*, 1985).

Bacterial disease is common in crustaceans though, as with viruses it is most commonly associated with aquaculture and other situations involving stressful conditions. Low level infestations of crustacean cuticles by bacterial epibionts occur naturally, such fouling being normal and of no detriment to the

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host. These bacterial growths are normally limited by combinations of environmental and host-related factors, such as water nutrient load and host grooming. However, under altered environmental conditions (particularly high organic nutrient load) heavy fouling may occur, and large bacterial mats can impair the behavioural and physiological functioning of the host (Fisher & Wickham, 1976, 1977; Young & Pearce, 1975). Such impairment can be serious ·if gill surfaces and chemoreceptor sites are involved, and may lead to mortality. High mortalities in captive rearing operations have been reported to be associated with bacterial fouling (Barkate *et al.*, 1974). Fouling of eggs and larvae has also been implicated in population declines of the dungeness crab, *Cancer magister* in the San Francisco Bay area (Fisher & Wickham, 1976; 1977).

No cuticle or internal changes have been reported due to severe epibiont infestations. In contrast, shell disease, involving ectoparasitic bacteria, can cause extensive damage to the cuticle and underlying tissues. Shell disease, also called rust disease, black spot and brown spot, affects many marine and freshwater decapods. The causative agents are various species of chitinovorous bacteria of several genera, including *Vibrio, Beneckea* and *Pseudomonas*. Incidences of shell disease in wild populations are more frequent than those of the other pathogens so far discussed, and infection rates as high as 76% have been reported in female Tanner crabs off the Oregon coast (Baross *et al.*, 1978). However, female Tanner crabs do not moult after the puberty moult, and hence this figure represents an overestimate of overall prevalence of disease (Baross *et al.*, 1978).

Shell disease at relatively high infection rates (up to 30%) has been noted in *Crangon septemspinosa, Homarus americanus* and *Cancer irroratus*, associated with organic pollution at sewage dumping sites. (Gopalan & Young, 1975; Young & Pearce, 1975). In severe cases, mortalities can occur if the cuticle is breached and the internal tissues become involved, especially if the host attempts to moult (Fisher *et al.*, 1976). Low level infections are completely

lost by moulting (Baross *et al.*, 1978), and hence younger, immature animals are less likely to suffer from serious infections. However, in cultured conditions, changes in environmental factors may allow larvae and postlarvae to suffer serious infections and mortality, as has been noted in *Homarus americanus* (Fisher *et al.*, 1976).

Bacterial infections are also involved in haemocoelic and enteric diseases of crustaceans. It has been reported that bacteria do not normally occur in the haemolymph of Crustacea (Bang, 1970; Lee & Pfeiffer, 1975; Johnson, 1976), and that bacterial disease arises only when the host's defences are compromised by injury and/or stress, allowing the invasion of facultative pathogens already present in the environment. Several authors, however, have reported the isolation of bacteria from the haemolymph of apparently healthy animals (Colwell *et al.*, 1975; Lightner, 1977) suggesting that the haemolymph is not normally sterile, but that the host's humoral and cellular defences are well able to cope with low levels of bacterial invasion (Brock & Lightner, 1990). Disease would then arise if intrinsic or extrinsic factors altered the ability of the host's defence systems to clear pathogens effectively from the haemocoel. In contrast, Lewis (1979) (cited in Brock & Lightner, 1990) has proposed that bacterial diseases are caused by obligate pathogens, normally present in host populations in a reservoir of a few individuals, but are able to cause epizootics when large numbers of animals become subject to stressing conditions.

Bacterial septicaemic diseases are commonly seen in captive and acute-to-chronically stressed marine Crustacea. The majority of these conditions are caused by the invasion of the haemocoel by gram-negative bacteria of the family Vibrionaceae, mainly of the genera *Vibrio* and *Aeromonas* (Lightner, 1977). Gram-negative *Pseudomonas* have been found to be pathogenic in penaeid shrimps (Lightner, 1977) and fresh water crayfish (Vey *et al.*, 1974). These conditions are known collectively as vibrioses, due to the predominance of *Vibrio* spp. *Vibrio* spp. have been reported in *Homarus americanus* (Bowser *et*

al., 1981), *Callinectes sapidus* (Johnson, 1976; Colwell *et al.*, 1975) and *Penaeus* spp. (Lightner & Lewis, 1975). The symptoms presented in animals affected by such vibrioses are many and varied, and hence are not diagnostic for the causative organisms. These symptoms include lethargy and weakness accompanied by prostration and lack of grooming behaviour; diffuse-to-focal opacity of striated muscle, visible particularly in the abdomen; acellular clots and debris in haemal sinuses and haemocoel; turbidity and reduced clotting of drawn haemolymph; and diffuse-to-focal lesions and necrosis of the gills, tegument and internal organs. (Lightner & Lewis, 1975; Johnson, 1976; Lightner, 1977).

A few gram-positive bacteria have also been implicated in disease epizootics, most notably *Aerococcus viridans* (var.) *homari*, the causative agent of gaffkaemia of American and European lobsters (*Homarus americanus* and *H. gammarus* respectively). Gaffkaemia presents a serious threat to captive lobster rearing operations but, although it is found in natural populations along the Atlantic coasts of North America and Europe, its impact on these populations is not known. Other decapod species have been found with natural, low level infections of *A. v.* (var.) *homari*, (Rabin & Hughes, 1968; Gallagher *et al.*, 1979), and a wide variety have been experimentally infected, but these suffered only mild (if any) disease (Cornick & Stewart, 1968, 1975). Gaffkaemia in homarid lobsters is almost invariably fatal and at high temperatures can cause death in as little as 2 days (Stewart *et al.*, 1969b). Infected animals appear moribund and weak, with thin, discoloured haemolymph which imparts a pinkish colour to the ventral abdomen. Haemolymph clotting is also impaired. The proliferation of *A. v.* (var.) *homari* in the haemocoel of infected animals results in a reduction in the number of circulating haemocytes, due to the formation of aggregations in the gills and other locations (Johnson *et al.*, 1981). Also associated with replication of the agent in the haemocoel is a reduction in haemolymph non-protein nitrogen (Stewart *et al.*, 1969a). However, these

authors state that L-lactate concentrations were unaffected and an observed hyperglycaemia was stress induced. Reductions in the levels of glycogen and ATP in abdominal muscles have also been reported (Stewart & Arie, 1973). Although haemolymph haemocyanin concentrations are not reported to be affected, the oxygen binding capacity of haemocyanin can be reduced by up to 50% (Rittenburg *et al.*, 1979). Death of infected lobsters has been variously attributed to bleeding to death of wounded animals due to impaired clotting ability of the haemolymph, to nutrient depletion (Stewart *et al.*, 1969a) and starvation of oxygen (and hence ATP) (Rittenburg *et al.*, 1979).

Fungal diseases are more significant in crustaceans than those caused by bacterial agents, particularly in wild populations. Populations of *Daphnia*, *Artemia*, amphipods and freshwater crayfish (*Austropotamobius pallipes*) have all been found to be infected by various species of yeasts (Brock & Lightner, 1990). Symptoms typically involve lethargy, and the host becoming white due to the production of large numbers of spores within the haemocoel. Fungal infections have also been implicated in outbreaks of shell disease, either alone or in conjunction with bacterially caused lesions. Death in these cases can often be due to smothering of, or damage to, the gills. Breaching of the cuticle (allowing penetration of internal tissues by the fungus itself or secondary infections by bacterial or other agents) may also lead to mortality.

Burned spot disease affects freshwater crayfish over large parts of Europe. The disease is caused by different fungal species in different crayfish, but the symptoms are very similar. Red or brown/black lesions appear as the fungus grows within the cuticle, which may be breached, allowing access to secondary bacterial infections, possibly resulting in host death. Black mat syndrome (BMS), caused by the Ascomycete *Trichomaris invadens* has for some time been recognised as a serious threat to commercially fished Tanner crabs of the genus *Chionoecetes* in Alaska (Brock & Lightner, 1990). BMS is very site specific within Alaskan fisheries and is totally absent from other Pacific and

Canadian Atlantic populations. The disease was initially regarded as no more than an economic nuisance, affecting the marketability of crab meats because of its appearance. It was thought to be an epibiotic encrustation of the coelomomycete *Phoma fimeti*. However, work by Sparks & Hibbits (1979) and Hibbits *et al.* (1981) revealed the true causative agent, and the fact that very early in infection the cuticle was perforated by fungal hyphae and the internal organs involved. Tissues closely associated with the cuticle are most heavily involved but, in massive infections, much connective tissue around major organs may be replaced by the fungus. Muscle (particularly cardiac) haemopoietic tissue and gut and blood vessel walls may also be severely affected. Necrosis of developing ova has also been observed in heavily parasitised females. Given that high percentages of mature barren females (up to 64%) are seen to be infected in some areas, and that black mat syndrome has also been shown to drastically reduce growth in smaller Tanner crabs, the implications for recruitment in some fisheries are serious. The disease appears to be host specific for the genus *Chionoecetes* (Hibbits *et al.*, 1981). *Chionoecetes bairdi* is the most severely affected species of the genus, whilst *C. opilio* and *C. tanneri* show less serious infections.

Perhaps the most dramatic example of the effects of a fungal disease on a wild crustacean population is 'Krebspest' (Crayfish plague). Krebspest is a virulent disease caused by *Aphanomyces astaci*, a phycomycete fungus of the family Saprolegniaceae. This disease was first recognised in 1860, when large numbers of dead and dying crayfish (*Astacus astacus*) were found in Lombardy, Italy. The epizootic then spread rapidly throughout Europe, reaching France and Germany by 1880, and Finland by 1910. The disease also affects other native European crayfish, *Astacus leptodactylus* and *Austropotamobius pallipes*, and as a result most populations of these species have been severely affected throughout their ranges (Johnson, 1983). All species of Australasian, Asian and European crayfish have been shown to be very susceptible to *A. astaci* infection,

whilst North American species show resistance (Unestam & Weiss, 1970), but have been found to act as hosts for *A. astaci*. This has prompted the suggestion that *A. astaci* originated in North America as a compatible parasite of native species of crayfish, and was introduced to European species with imported animals, thence causing epizootics in unresistant populations (Unestam, 1972; cited in Johnson, 1983). Hyphae of *A. astaci* grow within the cuticle of infected animals in areas adjacent to the site of entry (small lesions, caused either by the pathogen or already present). Heavy invasion of the internal tissues is rare, but hyphae are often associated with the connective tissues, particularly around the ventral nerve cord. The cause of death in susceptible crayfish is not certain but may be due to secondary bacteraemias, to toxins produced by the pathogen or as a result of host defence reactions. The alteration in behaviour and paralysis of the abdomen often seen in infected animals has led to the suggestion that neurotoxic agents may be involved in mortality (Unestam & Weiss, 1970).

With the possible exception of bacteraemias, the commonest cause of symptoms such as those presented by *Nephrops norvegicus* are infections by protistan pathogens. The most important protistan parasites of crustaceans are the microsporidians, with over 140 species reported to infect all orders (Brock & Lightner, 1990). Not all microsporidian infections are severely pathogenic to their hosts, for example *Thelohania heriditeria* infections of *Gammarus duebeni*. This parasite, however, appears to infect only females and has been implicated in the feminization of embryos when involved in oocyte infections (Bulnheim, 1975). Decapod microsporidean infections are considerably more pathogenic. Cotton or milky disease of penaeid shrimps and other decapods is caused by a number of microsporidean agents. The gross symptoms of this group of conditions are the whitening of the body musculature, particularly that of the abdomen (which has led to the alternative name of 'cotton tail'). This whitening is due to the invasion of muscle fibre cells by the pathogen after infection via the midgut epithelium. Once in the host cell, asexual schizogony occurs, and thence

spore formation. Depending on the agent, other tissues may also be involved. Infection by *Ameson nelsoni* has been shown to cause complete destruction of abdominal muscles in six species of penaeid shrimp, whereas *Pleistophora* sp. infections of penaeids, although not causing total muscle destruction, also affect the heart and hepatopancreas (Overstreet, 1973). Sporogenesis of *A. michaelis* in *Callinectes sapidus* results in almost total replacement of skeletal muscle with associated physiological changes, most notably a six to seven times increase in lactic acid concentrations in haemolymph and skeletal muscle and a terminal hypoglycaemia (Findley *et al.*, 1981).

Species of *Thelohania* are also implicated in cotton tail diseases in freshwater crayfish (Thelohaniasis) (Aldermann & Polglase, 1988), cancrid crabs (Vivarès & Cuq, 1981) and penaeids (Lightner, 1988). They are also responsible for so-called 'cottage cheese' diseases of lithodid king crabs involving the invasion of most major tissues by white curd-like debris and fluid (Sparks & Morado, 1985; cited in Brock & Lightner, 1990). All these infections appear to be fatal, but little information is available about the population effects of most. Exceptions are the infection and parasitic castration of 90% of white shrimp on the Louisiana coast by *Agmasoma penaei* (Sprague, 1970) and parasitic castration and high mortalities of *Palaemon* sp. caused by *Indosporus octospora* in France and England (Sprague, 1970).

Sarcomastigophoran parasites play a minor role in crustacean diseases, with the exception of *Paramoeba perniciosa*, the causative agent of Grey Crab disease in blue crabs (*Callinectes sapidus*) (Johnson, 1977a,b). The multiplication of this pathogen in the connective tissues and haemal spaces imparts a grey colouration to the venter of infected animals and, in terminal cases (when the amoebae invade the circulating haemolymph), an opacity to the haemolymph. Seasonal mortalities of blue crabs have been associated with *Paramoeba* infections along the eastern coast of the United States since the mid 1960's (Johnson, 1977b). Commercially significant natural mortalities (Sawyer *et*

al., 1970) and epizootics within holding tank operations (Johnson, 1988) have been reported, and handled crabs die rapidly. Some host response to infection has been reported; in some cases crabs were seen to be able to dispose of parasite cells within the haemolymph, but many of these crabs were seen to die, possibly due to the production of toxins by degenerating amoebae (Johnson, 1977a). Infected crabs probably die as a result of organ dysfunction, respiratory failure or nutrient deficiency. This is supported by the results of physiological studies of infected animals, which showed a reduction in clotting ability of haemolymph and serum haemocyanin and glucose compared with uninfected animals (Johnson, 1977b).

Ciliate and flagellate diseases are not common in crustaceans, and those which cause serious conditions are even rarer. The majority of reported conditions have been associated with intensive rearing operations, often complicated by other infections. *Leptomonas*-like infections (Lightner, 1988), *Zoothamnium* commensal infections of penaeid shrimps (Lightner, 1977) and *Parauronema* infections of penaeid shrimps have all been implicated in hatchery and holding tank mortalities (Lightner, 1977; Couch, 1983). Infections by the holotrichous ciliates of the genera *Anophrys* and *Paranophrys*, particularly *P.maggii*, are reported from *Cancer pagurus*, (Bang et al., 1972) and *Homarus americanus* (Sindermann, 1977), and have been noted in feral populations of *Cancer magister* (Armstrong et al., 1981) and *Carcinus maenas* (Poisson, (1930), cited in Armstrong et al., (1981)). The cause of death in infected crabs is probably major organ dysfunction due to massive proliferation of ciliates within the haemocoel, consuming haemocytes and organ tissues (Armstrong et al., 1981).

Until recently, although abundant in the marine habitat and known to be destructive to their hosts, parasitic dinoflagellate diseases of Crustacea had been studied by relatively few workers. Chatton and other workers (see Chatton, 1952) identified several genera of peridinean dinoflagellates infecting

Crustacea and other invertebrates. Chatton & Poisson (1931) found *Hematodinium perezi* in the haemolymph of *Carcinus maenas* and *Liocarcinus depurator* in European waters. In more recent years *Hematodinium* sp. has been found in *Callinectes sapidus* (Newman & Johnson, 1975), in two species of *Cancer* (*C. irroratus* and *C. borealis*) and in the portunid crab *Ovalipes ocellatus* (Maclean & Ruddell, 1978). A prominent feature of these infections was the low incidence of occurrence, below 5%. In *Callinectes sapidus* occurring off the Florida coast, however, infections can reach a peak incidence of 30% (Newman & Johnson, 1975). Moreover, in the past five years, seasonal *Hematodinium* sp. infections of *Necora* (=*Liocarcinus*) *puber* and *Cancer pagurus* have been reported around the north and west coasts of France associated with winter crab mortalities (Wilhelm & Boulo, 1988; Latrouite *et al.*, 1988). Peak infection rates in *N. puber* were observed to be as high as 87% at one site in early 1987, and a significant effect on stocks was postulated (Wilhelm & Boulo, 1988).

Hematodinium-like infections have also been reported in 13 species of benthic amphipods collected from the continental shelf of the northeast United States (Johnson, 1986). Johnson postulated that, in view of the high seasonal prevalences of infection observed in some species (up to 67%), these dinoflagellates may be involved in population regulation of some species. In addition, infections of commercially fished populations of Tanner crabs (*Chionoecetes bairdi* and, to a lesser extent, *C. opilio*) in south east Alaskan waters with a *Hematodinium*-like dinoflagellate (Bitter Crab Disease) have been reported, with peak infection rates of nearly 100% (Meyers *et al.*, 1987; Meyers, 1990; Meyers *et al.*, 1990; Eaton *et al.*, 1991).

Hematodinium-infected crabs generally appear moribund (Newman & Johnson, 1975), although Maclean & Ruddell (1978) reported that infected crabs were essentially normal in appearance. Dinoflagellate infections of Tanner crabs have a similar affect on their hosts, the majority of crabs being moribund, and killed by the additional stress of handling. All the known

haemocoel dinoflagellate infections are characterised by the milky-white appearance of the haemolymph, particularly in advanced infections, due to the presence of large numbers of vegetative parasite cells. In parasitised blue crabs, *Callinectes sapidus* (Newman & Johnson, 1975) and Tanner crabs, *Chionoecetes bairdi* (Meyers *et al.*, 1987) the internal tissues also take on a white, 'frosted' appearance. In addition, Tanner crabs show degraded muscle with a markedly bitter aftertaste (hence the name 'Bitter Crab Disease', or BCD), making them unsuitable for sale.

Damage to, or resorption of muscle tissue in crustaceans have been reported associated with the physical and nutritional stresses encountered due to starvation, long distance emigrations and moulting. Trendall & Prescott (1989) found increases in abdominal muscle water content after breeding migrations of *Panulirus ornatus* which they attributed to a loss of tissue, due to both reduced feeding and the distance travelled. Starvation also results in resorption of muscle tissue (Dall, 1974) presumably to serve as reserve energy supply. Muscle breakdown in the land crab *Gecarcinus lateralis* occurs during premoult, possibly to allow shedding of the old exoskeleton (Skinner, 1966). Body muscle opacity and necrosis, occasionally seen in cultured fresh-water shrimps of the genus *Macrobrachium* and in penaeid culture, have been attributed to environmental or physiological stresses, particularly high salinity or temperature (Sindermann, 1977).

Chapter 6 presents the results of a histopathological and physiological investigation into the cause of the condition seen in Clyde *Nephrops*, and into its effects on infected animals. The results of surveys of the distribution and prevalence of the condition around the Clyde sea area and the northwest coast of Scotland, and experiments to assess the rates of mortality due to it, are presented in Chapter 7.

CHAPTER 6. INVESTIGATIONS INTO THE NATURE AND PATHOLOGY OF THE DINOFLAGELLATE DISEASE INFECTING

Nephrops norvegicus (L.).

6.1 INTRODUCTION

During the past ten years, an increasing incidence of apparently diseased *Nephrops norvegicus* has been noted in catches taken from grounds in the Clyde sea area. These animals display the discoloured body fluids, lysis of body musculature, lethargy, and mortality induced by capture and handling typical of many crustacean diseases and stress responses (see General Introduction). Most notable of these are systemic protistan infections and bacterial septicaemias, both known to cause extensive mortalities in intensive rearing situations and, to a lesser extent, in natural populations.

The physiological effects of many protistan and bacterial infections are poorly (if at all) known. A few, however, have been studied in terms of their effects on the energy and respiratory metabolism of their hosts. *Ameson michaelis*, one of the microsporidian agents of the so-called 'cotton diseases', causes massive elevation of L-lactate concentrations in haemolymph and skeletal muscle of blue crabs, whilst also causing a terminal hypoglycaemia (Findley *et al.*, 1981). In contrast, infections of lobsters (*Homarus americanus*) by the gram-positive pathogen *Aerococcus viridans* (var.) *homari* (gaffkaemia) result in increased haemolymph D-glucose concentrations (Stewart *et al.*, 1969a), reduced abdominal muscle ATP concentrations (Stewart & Arie, 1973) and reduced haemocyanin oxygen binding capacity (Rittenburg *et al.*, 1979). Haemocyanin metabolism is also reported to be affected by *Paramoeba perniciosa*, where concentration of the respiratory pigment was reduced, as well as haemolymph D-glucose concentrations (Pauley *et al.*, 1975). In the light of these findings, an analysis of the haemocyanin oxygen carrying capacity, copper content, D-glucose and L-lactate concentrations in the haemolymph of affected *N. norvegicus* was undertaken as well as analyses of the ATP, L-lactate and D-glucose concentrations within the abdominal musculature.

Measurements of water content of the abdominal musculature were made in conjunction with light microscopical examination of deep flexor muscles to

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determine the cause of the apparent muscle lysis. The 'milky' appearance of the haemolymph of affected animals prompted an examination of total haemolymph cell numbers. In addition, a histopathological survey of the major organs and an electron microscopical examination of the cells in the haemolymph were undertaken to confirm the presence or absence of a pathogenic agent and to assess the effects of the condition on *N. norvegicus*. The possible presence of a bacterial agent was further investigated by microbiological examination of the haemolymph and certain internal tissues and organs.

These examinations have led to the discovery of non-motile, protistan parasites filling the haemal spaces. These parasites are dinoflagellates of the order Syndiniales, and resemble in particular *Hematodinium perezi* known to infect other species of decapod Crustacea. This chapter presents the results of these investigations into the cause of the condition observed in *N. norvegicus* collected from the Clyde sea area, and into the nature of the symptoms shown by affected animals.

6.2 MATERIALS AND METHODS

6.2.1 Experimental animals. The *N. norvegicus* used in laboratory investigations were obtained either by trawling during the course of prevalence and distribution investigations (see below), or were caught by creeling on grounds close to the Isle of Cumbrae, Firth of Clyde. Animals were maintained in aquaria in well aerated running seawater at the Zoology Department, University of Glasgow for up to 5 days. The temperature ranged between 10 and 13°C and the salinity between 33 and 34‰. Animals were fed *ad libitum* on squid and mussel flesh. Only animals in intermoult were used for experiments, as determined by the pleopod moult staging technique of Aiken (1980).
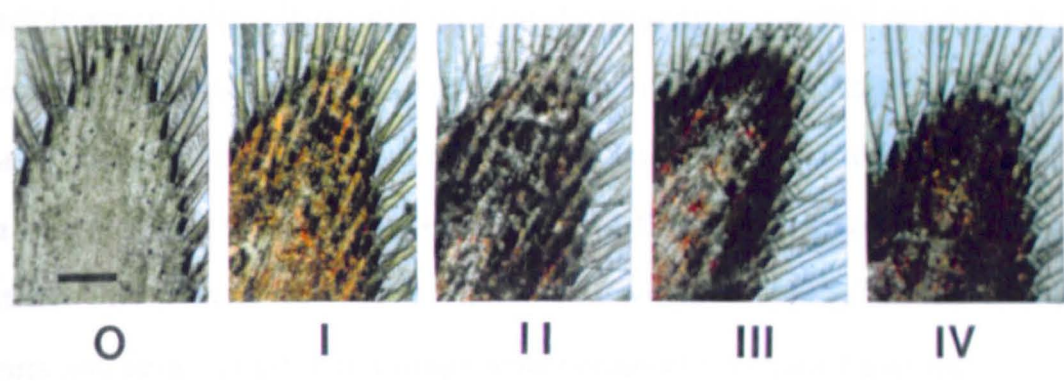
6.2.2 Diagnosis. Severely affected animals were easily recognised by their dull orange colouration (Figure 5.1) and 'milky' haemolymph, clearly visible through the translucent cuticle of the ventral abdomen. However, mild cases could not be reliably identified by this method alone. The condition was most easily and accurately diagnosed by microscopic examination of the pleopods where, under transmitted light, dense aggregations of cells in the haemolymph appeared as darkened areas (Figure 6.1). This method was found to be effective and consistent in identifying even slightly affected individuals, and was also useful in providing a measure of the severity of the condition. Severity was measured on a scale from I to IV (or 0 for unaffected animals), based on the degree of cell aggregation visible in the pleopod (Figure 6.1, Table 6.3).

6.2.3 Abdominal muscle and haemolymph metabolite concentrations. Haemolymph and abdominal deep flexor muscle tissue samples were taken from both affected and apparently healthy individuals as detailed in Chapter 2. All animals sampled in the laboratory were in a rested state. Care was taken to complete muscle and haemolymph sampling from each individual within 30 seconds, to minimise the risk of inducing stress-related hyperglycaemia (Telford, 1968). In addition, haemolymph samples only were taken from groups of affected and unaffected individuals immediately post-trawling, to examine

FIGURE 6.1. Light micrographs showing the appearance of pleopods of healthy and dinoflagellate infected *N. norvegicus*. The density of the layer of aggregated cells beneath the cuticle allows a measurement of the severity of infection on an arbitrary scale from I to IV. 0 = uninfected; I = slight infection; IV = heavy infection. Bar = 0.5mm.

O I II III IV

the effects of the additional stresses of trawling and handling on the energy metabolism of affected *N. norvegicus*. The D-glucose (and glucose-6-phosphate), L-lactate and ATP concentrations of abdominal muscle samples were determined by the enzymic methods given by Hill (1989), and detailed in Chapter 2, section 2.2.4. Similarly, D-glucose and L-lactate concentrations were measured in haemolymph samples.

6.2.4 Abdominal muscle water content. Samples of abdominal deep muscle were taken from affected and apparently normal animals for water content determination during removal of the abdomen for metabolite concentration determination. The two most caudal segments of the abdomen were removed and the deep extensor and flexor muscles separated from the cuticle. The muscle from each animal was weighed, oven dried at 100°C for 24 hours and re-weighed. Percentage water content was then calculated.

6.2.5 Haemolymph total cell counts. Haemolymph samples of 500μl were collected from the base of a 5th pereiopod using 1ml disposable syringes containing an equal volume of diluting fluid (as detailed by Stewart *et al.*, 1967). The samples were then diluted and the cells were counted by the method of Stewart *et al.* (1967). Both unaffected and affected animals of all degrees of severity were examined, but only animals in intermoult were used, as determined by the pleopod moult staging technique of Aiken (1980).

6.2.6 Stained haemolymph smears. Small quantities of fresh, undiluted haemolymph were withdrawn into 1ml disposable syringes as above. A small drop of each haemolymph sample was immediately placed on a clean glass slide, and a thin smear drawn out. These smears were allowed to dry in air, and then fixed and stained with a 0.2% w/v solution of Leishman's stain in methanol (BDH Chemicals Ltd. Poole England). Examination of these slides at 1000x magnification under oil immersion yielded estimates of the relative proportions of host haemocytes and parasite cells present. The numbers of host cells and

parasites in 10 fields of view were counted for each smear, and the counts of host haemocytes were further sub-divided into haemocyte types. The three types recognised, based on those described for the blue crab by Johnson (1980), were young/hyaline, semigranular and granular haemocytes. These types were identified on the basis of the presence and degree of cytoplasmic granulation, relative nuclear size and cell size.

6.2.7 Haemocyanin oxygen carrying capacity and copper concentration of haemolymph. The haemolymph of healthy *N. norvegicus* has a translucent blue colour, imparted by the presence of the oxygenated form of the copper-based respiratory pigment haemocyanin. This colour was not evident in the haemolymph of affected individuals, and consequently an analysis of the haemocyanin oxygen carrying capacity and copper concentrations of the haemolymph of these animals was undertaken. Following dilution with deionised water, the copper concentration of the haemolymph was determined using an atomic absorption spectrophotometer (Philips PU9200) calibrated with an appropriate range of copper standards. The haemocyanin oxygen carrying capacity of the haemolymph was determined using the method of Tucker (1967) as modified by Bridges *et al.*, (1979).

6.2.8 Microbiology. Haemolymph samples were taken aseptically from one affected female (staged at III by pleopod examination) and one apparently healthy, control male *N. norvegicus*, both in intermoult. Samples were taken via the arthrodial membrane of a 5th pereiopod, after sterilisation with 70% alcohol, using sterile disposable 1ml syringes. From each of these samples, 100μl subsamples of serum were taken and plated onto marine agar (Zobell 2216E), and incubated at 10°C for one week. After this period the plates were examined and the numbers of colony-forming units (cfu) counted and compared. In addition, during the course of the aseptic dissection of an intermoult male suffering from stage III symptoms (for samples of internal organs for histopathology) the following tissues were removed for

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microbiological analysis; pleopod, haemopoietic tissue, heart, hepatopancreas and antennal gland. These samples were dispersed in 1ml of tryptone saline (1% tryptone in 3.2% NaCl), or 10ml in the case of hepatopancreas. From these dispersed samples, 100μl aliquots were plated onto marine agar, and incubated at 10°C for one week. Thereafter, the plates were examined and the numbers of colony-forming units counted.

6.2.9 Histopathology. Two male *N. norvegicus* exhibiting severe symptoms of the condition (staged at III by pleopod examination) were dissected to provide samples of the major organs. Two further individuals (one male and one female), both confirmed to be healthy by pleopod examination, provided samples of unaffected tissues from the same organs. All animals were in intermoult, and were caught by trawling during routine sampling in the Clyde. The organs removed were; hepatopancreas, antennal gland, midgut, abdominal muscle, haemopoietic tissue, heart, gills and pleopod. Upon removal, tissues were fixed in Helly's mercuric chloride fixative (Johnson, 1980) and embedded in paraffin wax. Thin sections (6μm) were treated with Lugol's iodine solution to remove mercury, and stained with haematoxylin and eosin (H & E) (Johnson, 1980).

6.2.10 Electron microscopy. Haemolymph was taken from one severely affected male (stage III) and one healthy male *N. norvegicus* for examination of constituent cells by electron microscopy. To minimise sampling artefacts by mechanical stresses and clotting, haemolymph was allowed to flow from a cut walking leg directly into fixative (2% paraformaldehyde, 1% glutaraldehyde, 4% sucrose in 0.1M phosphate buffer). Cells for examination by transmission electron microscopy (TEM) were rinsed twice in chilled phosphate buffer (0.1M PO_4, 8% sucrose), post fixed in 1% OsO_4 in phosphate buffer and pelleted by centrifugation. Pellets were stained with 0.5% uranyl acetate, dehydrated through an alcohol series and embedded in Araldite. Thin sections (60-70 nm) were stained with uranyl acetate and lead citrate and examined using a Philips

301 TEM. Cells for scanning electron microscope (SEM) examination were dehydrated through an acetone series, resuspended and filtered through 0.2 μm microfilters after uranyl acetate staining. Cells retained on filters were then critical point dried, coated with gold and examined using a Philips 500 SEM.

6.3 RESULTS

6.3.1 Gross appearance. Infected animals sustained varying degrees of lethargy, which seemed to worsen with increased severity of infection. Severely affected individuals were moribund and often had difficulty in walking or even supporting themselves. The 'milky', yellow-white appearance of the haemolymph appeared to be due mainly to the presence of increased cell numbers, but also to quantities of suspended 'debris'. Examination of the cephalothoracic cavity showed that the internal organs were coated in deposits of the thick 'creamy' haemolymph, which appeared reduced in volume and of increased viscosity. The haemopoietic tissue, the heart and pericardium were swollen and yellow with collected haemolymph. Other organs were shrunken, most strikingly the hepatopancreas, which also had a very obvious green colouration instead of the normal brown.

6.3.2 Abdominal muscle water content. The water content of the abdominal muscles of infected animals was found not to vary significantly from that of apparently healthy ones, sampled at the same time of year (Table 6.1). This was true of all three samples, taken in February and March 1989, and March 1990.

It is also interesting to note that there was a significant decrease in muscle water content in both infected and apparently healthy animals between the February and March 1989 samples, whereas those in the March samples of both years showed no significant difference from one another.

6.3.3 Abdominal muscle and haemolymph metabolite concentrations. Table 6.2 shows that ATP concentrations in the abdominal musculature of affected animals were significantly lower than those of apparently healthy *N. norvegicus* ($P<0.05$, t-test). Similarly, the glucose-6-phosphate concentrations in affected individuals were also reduced ($P<0.05$, t-test). Changes in muscle L-lactate and D-glucose concentrations were not significant at the $P=0.05$ level, although D-glucose concentrations appeared raised, with a significance of

TABLE 6.1. Mean abdominal muscle water content expressed as a percentage of total wet weight of tissue, of infected and apparently healthy *N. norvegicus* from three samples taken in the Clyde in February and March 1989, and March 1990. The upper half of the table compares infected and healthy animals, whilst the lower half shows the significance of seasonal muscle water content variations (pooled for healthy and infected animals). N.S. = not significant. All comparisons were made using *t*-tests, after arcsine transformation. (Figures in parentheses are sample sizes).

	February '89	March '89	March '90
Healthy	80.49 (13)	78.20 (10)	77.77 (11)
	N.S.	N.S.	N.S.
Infected	80.16 (11)	78.33 (20)	78.39 (17)
Overall	80.35	78.31	78.26
Feb. '89	-	P <0.001	P <0.001
Mar. '89	-	-	N.S.

TABLE 6.2. Mean concentrations of energy metabolites in abdominal musculature (μmol g-1 wet weight) and haemolymph (μmol ml-1) of infected and apparently healthy *N. norvegicus* taken in the Clyde. N.S. = not significant. All comparisons were made using twosample t-tests. Figures in parentheses are standard deviations around the means. (n = 23 healthy animals, 31 infected animals, except for post-trawl blood metabolite determinations, where n = 22 healthy animals, 12 infected animals).

Metabolite	Metabolite concentration			
	In rested animals		Post-trawling	
	Healthy	Infected	Healthy	Infected
Muscle ATP	6.43 (2.31)	4.20 (2.67)	-	-
		P <0.005		
Muscle Glucose- 6-phosphate	0.94 (0.61)	0.57 (0.57)	-	-
		P <0.05		
Muscle D-Glucose	0.86 (0.80)	1.34 (1.05)	-	-
		0.05<P<0.06		
Muscle L-Lactate	10.89 (7.46)	13.03 (7.45)	-	-
		N.S.		
Haemolymph D-Glucose	0.20 (0.15)	0.34 (0.24)	0.51 (0.25)	0.57 (0.37)
		P <0.05		N.S.
Haemolymph L-Lactate	0.09 (0.05)	0.16 (0.25)	4.88 (2.10)	2.87 (2.08)
		N.S.		P <0.001

0.05<P<0.06, t-test). This may indicate that some alteration of D-glucose concentrations had occurred due to the condition, especially as haemolymph D-glucose concentrations showed a significant increase in affected animals above those of control animals (P<0.05, t-test). Haemolymph L-lactate concentrations, however, remained unchanged. In contrast to this, the haemolymph L-lactate concentrations measured in affected animals immediately post-trawling were significantly lower than those measured in the control group (P<0.01, t-test). Despite this, the concentrations of L-lactate in the haemolymph of both affected and control groups were significantly higher than those seen in any animals sampled when at rest in the aquarium.

6.3.4 Total haemolymph cell counts.

Total haemolymph cell counts confirmed that the abnormal colouration of the haemolymph in severely affected animals was due to an increase in total cell numbers (both host haemocytes and parasite cells) (Table 6.3). Cell counts in mild infections (pleopod stages I and II; $1.35 \times 10^4 \pm 0.93 \times 10^4$ and $2.02 \times 10^4 \pm 1.34 \times 10^4$ cells per mm^3 respectively) were not significantly different from those of healthy individuals ($1.35 \times 10^4 \pm 0.56 \times 10^4$ cells per mm^3), but stage III infections were characterised by a significant, more than fourfold increase in cell numbers above stage II numbers (to $8.55 \times 10^4 \pm 5.65 \times 10^4$ cells per mm^3, P<0.005, one-way ANOVA). Interestingly, more severe (stage IV) infections showed a return towards near normal numbers of circulating cells.

6.3.5 Stained haemolymph smears.

Stained smears revealed that the percentage of the total cell content of infected haemolymph contributed by parasite cells ranged from 3% to 89%. This indicates that there is probably a progression of infection involving an increase in parasite numbers within the haemolymph, although percentages could not be related to severity (based on the arbitrary pleopod scale used). However, the stained smears were useful in assessing another possible effect of infection on haemocyte 'dynamics'. Comparison of the differential haemocyte counts of infected and uninfected

TABLE 6.3. Variation of total haemolymph cell counts of *N. norvegicus* in relation to degree of severity of infection, assessed by pleopod staging on an arbitrary scale from I to IV, 0 = apparently uninfected (see Figure 6.1). Samples compared by Oneway ANOVA, * = significantly higher at P<0.005.

Pleopod Stage	Description	Number of Animals	Haemolymph Cell Count $(\times 10^4$ per mm^3)	
			Mean	S.D.
0	A few cells visible, no aggregation, pleopod clear	11	1.348	0.563
I	Clumps of aggregated cells around pleopod periphery	12	1.346	0.932
II	Cell aggregation continuous around pleopod periphery	12	2.017	1.337
III	Layer of aggregated cells expanded to more central areas of pleopod	7	8.551	5.650*
IV	Pleopod interior almost totally obscured by masses of aggregated cells	4	3.331	1.171

animals revealed no significant differences in the relative percentages of the three haemocyte types between the two groups.

6.3.6 Haemocyanin oxygen carrying capacity and copper concentration of haemolymph.

Copper concentrations in the haemolymph of affected animals were found to be significantly lower than those of apparently healthy individuals (0.61 ± 0.29 mmol l^{-1} compared with 0.94 ± 0.34 mmol l^{-1}, P<0.001 t-test). The effects of the condition were further illustrated by the analysis of the haemocyanin oxygen carrying capacity of the haemolymph of affected animals. This showed the carrying capacity of haemocyanin in abnormal haemolymph to be significantly lower than in control animals (0.25 ± 0.21 mmolO_2 l^{-1} compared with 0.44 ± 0.17 mmolO_2 l^{-1}, P<0.001, t-test).

6.3.7 Microbiology.

Examination of the plated-out haemolymph samples revealed the following results; serum from the affected animal yielded 38 large cream-coloured colonies of 5-6mm diameter, and approximately 320 small yellow colonies of 1-2mm diameter; control serum from the apparently healthy individual yielded similar results, 11 large colonies as above and 410 small colonies as above. These numbers correspond to approximately 4 x 10^3 cfu ml^{-1}. Marine agar plates of tissue samples taken from an affected animal yielded a single uniform colony type, probably a pseudomonad. The concentrations encountered corresponded to the numbers of cfu ml^{-1} given in Table 6.4.

Both the bacterial numbers observed in the haemolymph samples, and those of internal organs and tissues were all relatively low and probably not indicative of any serious bacterial infection. The numbers of cfu encountered in cultures taken from pleopod tissue were considerably greater than those obtained from either haemolymph or other tissue samples, and this may be due to the presence of contaminating bacteria on the exterior surfaces of the pleopod cuticle remaining after incomplete sterilisation.

TABLE 6.4. Numbers of bacterial colony forming units per ml estimated from the plating-out and incubation of samples of various organs and tissues from infected *N. norvegicus*.

Tissue	Numbers cfu ml^{-1}
Pleopod	10,000
Haemopoietic tissue	50
Heart	120
Hepatopancreas	230
Antennal gland	190

6.3.8 Microscopical appearance. Examination of fresh, unfixed affected haemolymph by phase contrast microscopy revealed the abnormal turbidity to be due to large numbers of non-motile cells superficially similar in size and appearance to host haemocytes (between 5 and 14μm in diameter)(Figure 6.2). Whether or not host haemocyte numbers are changed absolutely in affected animals has not yet been determined. However, there was no suggestion of impairment of clotting ability, and haemocytes in fresh smears of affected haemolymph appeared to spread and aggregate normally (Figure 6.2).

In fixed, sectioned and stained material, the parasite was seen to have infiltrated the haemal spaces of all the major organs, as well as the haemocoel. The majority of parasite cells were uninucleate, many undergoing mitosis (Figure 6.3). These cells typically displayed a weakly staining reduced cytoplasm surrounding a densely stained nucleus. The mitotic figures revealed the very distinctive 'V'-shaped chromosomes typical of the dinokaryon-type nucleus of dinoflagellates. Multi-nucleate 'plasmodial' cells were also common in the haemal spaces (Figure 6.3), typically with between two and five nuclei, again often showing mitotic figures. Also present, but apparently attached to cardiac muscle within the lumen of the heart, were many multi-nucleate 'vermiform' cells (Figure 6.4). These also displayed up to 5 nuclei in various mitotic stages.

6.3.9 Ultrastructure of parasite. SEM examination of infected haemolymph showed the irregularly shaped cells to be of a similar size to host haemocytes and to be without flagella. The cell surface was irregular, showing bulges and some form of extrusion (possibly of trichocyst material) similar to that seen by Meyers *et al.* (1987) in the Bitter Crab Disease parasite (Figure 6.5).

TEM observation of the irregularly shaped cells from infected haemolymph showed them to be quite unlike host haemocytes. Cells were between 6 and 10μm in diameter. They were bounded by a characteristic amphiesma, showing amphiesmal alveoli with a

FIGURE 6.2. Light micrograph of uni-nucleate dinoflagellate cells in fresh haemolymph of *N. norvegicus*. Note the apparently normal spreading of host haemocytes (Hc). Phase contrast. Bar = 10μm.
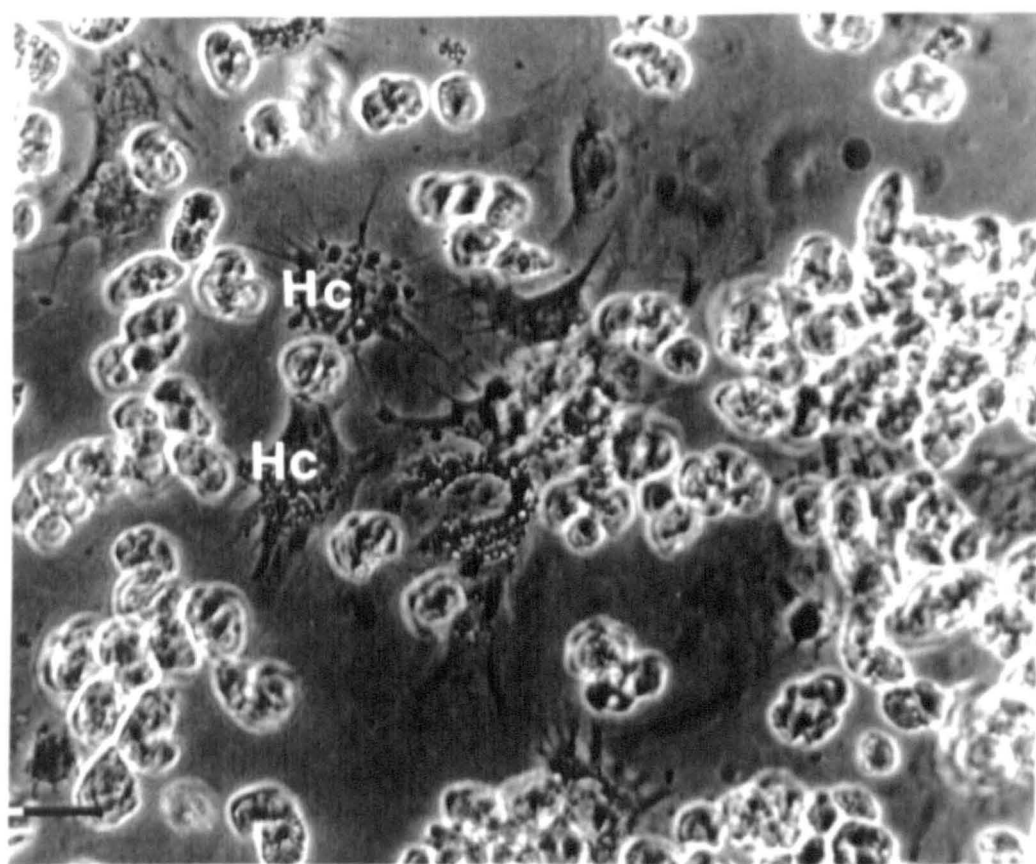
FIGURE 6.3. Light micrograph of uni-nucleate and multi-nucleate plasmodial dinoflagellate cells in fixed, sectioned haemolymph of infected *N. norvegicus*. Note prominent V-shaped chromosomes. P = plasmodial cell. H & E. Bar = 10μm.

FIGURE 6.4. Light micrograph of multi-nucleate vermiform dinoflagellate cells apparently attached to heart tissues of infected *N. norvegicus*. V = vermiform cells; M = myocardial muscle. H & E. Bar = 10μm.
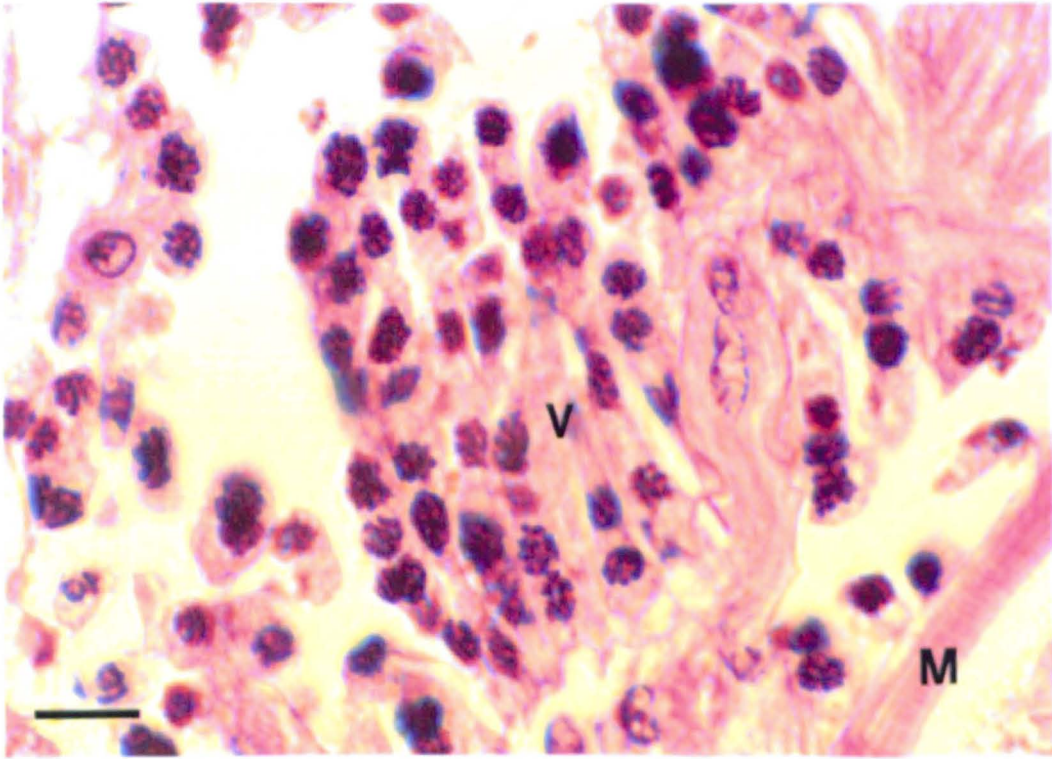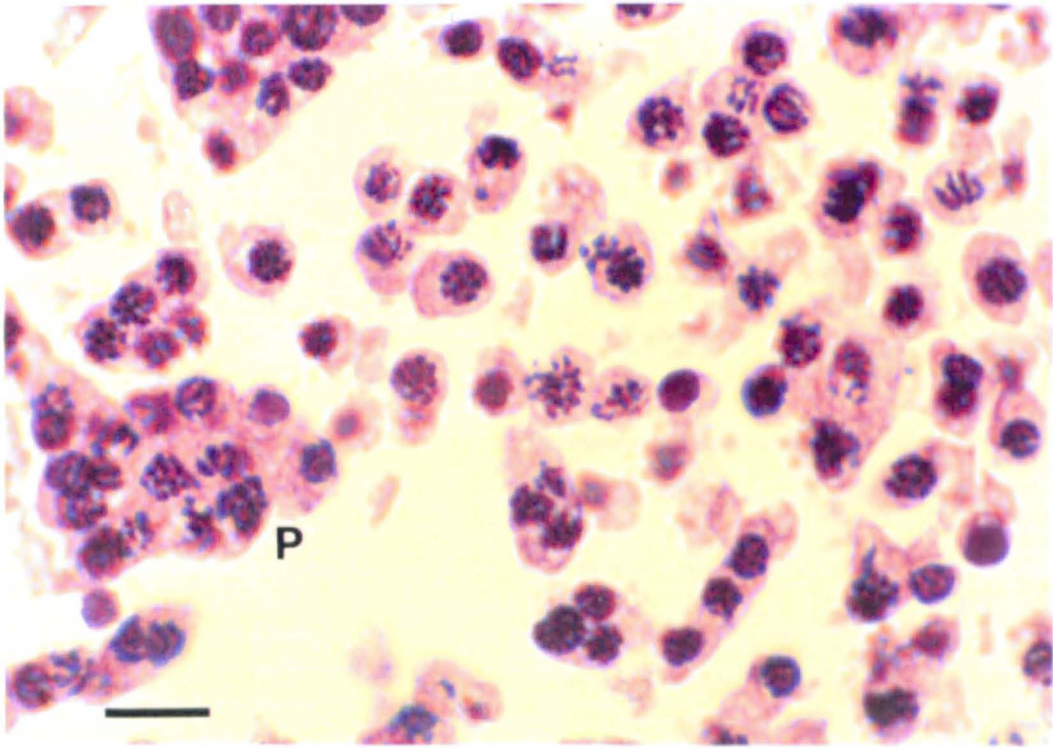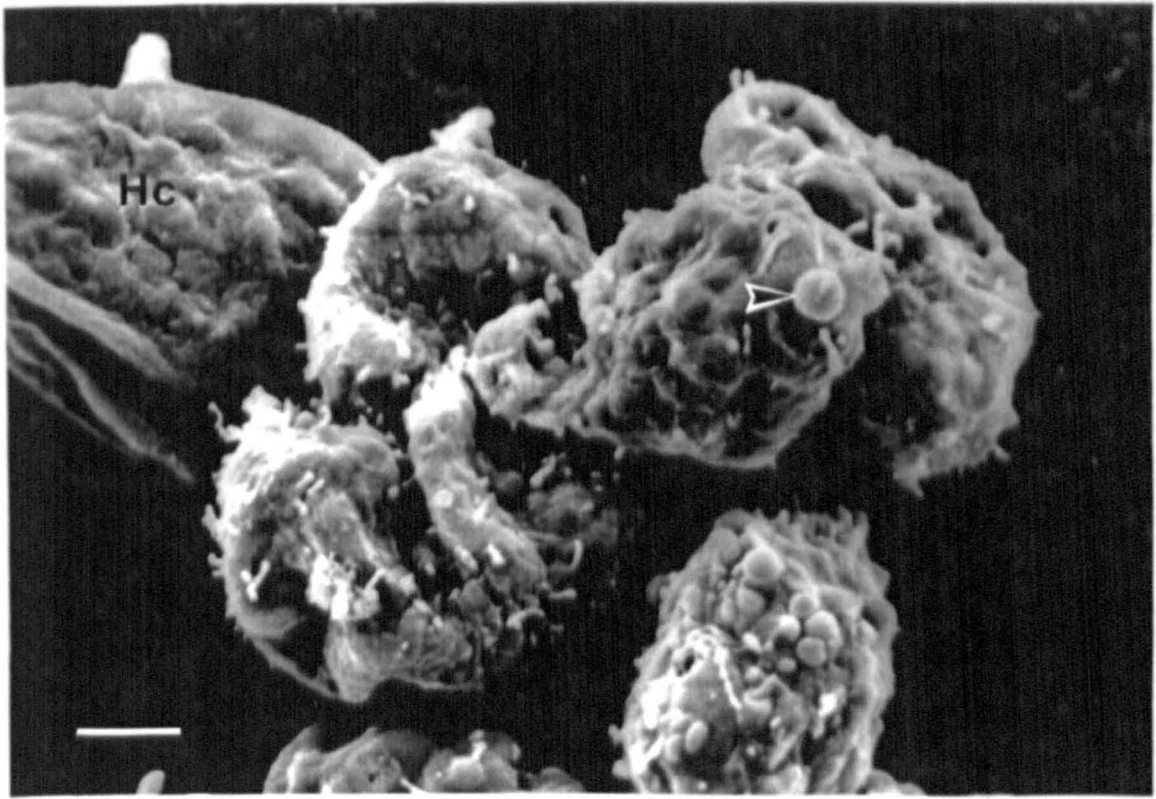
FIGURE 6.5. Scanning electron micrograph of dinoflagellate cells in the haemolymph of infected *N. norvegicus*. Note irregular cell shape and exudate globules (arrow). Hc = host haemocyte. Bar = 2μm.

three-membraned structure (Figures 6.6 & 6.7). There was no indication of thecal plates associated with the amphiesma, nor of microtubules reinforcing the innermost membrane. Nuclei were bounded by a double membrane nuclear envelope and showed chromosomal profiles and nucleoli (Figures 6.6 & 6.7). The cytoplasm showed large numbers of lipid globules, membrane-bound inclusion vesicles and many membrane-bound trichocysts. Rough endoplasmic reticulum was present around the trichocysts. The abundant mitochondria showed the tubular cristae characteristic of dinoflagellates (Figure 6.7). Chloroplasts were absent, and flagellated stages have not been identified as yet. Some evidence of flagellar structures, however, was seen. In some cells, groups of distinctive, membrane-bound vesicles were seen associated with the golgi apparatus, close to the nucleus (Figures 6.7 & 6.8). These vesicles contained material of a fibrous appearance, and usually occurred in groups of three or four. They resembled in particular the large perinuclear vesicles reported by Dodge (1987) in other dinoflagellates, associated with the production of flagellar hairs, and suggest the possibility that a flagellate stage exists in this particular organism.

The presence of prominent chromosomes and trichocysts, a pellicle of amphiesmal structure, tubulocristate mitochondria and the absence of chloroplasts and flagella support the identification of the parasite as the vegetative stage of a parasitic dinoflagellate, probably belonging to the botanical order Syndiniales and possibly related to *Hematodinium* sp.

6.3.10 Histopathology of condition. The histopathological effects of infection of *N. norvegicus* by this dinoflagellate have been investigated by comparison of the histology and structure of various major organs from infected and apparently healthy individuals. The terminology used in the description of organs follows that used by Johnson (1980).

I) Hepatopancreas.

The hepatopancreas (or midgut gland) of healthy animals consists of

FIGURES 6.6. Transmission electron micrograph of vegetative dinoflagellate cell from infected *N. norvegicus* haemolymph. A = amphiesmal alveolus; M = mitochondrion; G = golgi apparatus; In = inclusion vesicles; Tr = trichocysts; L = lipid globules; N = nucleus; C = chromosomal profile. Bar = 1μm

FIGURES 6.7. Transmission electron micrograph of vegetative dinoflagellate cell from infected *N. norvegicus* haemolymph showing details of cell surface and cytoplasmic inclusions. A = amphiesmal alveolus; M = mitochondrion; G = golgi apparatus; In = inclusion vesicles; Tr = trichocysts; L = lipid globules; N = nucleus; C = chromosomal profile; F = flagellar hair vesicles. Bar = 0.5μm.
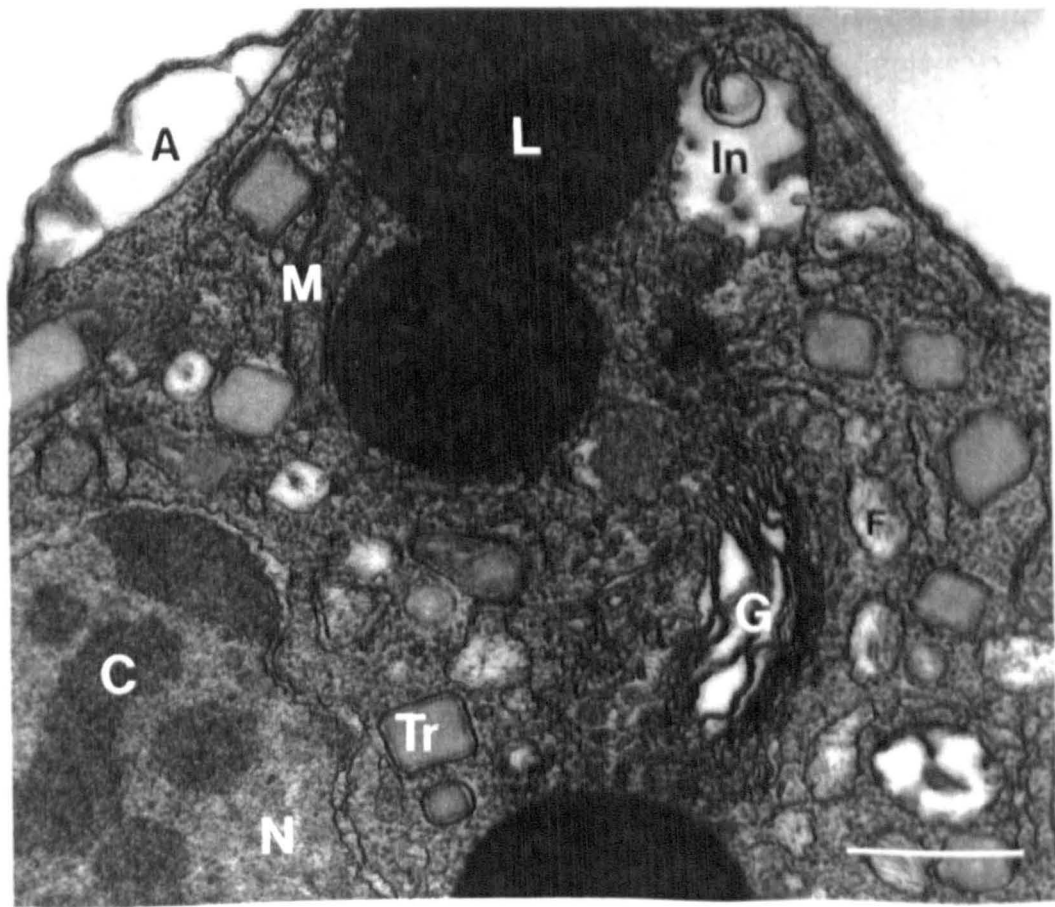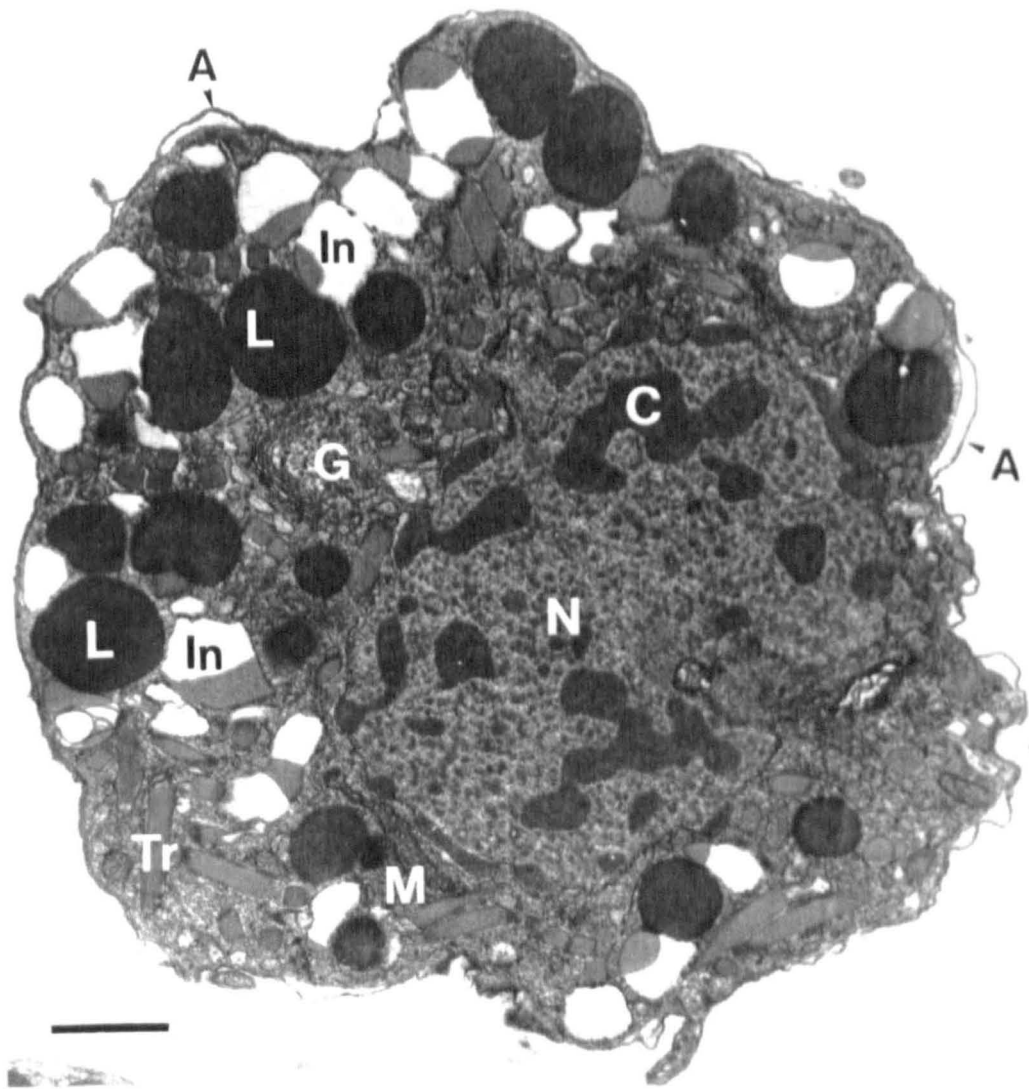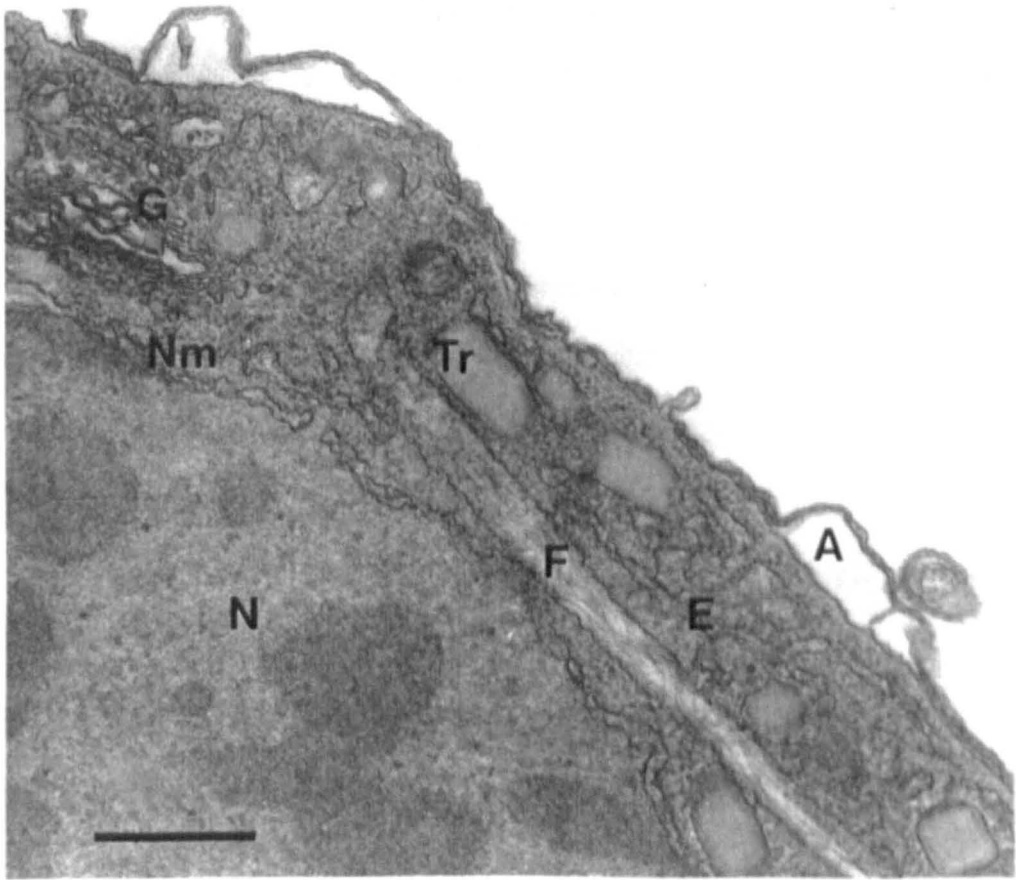
FIGURE 6.8. Transmission electron micrograph of vegetative dinoflagellate cell from infected *n. norvegicus* haemolymph showing detail of possible flagellar hair vesicle associated with golgi apparatus. F = flagellar hair vesicle; N = nucleus; Nm = nuclear membrane; G = golgi apparatus; E = rough endoplasmic reticulum; Tr = trichocyst; A = amphiesmal alveolus. Bar = 0.5μm.

many blind-ended tubules, internally bordered by a brush border of microvilli, and surrounded by haemal spaces (Figure 6.9). Within the haemal spaces occur what appear to be blood vessels, often surrounded by groups of fixed phagocytes (Figures 6.9 & 6.10)(Johnson, 1980). The tubule epithelium comprises four cell types, whose distributions vary with position in the tubule. The apical epithelium of tubules is composed of undifferentiated cuboidal to low columnar 'E-cells' (Figure 6.11). Distal to this occurs a region of mixed cell types (Figure 6.12); columnar 'F-cells' with a darkly staining granular cytoplasm and a large darkly staining central to basal nucleus; narrow columnar 'R-cells', sometimes bi-nucleate, but usually displaying one small, weakly stained basal nucleus (the bi-nucleate state may in fact be common or the rule, but is not always apparent in thin sections) and many cytoplasmic vacuoles; larger, more cuboidal 'B-cells' with a compressed, basal, darkly staining nucleus, and a large vacuole occupying most of the cytoplasm, sometimes accompanied by a few smaller vacuoles.

The hepatopancreatic structure of infected animals shows several marked differences from the normal situation. Firstly, the haemal spaces between the tubules are much enlarged, and many parasite cells are visible, some being associated with the tubule walls (Figure 6.13). Secondly, much lysis, both between the epithelial cells, and in the tubule walls, is evident (Figure 6.14). It is possible that at least some of this lysis may be the result of prefixation artefacts (Johnson, 1980), since lysis is sometimes apparent in the hepatopancreas of healthy animals. However, in these cases this is restricted to breakdown of epithelial cells and tubule walls are not breached. In addition, although it has not been investigated quantitatively, an increase in the number of 'B-cells' is apparent (Figure 6.14).

II) Antennal gland.

The paired antennal glands of Crustacea (green glands, excretory glands, antennary glands) are, along with the gills and gut, the sites of water and

FIGURE 6.9. Light micrograph of transverse section of the hepatopancreatic tubules of healthy *N. norvegicus*. Hs = haemal space; Tl = tubule lumen; Bv = blood vessel. H & E. Bar = 100μm.

FIGURE 6.10. Light micrograph of transverse section the hepatopancreas of healthy *N. norvegicus* showing blood vessels within haemal spaces. E = epithelium of blood vessel; Fp = fixed phagocytes; H = haemocytes within lumen of blood vessel; Te = hepatopancreatic tubule epithelium. H & E. Bar = 20μm.
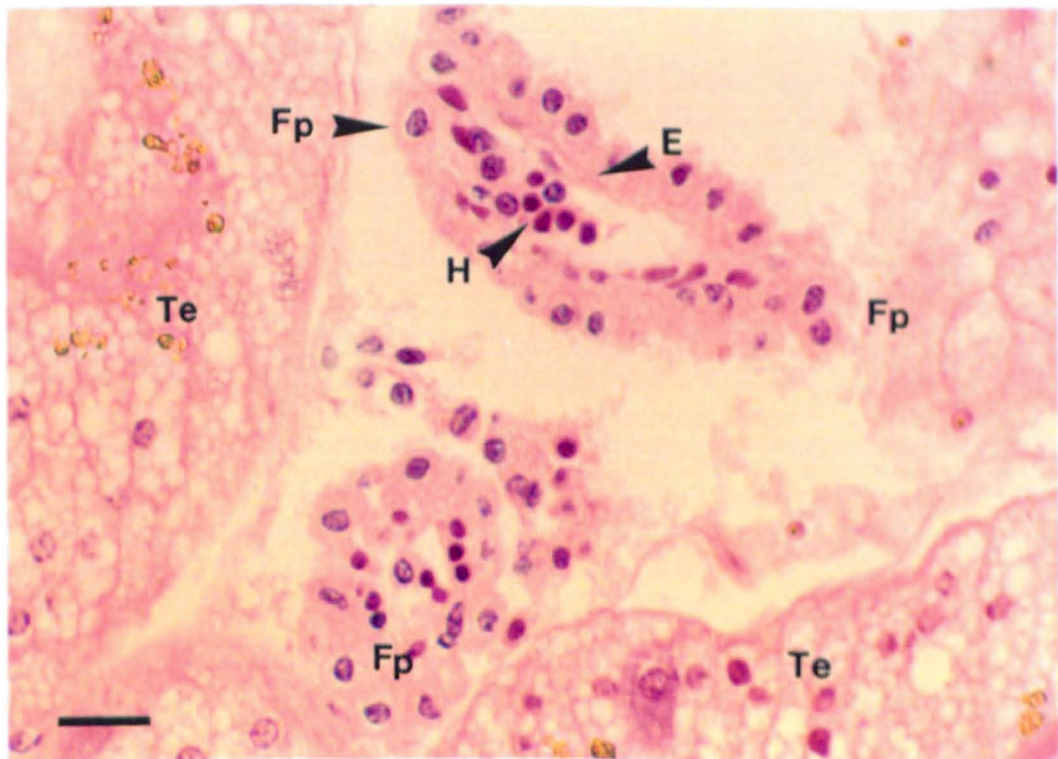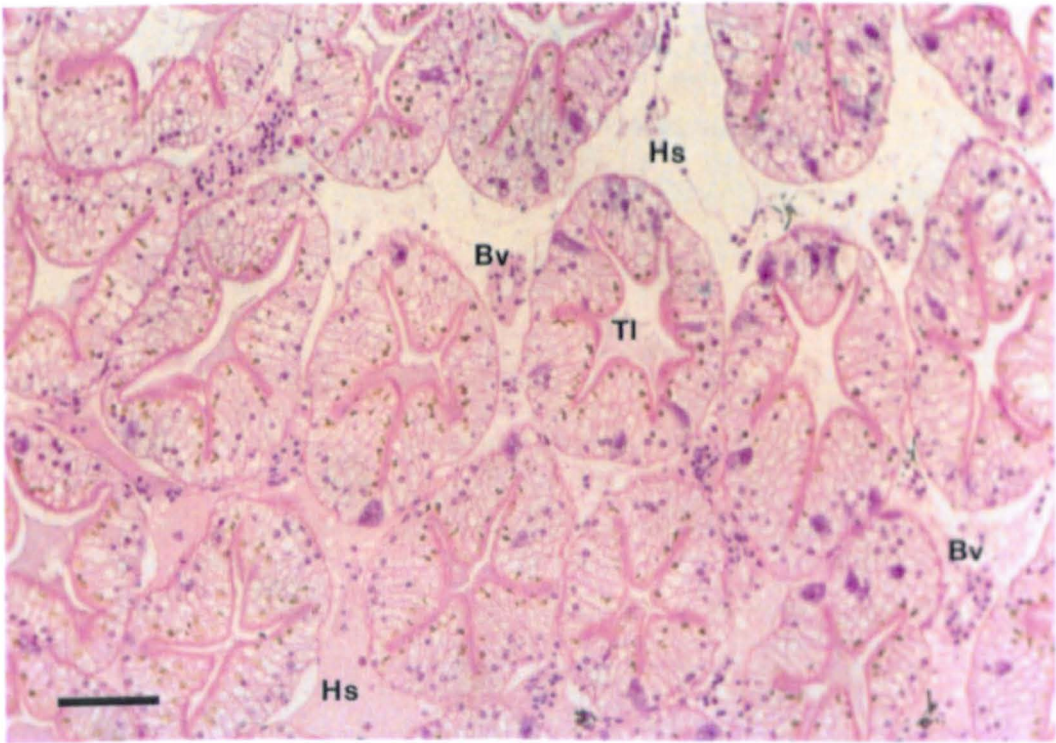
FIGURE 6.11. Light micrograph of longitudinal section of hepatopancreatic tubule of healthy *N. norvegicus* showing the appearance of the apical epithelium. E = 'E'-cell zone; H = haemocyte in haemal space; L = lumen of tubule. H & E. Bar = 20μm.

FIGURE 6.12. Light micrograph of transverse section of hepatopancreatic tubules of healthy *N. norvegicus* showing the various cell types present in the distal regions of the tubule epithelium. F = 'F'-cell; R = 'R'-cell; B = 'B'-cell; Tl = tubule lumen; Hs = haemal space; Bv = blood vessel. H & E. Bar = 40μm.
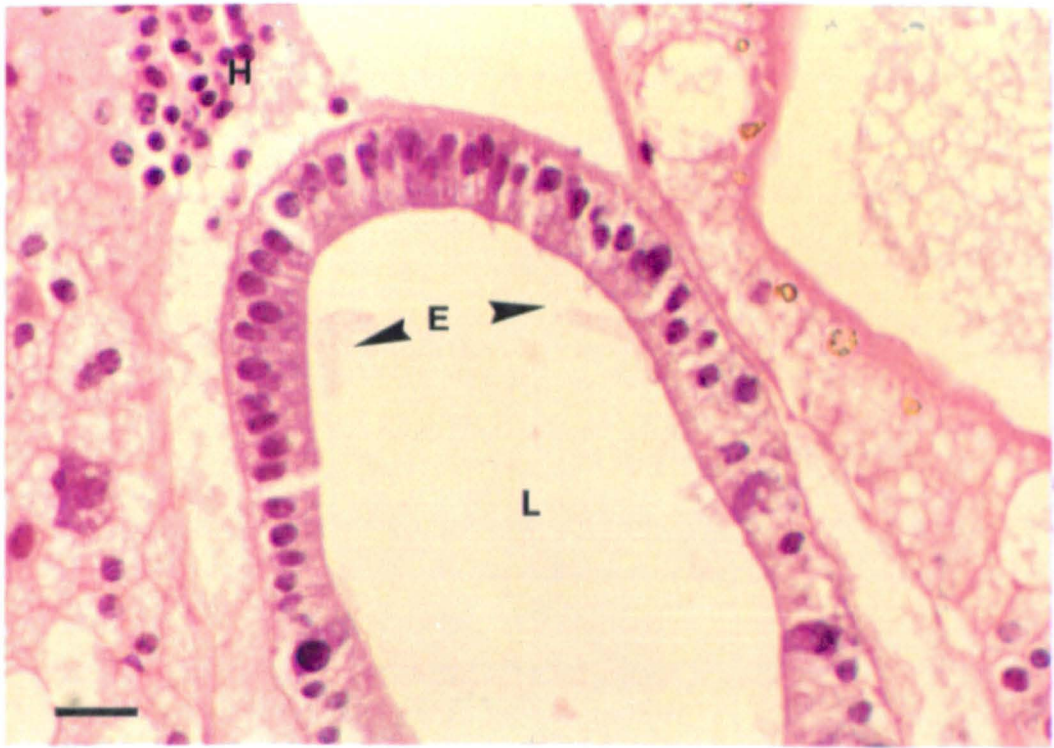
FIGURE 6.13. Light micrograph of transverse section of hepatopancreatic tubules of dinoflagellate infected *N. norvegicus* showing enlargement and distention of haemal spaces between tubules with parasite cells, and increased vacuolation of tubule epithelium. Tl = tubule lumen; Hs = haemal space filled with parasite cells. H & E. Bar = 100μm.

FIGURE 6.14. Light micrograph of transverse section of hepatopancreatic tubules of dinoflagellate infected *N. norvegicus* showing detail of lysis of tubule epithelium, the increase in the number of 'B'-cells and the increase in the size of 'B'-cell vacuoles. Note the presence of large numbers of parasite cells in haemal spaces and a few host haemocytes (arrows). Tl = tubule lumen; B = 'B'-cells; D = parasite cells. H & E. Bar = 40μm.
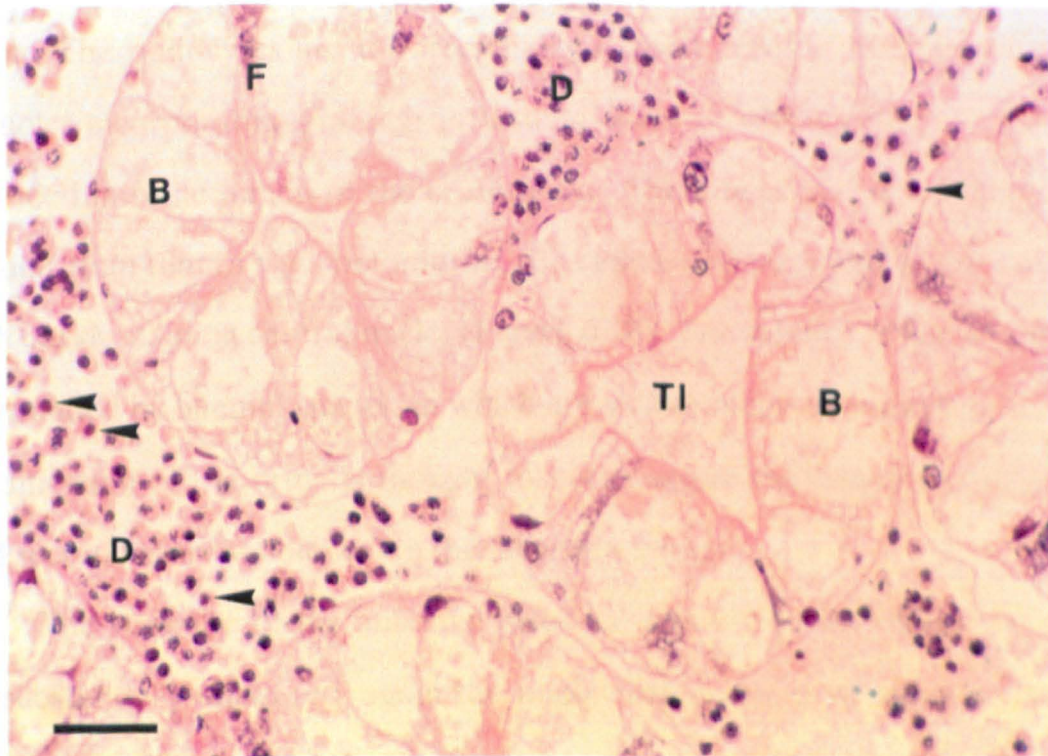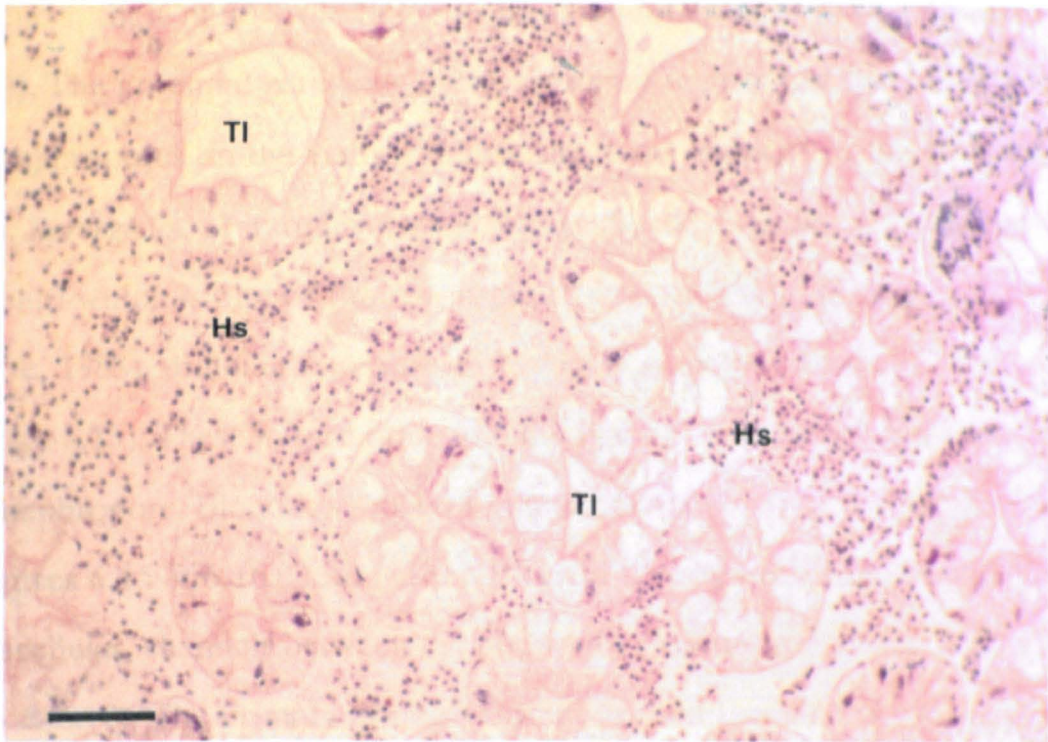
ionic balance, and nitrogenous waste excretion (Phillips *et al.*, 1980). In marine decapods, the antennal glands play only a minor role in the disposal of waste products, their major role being in the maintenance of ionic balance (Parry, 1960). The antennal glands lie in the antennal segment of the head, and open via nephropores on the ventral side of the basal segment of the first antennae. In *N. norvegicus*, as in other marine decapods, they consist of a coelomosac (or endsac) which is one of the few organs truly coelomic in origin (Holdich & Reeve, 1988), draining into a series of highly convoluted tubules, the labyrinth (Figure 6.15). These in turn drain into the bladder and thence from the body via the nephropore. Blood is supplied to the antennal gland by the antennary artery (Figures 6.15 & 6.16), and is then filtered through the coelomosac wall, via intracellular channels between the podocytes (Mantel & Farmer, 1981)(Figures 6.16 & 6.17). The primary urine so formed is then further elaborated during its passage through the labyrinth to the bladder.

The epithelium of the coelomosac consists of a single layer of podocytes (Figure 6.17), separating the coelomosac lumen from the haemolymph in narrow haemal spaces, forming a barrier through which primary filtration may occur. The podocytes are tall irregularly shaped cells, often containing large vacuoles incorporating granular inclusions, some of which appear to have been extruded into the lumen of the coelomosac. The labyrinthal epithelium is more regular than that of the coelomosac, the cells being cuboidal to low columnar, with darkly staining central nuclei (Figure 6.18). The tubules of the labyrinth are surrounded by haemal spaces, in which are visible many haemocytes. Secondary modification of the urine is thought to occur across the labyrinthal epithelium, and secretions are visible within the labyrinth lumen.

The structure of the antennal gland of infected *N. norvegicus* was seen to be substantially unaltered, again the major difference in appearance being the presence of large numbers of parasite cells within the haemal spaces surrounding the labyrinth, coelomosac and within branches of the antennary

FIGURE 6.15. Light micrograph of the antennal gland of healthy *N. norvegicus* showing the labyrinth, coelomosac and a branch of the antennal artery. L = labyrinth; C = coelomosac; A = antennal artery; W = wall of antennal gland. H & E. Bar = 100μm.

FIGURE 6.16. Light micrograph of the antennal gland of healthy *N. norvegicus* showing detail of the coelomosac and antennal artery. L = labyrinth; Cl = coelomosac lumen; Ce = coelomosac epithelium; P = podocyte; In = granular inclusion in podocyte vacuole; Al = antennal artery lumen; Ae = antennal artery epithelium; Hs = haemal space containing haemocytes. H & E. Bar = 40μm.
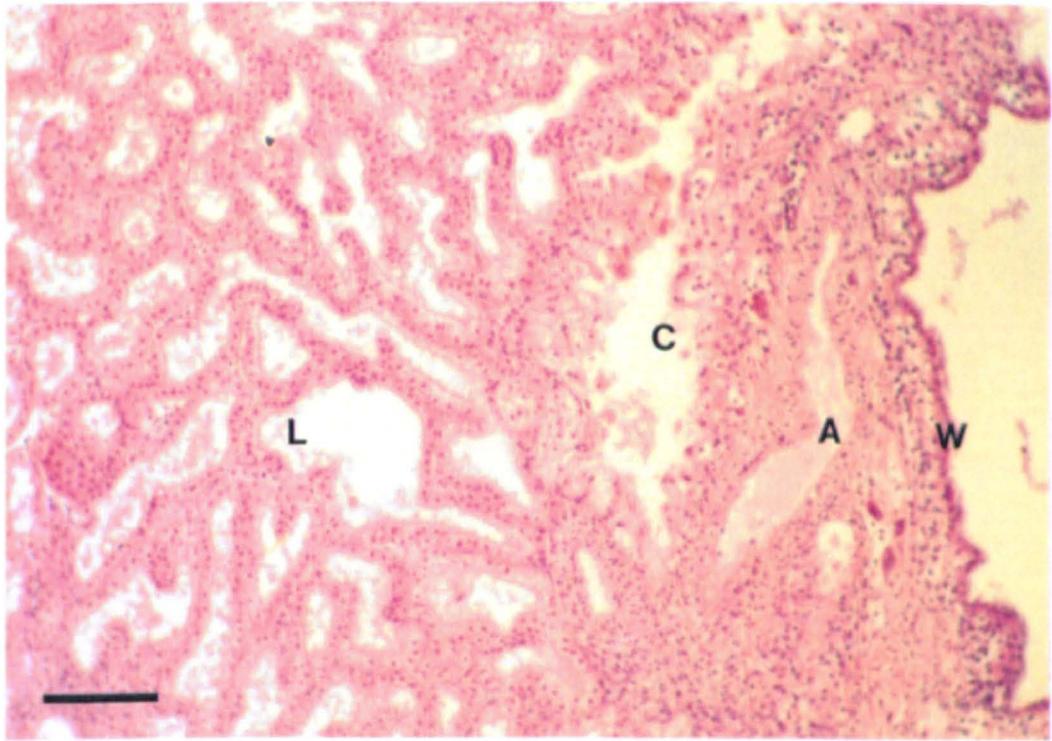
FIGURE 6.17. Light micrograph of the antennal gland of healthy *N. norvegicus* showing detail of the podocytes of the coelomosac epithelium. P = podocyte; In = inclusion in podocyte vacuole; Ic = intracellular channel between podocytes; Cl = coelomosac lumen. H & E. Bar = 20μm.
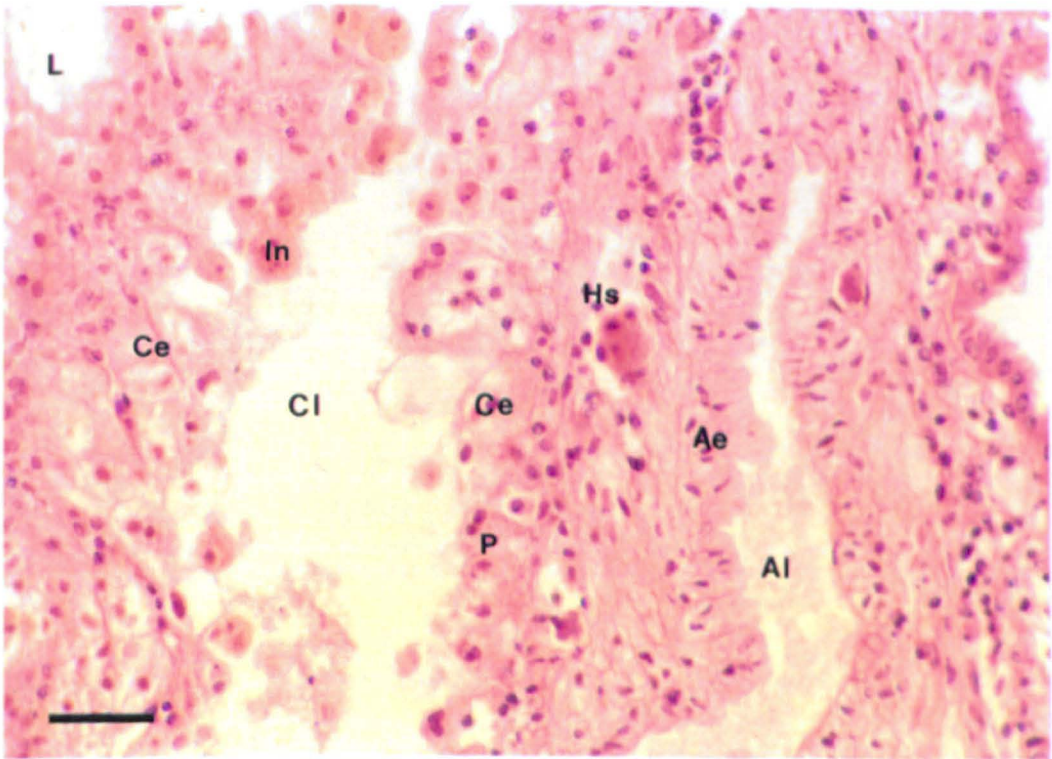
FIGURE 6.18 Light micrograph of the antennal gland of healthy *N. norvegicus* showing detail of the labyrinth. Le = labyrinth epithelium; Bb = apical brush border of labyrinth epithelium; Ll = labyrinth lumen containing secreted material; G = granular extrusion body; Hs = haemal space containing haemocytes. H & E. Bar = 40μm.
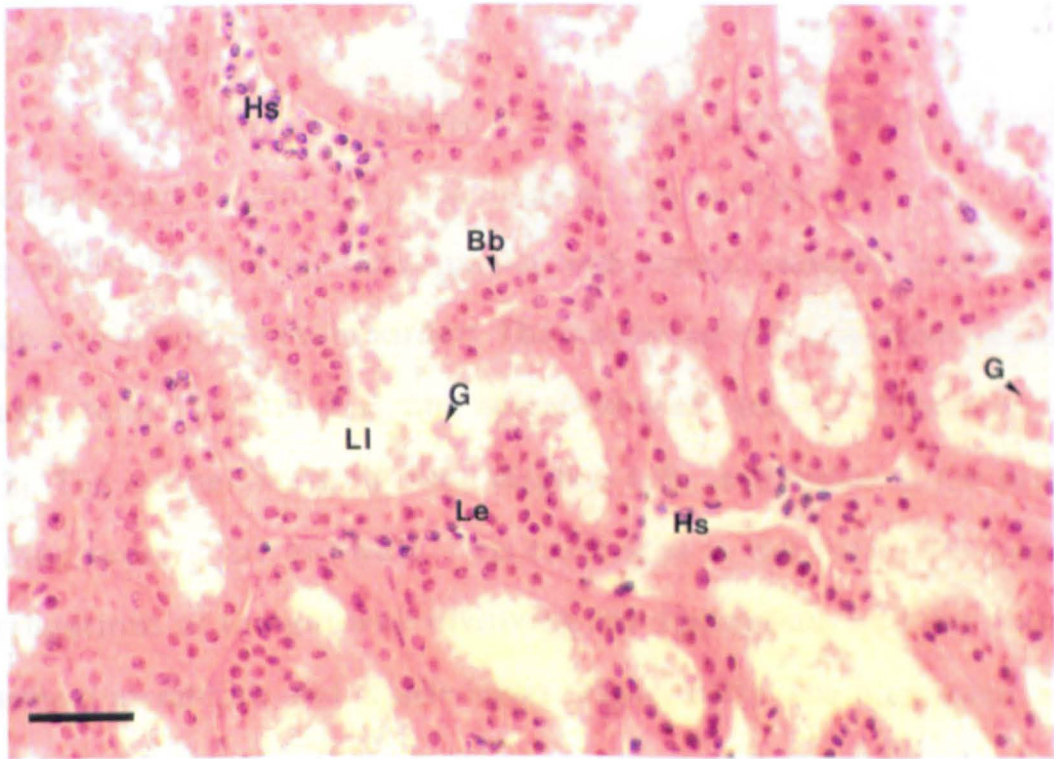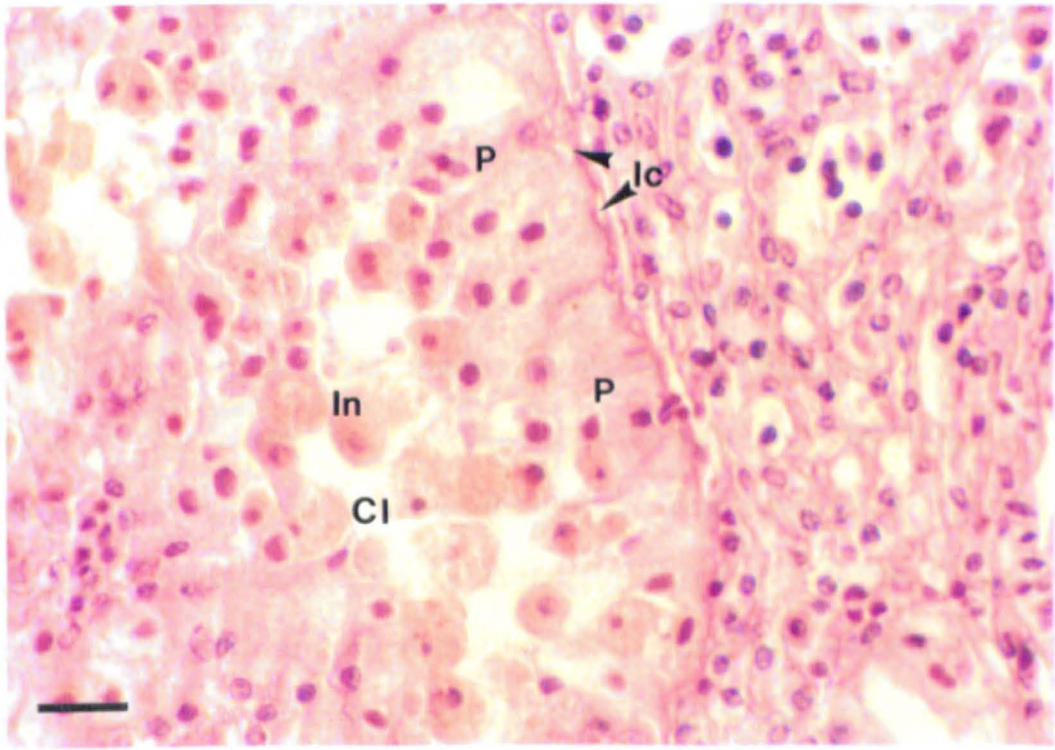
artery (Figure 6.19). As in the haemal spaces of the hepatopancreas, the majority of the parasite cells were uninucleate, but also present in the antennal gland were many plasmodial and vermiform multinucleate forms. The vermiform cells were often attached to the basal side of the labyrinthal epithelium (Figure 6.20). The coelomosac epithelium appeared unchanged, save for the presence of parasite cells in the narrow haemal spaces bounding it (Figure 6.19). The labyrinthal epithelium, however, did exhibit alterations from the normal state. Although substantially intact, some areas of the epithelium were disrupted and parasite cells were present in the lumen of the labyrinth itself (Figure 6.19). In addition, the cells of the intact epithelium showed a degree of vacuolation not observed in those of healthy animals (Figures 6.19 & 6.20). The labyrinth epithelium of both the normal and infected animals investigated showed some degree of secretory activity (Figures 6.18 & 6.19), though this was more marked in the healthy antennal gland, with the apical brush border of cells often showing extruded granular bodies (Figure 6.18), similar to those seen in the antennal gland of *Astacus* and thought to be involved in water secretion (Parry, 1960).

III) Midgut.

The midgut of healthy *N. norvegicus* comprises four layers. The innermost layer (Figure 6.21), adjoining the lumen of the gut, is the epithelium. The cells of this layer are tall columnar, with central, relatively large nuclei, and often a prominent brush border. The epithelial cell cytoplasm is generally weakly stained with H & E, and apical vacuoles are not uncommon (Figure 6.22). The epithelium itself often displays villi, in addition to the prominent folding seen in the gut wall. This folding is in the form of longitudinal corrugations of the gut wall involving the epithelium and the underlying basement membrane and muscle layers (Figure 6.21). The epithelium is usually separated from the underlying tissues as an artefact of fixation. Peripheral to the epithelium lies the basement membrane, a thin, darkly staining layer of non-

FIGURE 6.19. Light micrograph of the antennal gland of dinoflagellate infected *N. norvegicus* showing detail of the labyrinth epithelium. Note many uni-nucleate parasite cells in haemal spaces, disruption of the epithelium and invasion of the labyrinth lumen by parasite cells. Le = labyrinth epithelium; Hs = haemal space containing numerous uni-nucleate parasite cells; V = vacuoles in epithelial cells; Ll = labyrinth lumen with invading parasite cells; S = labyrinth secretion. H & E. Bar = 40μm.

FIGURE 6.20. Light micrograph of the antennal gland of dinoflagellate infected *N. norvegicus* showing detail of vermiform multi-nucleate parasite cells within haemal spaces of labyrinth. Le = labyrinth epithelium; V = vermiform parasite cells; Ll = labyrinth lumen; Va = vacuole in epithelial cell; P = uni-nucleate parasite cell invading labyrinth lumen. H & E. Bar = 20μm.
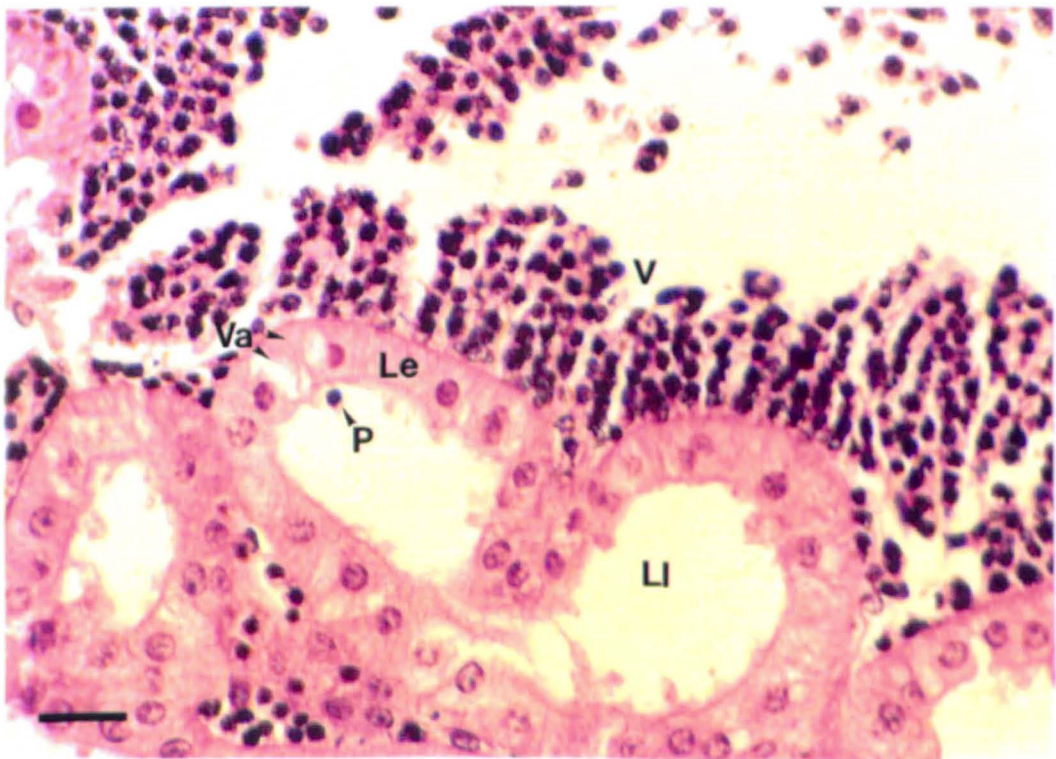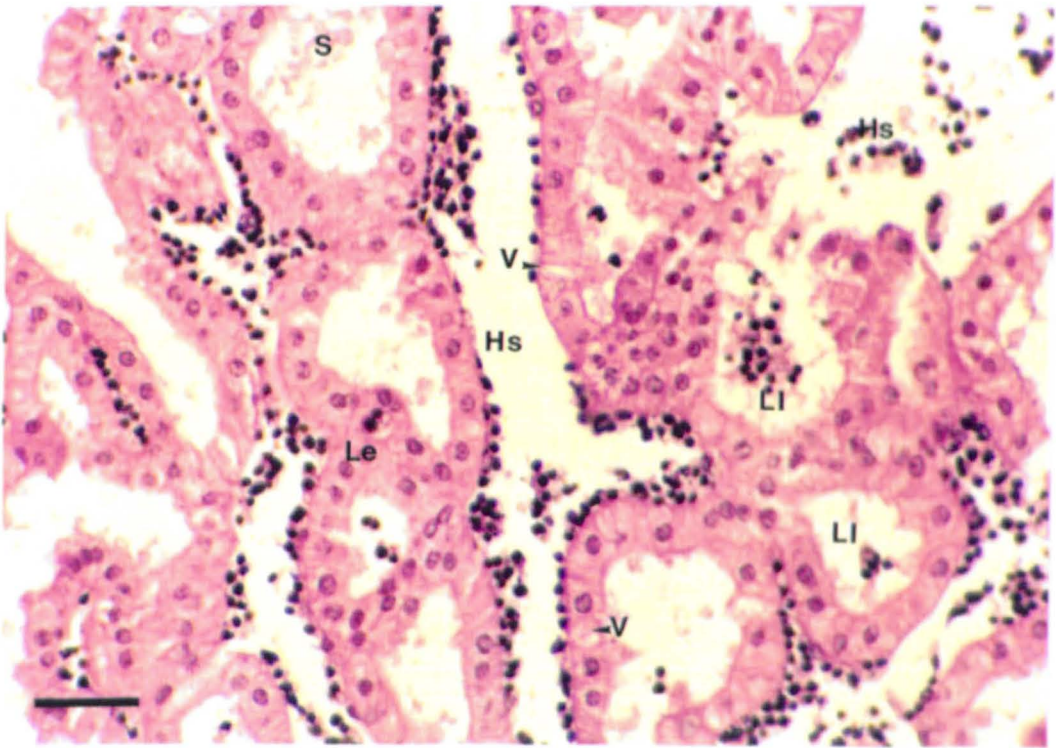
FIGURE 6.21. Light micrograph of transverse section of the midgut of healthy *n. norvegicus.* E = epithelium; L = lumen; B = basement membrane; m = muscle layers surrounded by connective tissue. H & E. Bar = 100μm.

FIGURE 6.22. Light micrograph of transverse section of the midgut of healthy *N. norvegicus* showing detail of epithelial cells. E = epithelial cells; V = vacuoles; L = lumen; B = basement membrane; C = connective tissue of muscle layers. H & E. Bar = 20μm.
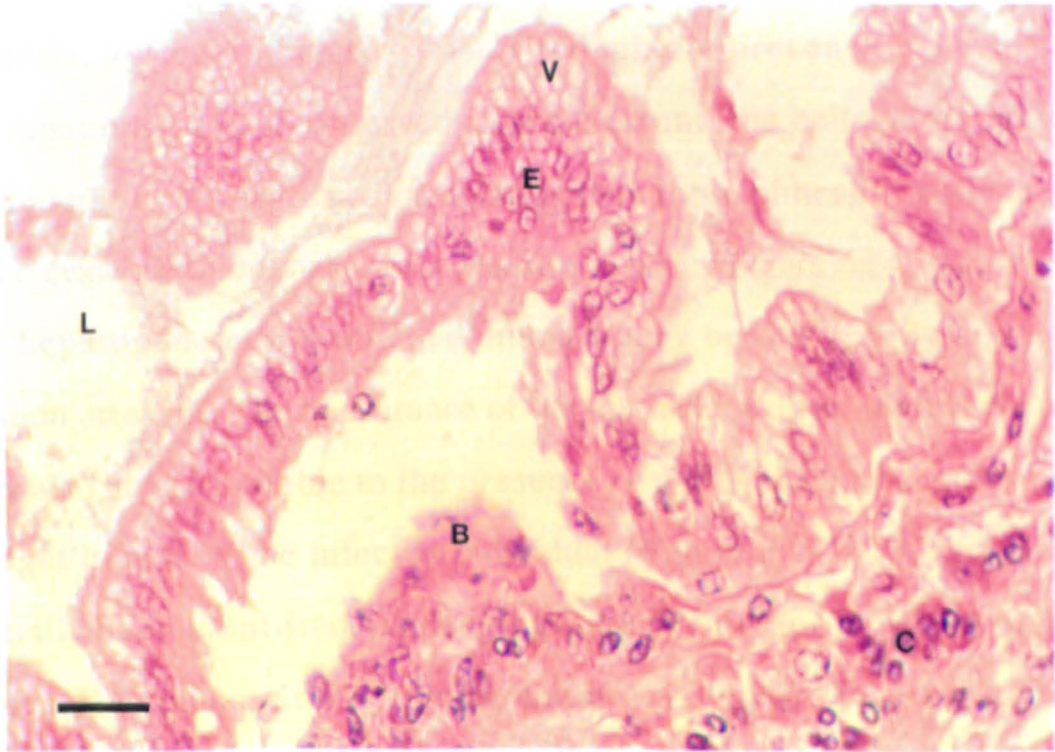
cellular material which overlies layers of longitudinal and circular muscles (Figures 6.21 & 6.22). Beneath the muscular layers of the gut wall is found a layer of connective tissue, infiltrated by haemal sinuses and blood vessels.

The midgut exhibits the most marked changes as a result of infection by the parasitic dinoflagellate of any organ of *N. norvegicus* yet examined. The midgut wall showed large scale infiltration and disruption by the parasite cells. The connective tissue of the outer layers of the gut wall were seen to have largely disappeared, and been replaced by large numbers of both plasmodial and vermiform cells (Figure 6.24). Vermiform cells were again seen to be attached to host tissue, in this case the remnants of muscle tissue in the connective tissue layer. Muscle tissue, although still present, appeared somewhat fragmented and reduced (Figure 6.24). Haemal spaces and blood vessels were apparently totally disrupted in the connective tissue layer, and no host haemocytes have so far been observed in the haemal spaces of the midgut of an infected animal. The basal membrane of infected midgut did not show any overt alteration at the light microscope level (Figures 6.23 & 6.24). Evidence of possible alteration of the midgut epithelium structure was seen in infected animals, however (Figure 6.24). Although the presence of both villi and corrugations was still observed to some extent, the cells of the epithelium exhibited a lesser degree of vacuolation than those of healthy epithelium, and some evidence of epithelial lysis was also present (Figure 6.24). This lysis, as in the hepatopancreas of infected animals, may be due (partly or wholly) to fixation artefacts. The appearance of the epithelial cells in the tissue examined may also be attributable to the presence of a gregarine infection within the midgut lumen of one infected individual (Figure 6.24). This gregarine has initially been identified as a Eugregarine (Professor K. Vickerman, pers. comm.) but observation of living material is required before a definitive description can be given.

FIGURE 6.23. Light micrograph of oblique section of the midgut of dinoflagellate infected *N. norvegicus* showing invasion of the muscle layer and connective tissue by large numbers of parasite cells, and the presence of a gregarine infection of the lumen. E = epithelium; Vi = villus; L = lumen; G = gregarine; B = basement membrane; M = connective tissue and muscle layer. H & E. Bar = 100μm.

FIGURE 6.24. Light micrograph of oblique section of the midgut of dinoflagellate infected *N. norvegicus* showing detail of muscle and connective tissue layers invaded by parasites. E = epithelium; L = lumen; G = gregarine cells; B = basement membrane; V = vermiform parasite cells; P = plasmodial parasite cells; M = muscle. H & E. Bar = 40μm.
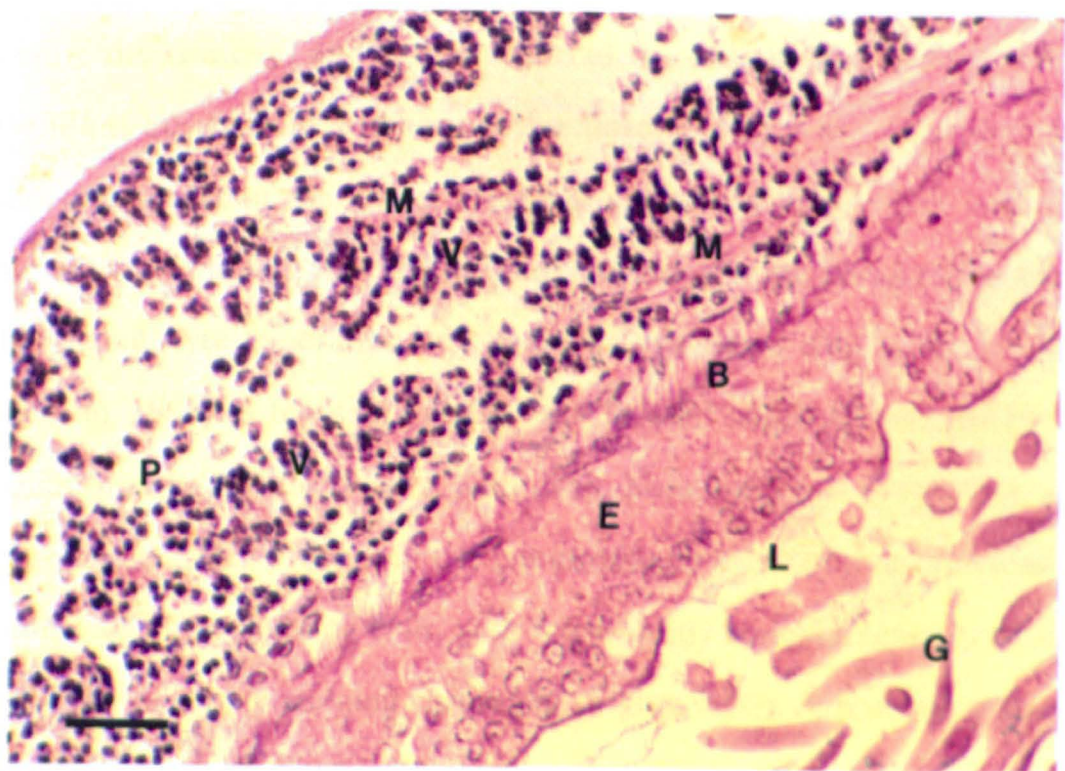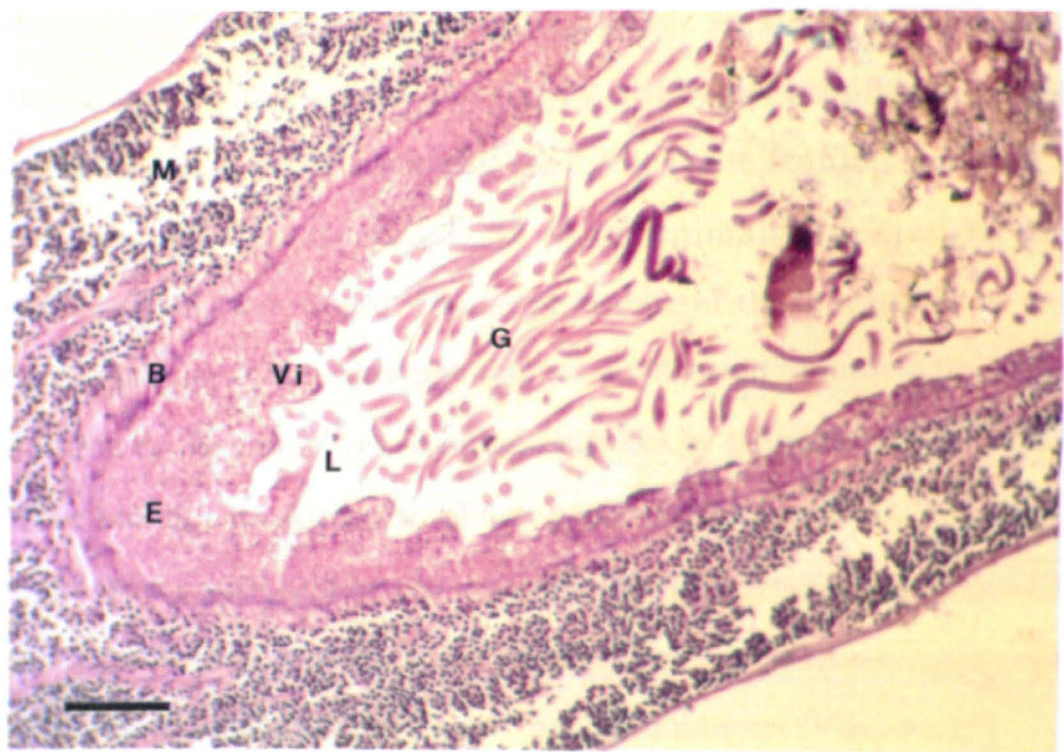
IV) Abdominal muscle.

The abdominal muscle of healthy *N. norvegicus* displays a structure typical of crustacean homogeneous fast phasic muscle. The fibres have short sarcomere length (2-4μm) and lightly staining sarcoplasmic nuclei, with peripherally dense chromatin (Figure 6.25). These features are unchanged in the abdominal deep muscle of those animals infected by parasitic dinoflagellates examined. The appearance of these muscles is, however, different (Figure 6.25). Although there is no invasion of muscle fibre cells, there is invasion of the interstices of muscle bundles by parasite cells, dispersed areas of fibre bundles do appear to be lysed and parasite cells are numerous in the haemal spaces surrounding the muscles. This lysis is manifested as an increase in the separation, and decrease in the number, of fibres apparent within bundles, more obvious in peripheral regions of muscles (Figure 6.25).

V) Haemopoietic tissue.

The haemopoietic tissue of *N. norvegicus* is a thin sheet of cells located in the connective tissue on the dorsal surface of the gastric mill (cardiac stomach). The haemopoietic tissue of healthy animals was difficult to locate despite the removal and examination of the entire gastric mill roof with its attendant epithelium and connective tissues. This was probably due to the indistinct nature of the tissue, often being distributed as small nodes of cells, coupled with the fact that the animals examined were dissected in the winter, when haemocyte generation and haemopoietic tissue activity are probably low (Johnson, 1980). A few small nodes were located which showed the presence of a small number of stem cells, with dark, homogeneous cytoplasm, and very dense nuclei. Also present in the haemopoietic node of one individual were a number of differentiating cells, with more lightly staining heterogeneous nuclei and a rim of darkly staining chromatin (Figure 6.26). A few young haemocytes were also observed in this animal.

In contrast, the haemopoietic tissue of the infected animals examined was

FIGURE 6.25. Light micrograph of abdominal deep flexor muscle of dinoflagellate infected *N. norvegicus* showing invasion of muscle bundle interstices by parasite cells and lysis of muscle cells. P = parasite cells; L = areas of lysis; S = sarcoplasmic nucleus. H & E. Bar = 20μm.

FIGURE 6.26. Light micrograph of haemopoietic node of healthy *N. norvegicus*. S = stem cell; D = differentiating cell; C = connective tissue. H & E. Bar = 10μm.
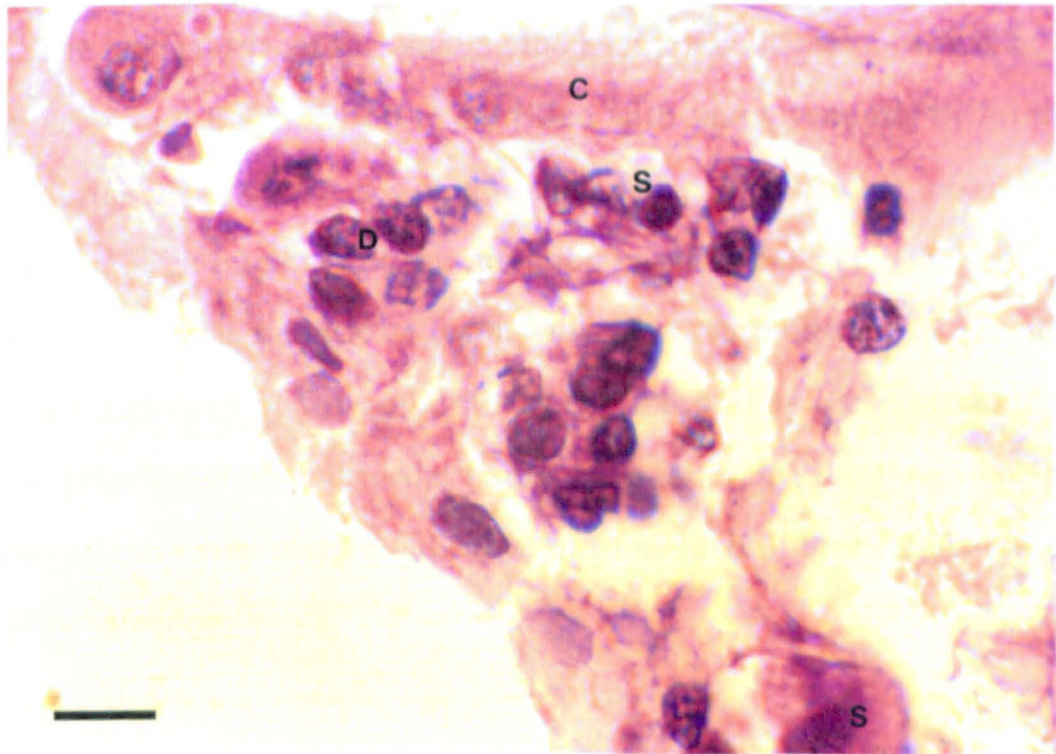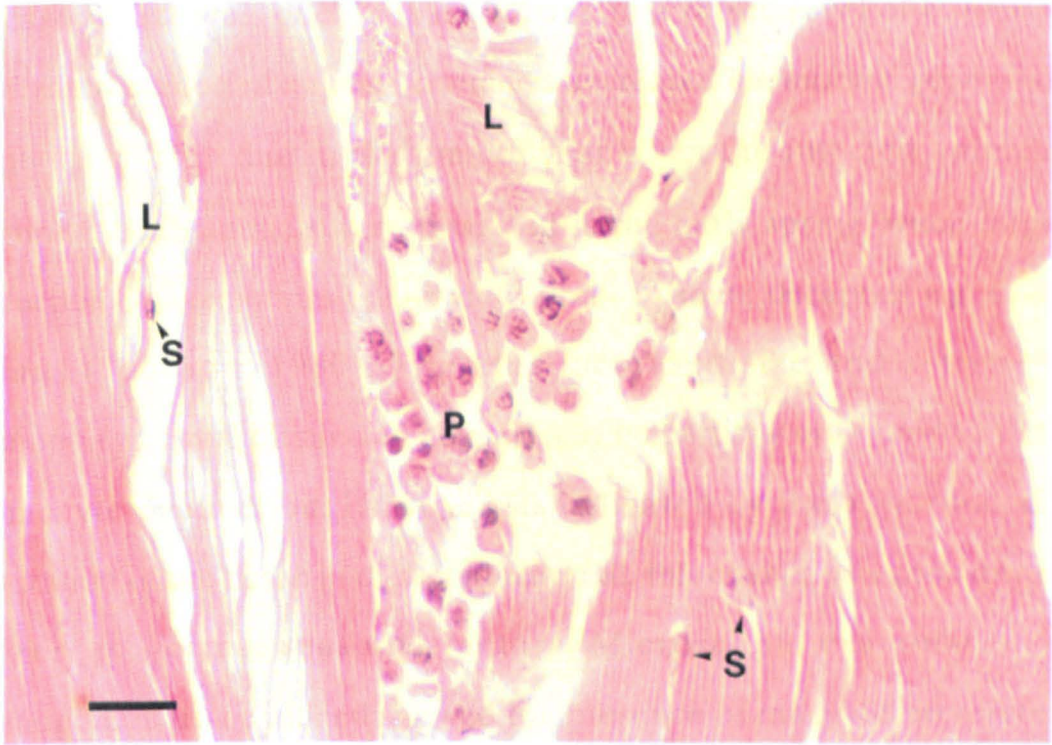
markedly different. In its macroscopical appearance, the haemopoietic tissue of such animals was very much larger than that of control specimens. This increase in size was due, at least in part, to a raised level of haemopoietic activity in the infected animals examined. Increased haemocytic generation may simply have been due to the time of year when the infected animals were examined. Both animals were dissected in the early spring, when large scale production of haemocytes is known to occur in some decapods e.g. *Callinectes sapidus* (Johnson, 1980). Thus the increased size and activity of the haemopoietic tissue of infected animals may be partially attributed to this spring haemocyte production. However, as indicated by the abnormal size and colouration of the tissue in dissected material, this change was probably not due entirely to normal seasonal factors. Microscopic examination of the haemopoietic tissue indeed showed the presence of many differentiating cells and also many cells similar in appearance to stem cells as identified by Johnson (1980) in *Callinectes sapidus*(Figure 6.27). However, very few haemocytes were in evidence. This paucity of host haemocytes despite haemopoietic activity was noted in other tissues and may imply destruction of haemocytes by the invading cells. The haemal spaces surrounding the haemopoietic tissues which, in normal animals, would be expected to be filled with many recently differentiated haemocytes (as in *Callinectes sapidus* (Johnson, 1980)), were observed to be filled with large numbers of the infecting parasite cells (Figure 6.27). These were mainly of the uninucleate form, with a few plasmodial cells. The presence of large numbers of foreign cells in the haemal spaces surrounding the haemopoietic tissue, and in the haemocoel generally, suggests that the high activity of this tissue may be indicative of some kind of host cellular reaction to parasitic infection.

VI) Heart.

The heart of *N. norvegicus* is situated dorsally in the thorax and is enclosed within the pericardium. The healthy heart comprises two layers of

FIGURE 6.27. Light micrograph of haemopoietic tissue of dinoflagellate infected *N. norvegicus* showing increased activity, evident in the number of differentiating cells, and packing of the haemal spaces with parasite cells. Note the paucity of host haemocytes (arrows). U = uni-nucleate parasite cells; P = plasmodial parasite cells; D = differentiating cell in haemopoietic tissue; S = haemopoietic stem cell. H & E. Bar = 20μm.

FIGURE 6.28. Light micrograph of heart of healthy *N. norvegicus* showing myocardial and epicardial layers. M = myocardium; E = epicardium. H & E. Bar = 100μm.
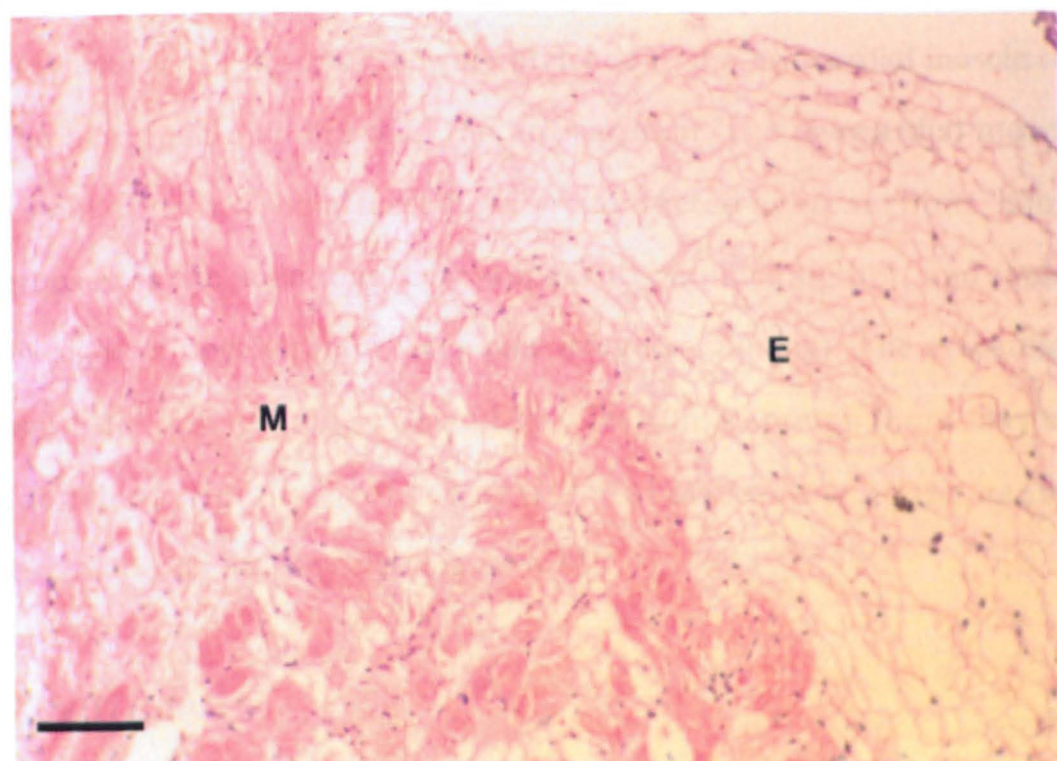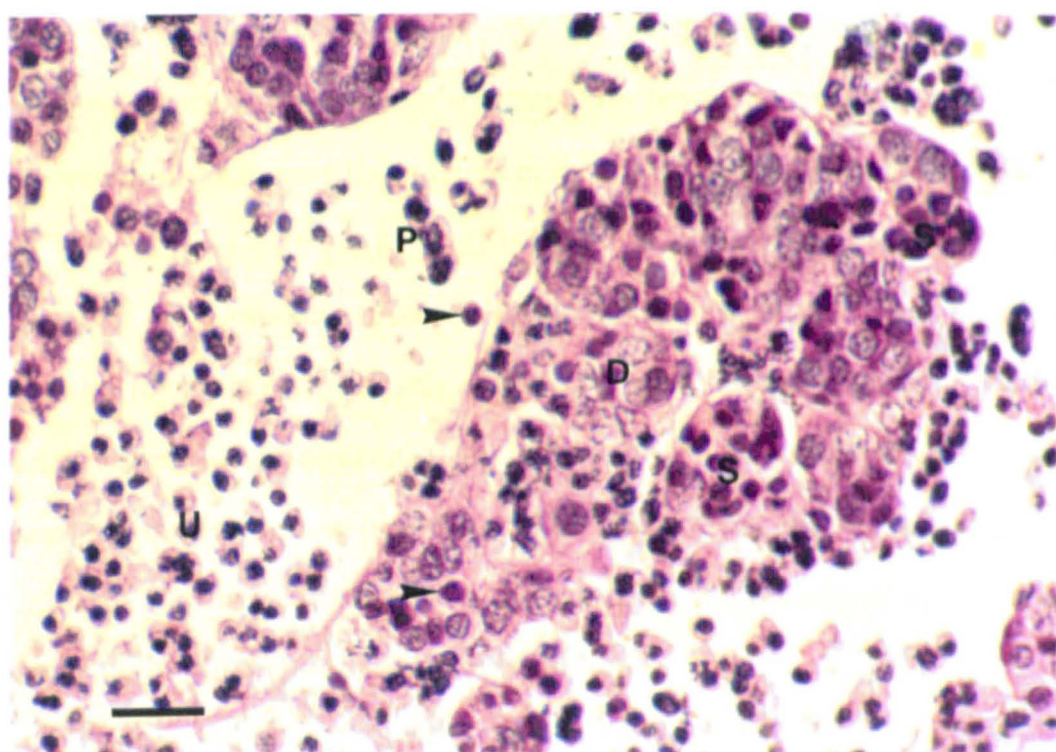
tissue (Figure 6.28). Bounding the lumen, which is often indistinct due to the irregular shape of the heart, is the myocardium (Figure 6.29). This consists of myocardial muscle cells which span the myocardium, inserting on the basement membrane which separates the myocardium from the outer layer of the heart, the epicardium. Myocardial muscle cells are often multinucleate, nuclei being finely granular, with a peripheral layer of denser chromatin. These cells are surrounded by connective tissue. Within the lumen, and smaller haemal spaces within the myocardium, densely stained, clotted haemolymph and haemocytes, often forming loose aggregations, are visible. Also, nerve cells were often present within the myocardial tissue. The epicardium (Figure 6.30) is comprised of uniform connective tissue, of a distinctive 'honeycombed' appearance. This contains no haemal spaces or nervous tissue.

The general structure of the heart remains unchanged by infection with the dinoflagellate cells, the most obvious effect again being the invasion of all haemal spaces by the parasite (Figure 6.31). The lumen, the haemal spaces and the connective tissue of the myocardium are massively infiltrated by parasite cells of multi- and uninucleate forms, and vermiform cells are again attached to host structures. In this case they are attached to the myocardial muscle cells and to the basement membrane of the myocardium. The myocardial muscle itself does not appear to be affected. As in uninfected heart sections, haemocyte aggregations are seen, but these appear to be much tighter, and their nuclei show signs of degeneration (very pale irregular staining). The epicardial tissue shows little sign of being affected by infection, although there may be some evidence of slight lysis of the peripheral areas close to the haemocoel, associated with parasite cells (Figure 6.32).

VII) Gills.

The gills of lobsters are the major organ responsible for oxygen uptake, the pleopods accounting for just 3% of the total (Phillips et al., 1980).

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FIGURE 6.29. Light micrograph of heart of healthy *N. norvegicus* showing detail of the myocardium. L = lumen; H = haemocytes in lumen; M = myocardial muscle; Mn = myocardial muscle cell nuclei; C = myocardial connective tissue; N = nervous tissue. H & E. Bar = 40μm.

FIGURE 6.30. Light micrograph of the heart of healthy *N. norvegicus* showing detail of the epicardium. H & E. Bar = 40μm.
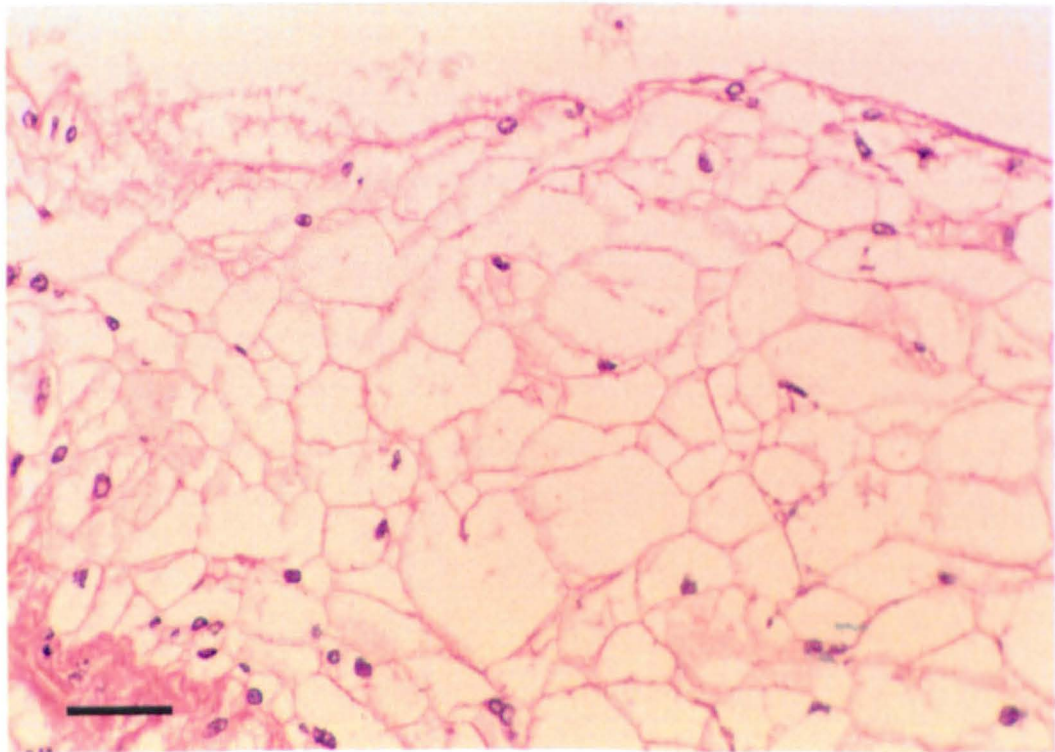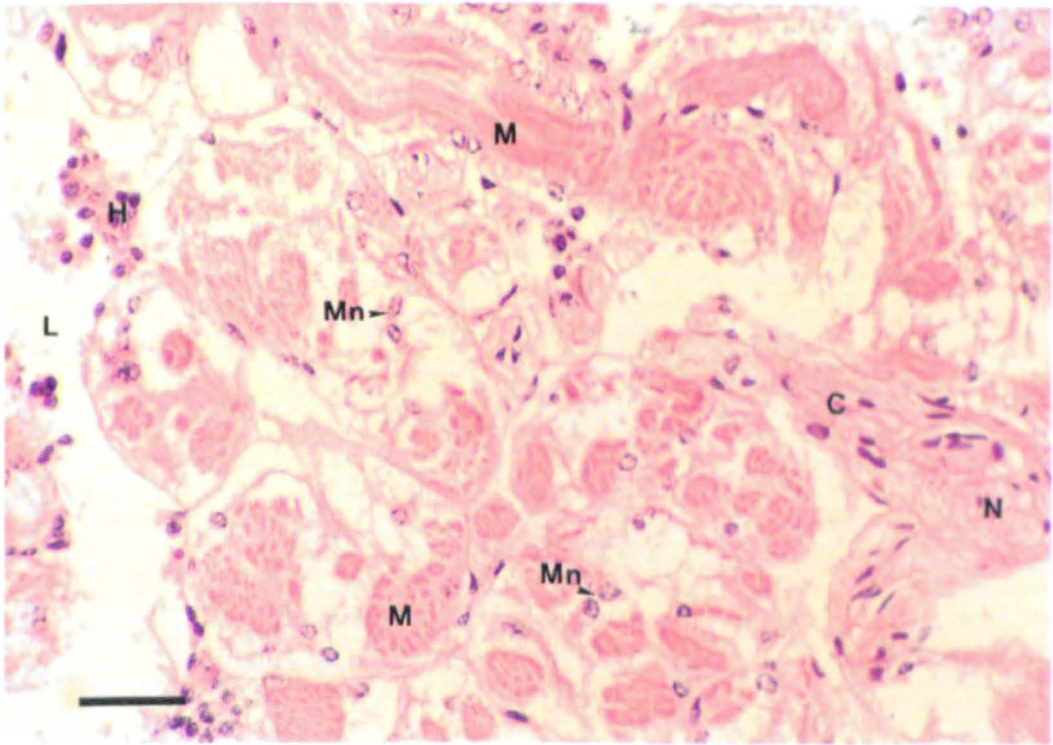
FIGURE 6.31. Light micrograph of the heart of dinoflagellate infected *N. norvegicus* showing invasion of haemal spaces and lumen of myocardium by numerous uni- and multi-nucleate parasite cells. L = lumen; M = myocardial muscle; H = haemal space; U = uni-nucleate parasite cells; P = plasmodial multi-nucleate parasite cells; V = vermiform multi-nucleate parasite cells; N = nervous tissue; Ha = haemocyte aggregation. H & E. Bar = 40μm.

FIGURE 6.32. Light micrograph of the heart of dinoflagellate infected *N. norvegicus* showing detail of possible lysis of peripheral areas of epicardium, associated with parasite cells. E = epicardium; M = myocardium; P = parasite cells. H & E. Bar = 20μm.
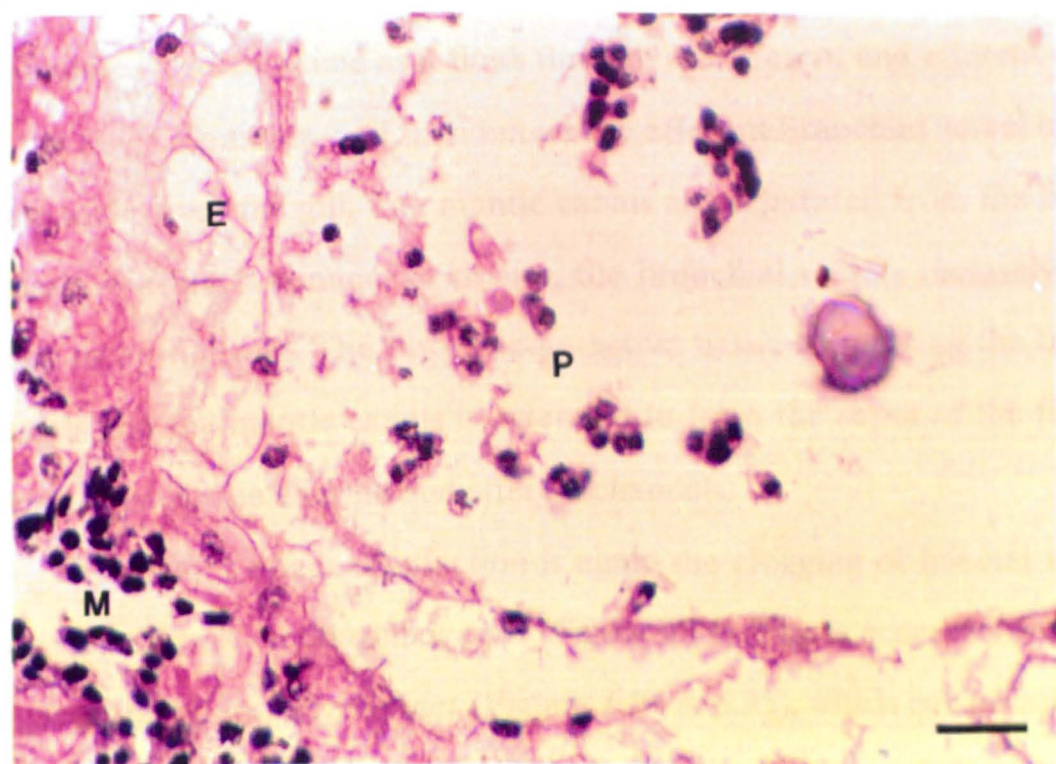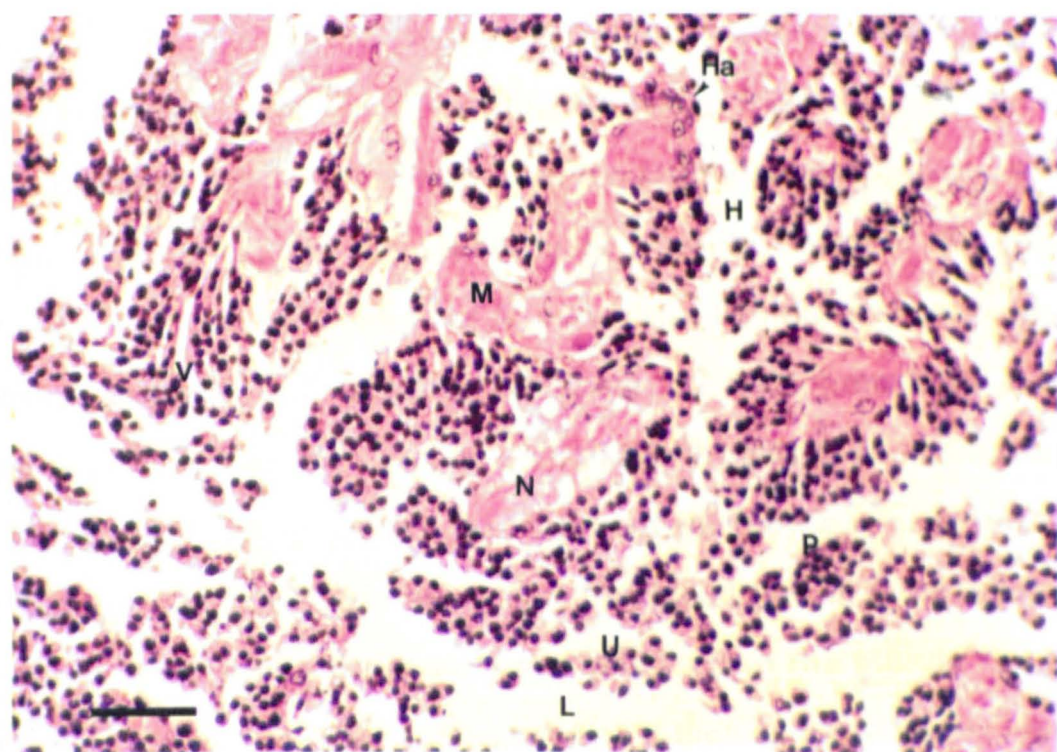
They also have roles in the excretion of nitrogenous wastes, and the balancing of water and electrolytes (Phillips *et al.*, 1980). Nephropid lobsters possess 20 gills on each side of the body (Barnes, 1974), of three types, differentiated by their site of implantation. The pleurobranchs arise from the inner wall of the branchial chamber, arthrobranchs from the arthrodial membranes at the bases of the pereiopods, and the podobranchs from the coxae of the pereiopods (Phillips *et al.*, 1980). The gills of *N. norvegicus*, like those of other nephropid lobsters and indeed all asticidean Decapods, are trichobranchiate in structure (Barnes, 1974). The fine structure seen in healthy gills of *N. norvegicus* (Figure 6.33) appears very much like that reported for the Cape rock lobster *Jasus lalandii* by Paterson (1968). She reports the blood flow in *Jasus lalandii* gills as follows; deoxygenated haemolymph passes into the gill via the afferent branchial vessel in the gill stem, from which it then enters the outer filaments of the gill, where oxygenation occurs. The haemolymph passes outwards along the filaments within the afferent channels, and then returns to the gill stem within the efferent channels. In the gill stem the blood then enters the lateral mantle canals, which direct it to the inner filaments. The haemolymph is then oxygenated a second time as it flows through the afferent and efferent channels of the inner filaments, and then enters the efferent branchial vessel of the gill stem and leaves the gill. The mantle canals are separated from the branchial vessels by fibrous connective tissues, the branchial vessels themselves being lined by epithelium. The fibrous connective tissue separating the branchial vessels and the mantle canals is extended to form the septa of the filaments, which separate the afferent and efferent channels.

The major effect of infection is again the clogging of haemal spaces by large numbers of dinoflagellate cells (Figure 6.34). This is accompanied by some aggregation of host haemocytes (Figures 6.34 & 6.35), which can occasionally be seen blocking gill filaments. Some internal structures of the gills, and in particular the septa of the filaments, are disrupted by the parasite cells. This is

FIGURE 6.33. Composite light micrograph of transverse section of the gill of healthy *N. norvegicus*. Ab = afferent branchial vessel; O = outer filaments; Ac = afferent channel of filament; Ec = efferent channel of filament; M = mantle canal; I = inner filament; Eb = efferent branchial vessel; F = fibrous connective tissue; Epi = epithelium; S = septum; C = cuticle. Labelling follows the scheme of Paterson (1968) for *Jasus lalandii*. H & E. Bar = 100μm.
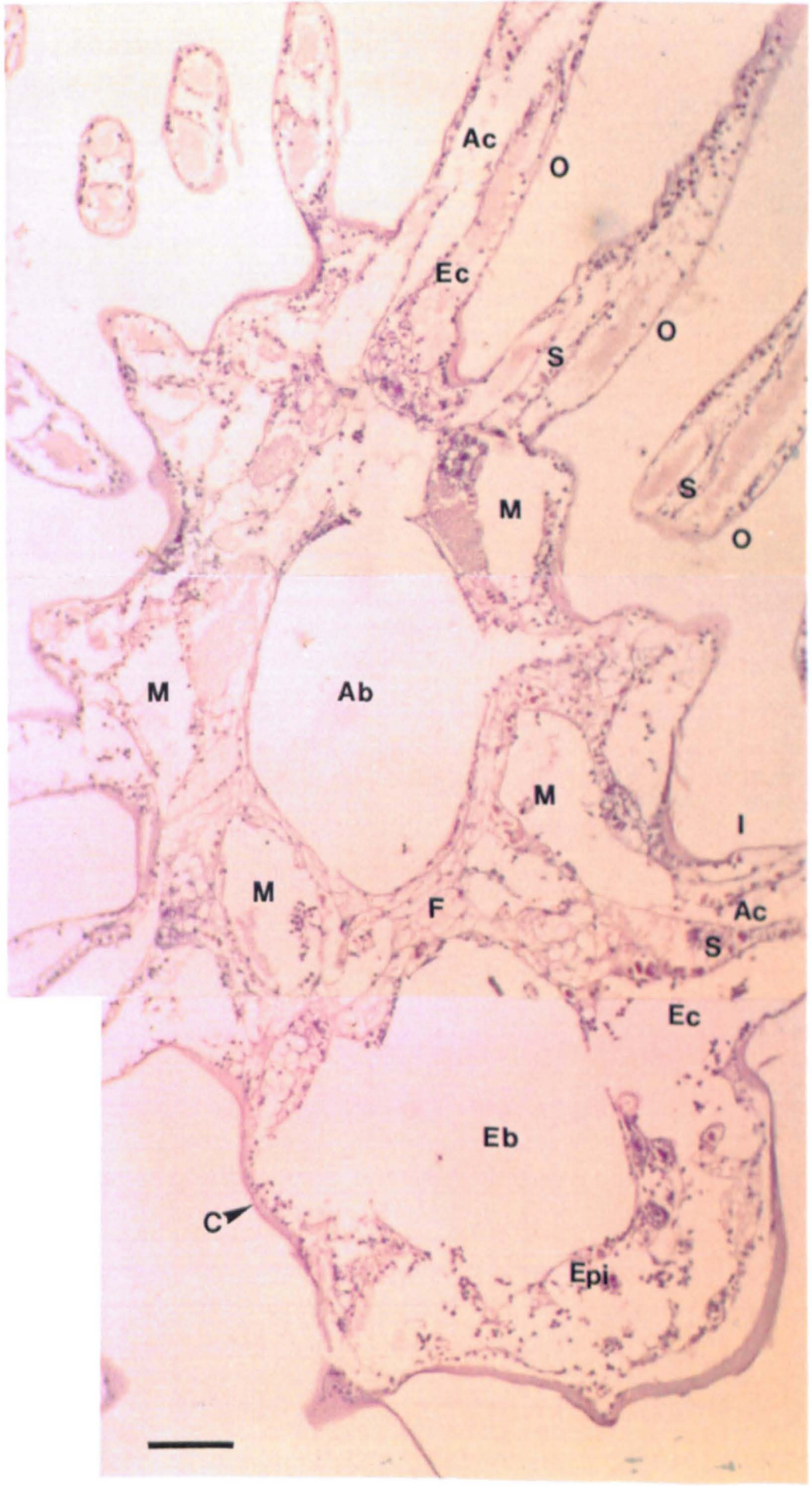
FIGURE 6.34. Light micrograph of longitudinal section of the gill of dinoflagellate infected *N. norvegicus* showing large numbers of parasite cells and haemocyte aggregations packing filaments. P = parasite cells; H = haemocyte aggregation; S = septum; C = cuticle. H & E. Bar = 100μm.

FIGURE 6.35. Light micrograph of longitudinal section of the gill of dinoflagellate infected *N. norvegicus* showing detail of haemocyte aggregation apparently blocking gill filament. Note appearance of apparently degenerating nuclei. C = cuticle. H & E. Bar = 10μm.
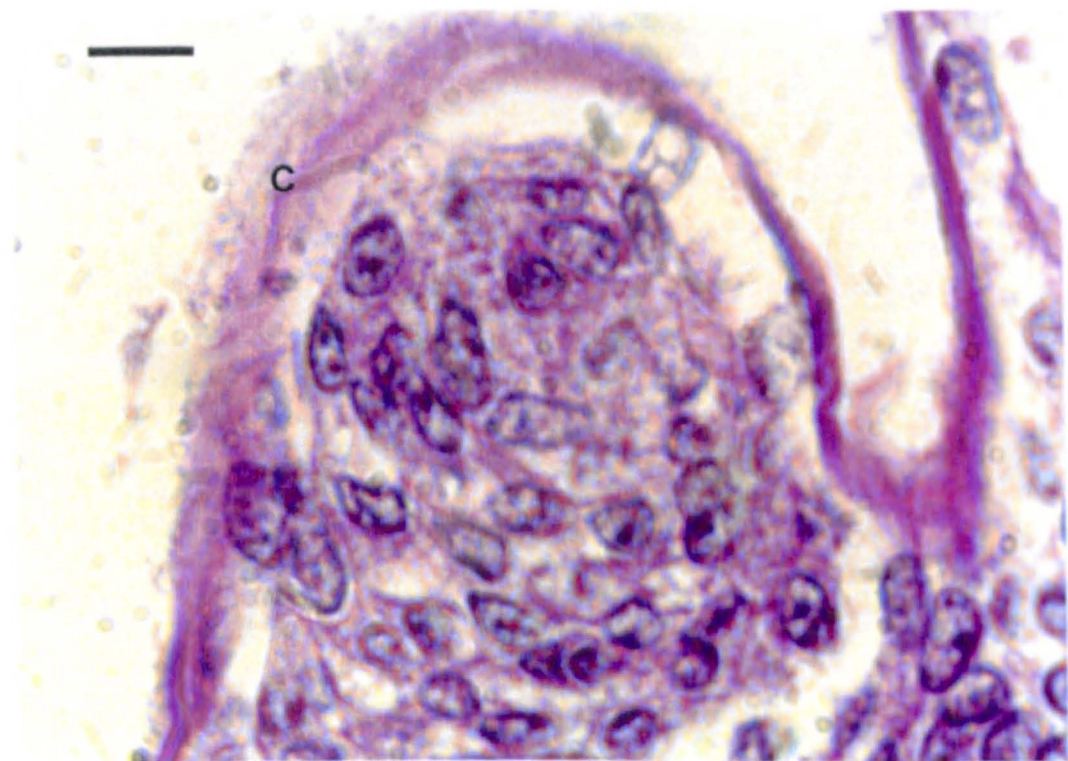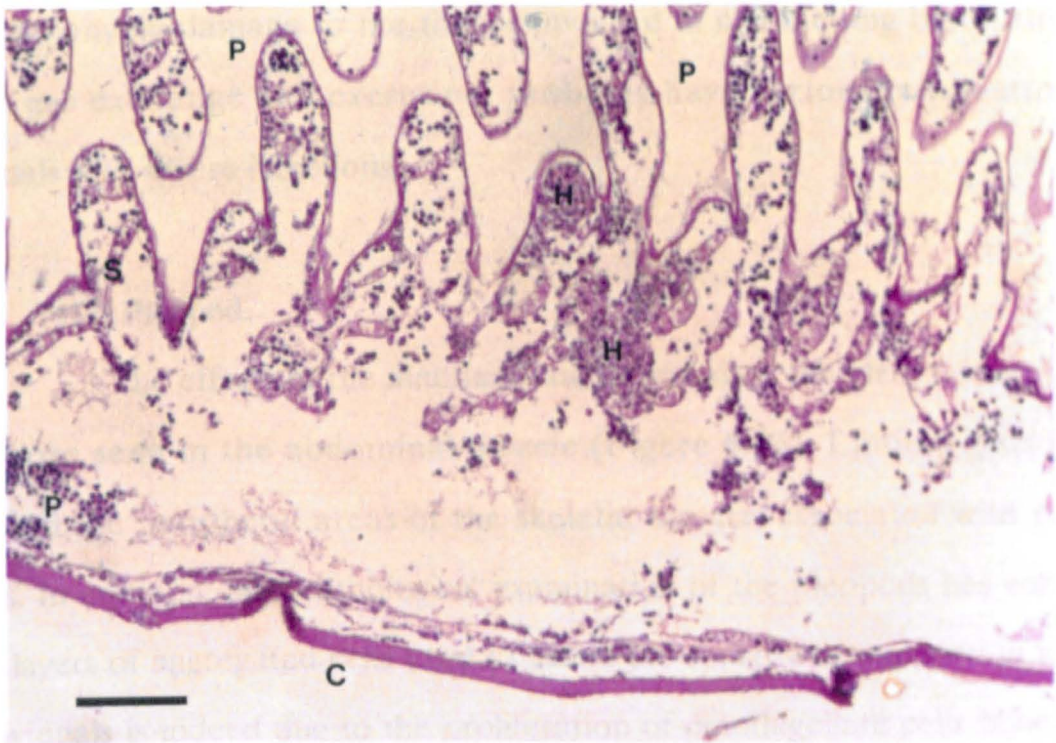
probably the result of the packing of narrow channels with large numbers of cells. The restriction of the flow of haemolymph through the gills, with the accompanying damage to the tissues involved in channelling blood flow and with gas exchange and excretion, probably have serious implications for animals with severe infections.

VIII) Pleopod.

The effects of the dinoflagellate infection on the pleopods are similar to those seen in the abdominal muscle (Figure 6.36). Limited lysis is seen around the peripheral areas of the skeletal muscle, associated with parasite cells. In addition, histopathological examination of the pleopods has confirmed the layers of aggregated cells used to assess the severity of infection in affected individuals is indeed due to the proliferation of dinoflagellate cells beneath the cuticle and epidermis (Figure 6.36), with some associated host haemocyte aggregation.

FIGURE 6.36. Light micrograph of transverse section of the pleopod of dinoflagellate infected *N. norvegicus* showing the layer of parasite cells beneath cuticle. C = cuticle; P = parasite cells; M = muscle. H & E. Bar = 100μm.
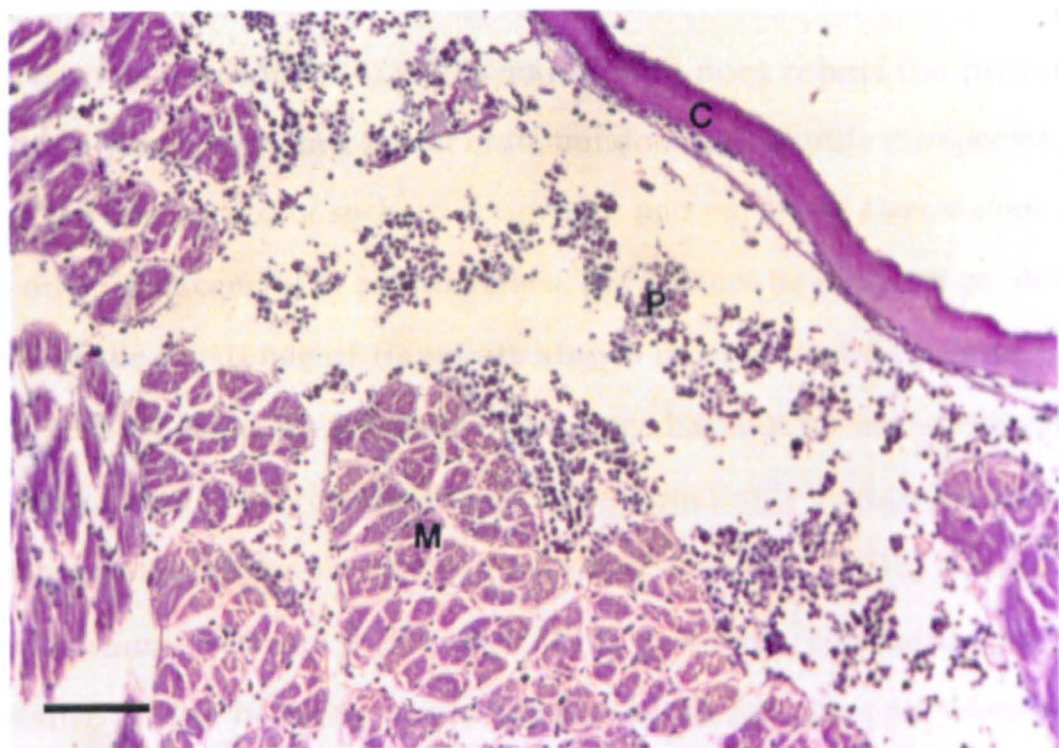
6.4 DISCUSSION

An infecting organism associated with the diseased condition of *N. norvegicus* has been identified as a parasitic dinoflagellate, resembling *Hematodinium perezi* Chatton & Poisson. This identification has been made on the basis of the characteristic amphiesma structure of the cell surface, the presence of prominent trichocysts, tubular cristae in the mitochondria, and the very striking appearance of the large chromosomes (Cachon & Cachon, 1987). The exact identity of the parasite, however, remains uncertain. The gross pathology, the parasite morphology and its effects on the host are similar to those reported in *Hematodinium* sp. infections of blue crabs, *Callinectes sapidus* (Newman & Johnson, 1975). Newman (1988) does report the presence of flagellate 'dinospores' in a spider crab, but does not identify the species. Since this is the only record of such an occurrence and indeed of *Hematodinium* in a crab other than cancrid or pagurid crabs, this cannot be regarded as definitive proof of the existence of flagellate stages in *Hematodinium* until further evidence is available. No flagellate spore stage has thus far been observed in *N. norvegicus*, and the three distinct forms of apparently vegetative cells which have been seen resemble closely Chatton & Poisson's (1931) description of *Hematodinium*. Newman & Johnson (1975), however, reported the vermiform vegetative cells to be highly motile and free in the haemolymph, whereas these forms were observed only in the heart, antennal gland, midgut wall and haemopoietic tissue of *N. norvegicus*, and were apparently non-motile and attached to host tissue. The ultrastructure briefly reported for *Hematodinium* by Newman & Johnson (1975) was also very similar to that of the dinoflagellate in *N. norvegicus*; differences in nuclear structure (indistinct nucleoli and nuclear membranes in the blue crab parasites) may have been the result of processing for TEM.

The histopathological consequences of this disease also bear considerable resemblance to those reported by Meyers *et al.* (1987) for Bitter Crab Disease

(BCD) in Alaskan tanner crabs (*Chionoccetes bairdi*). Most notable are the disruption of the muscle layers of the midgut wall, the lysis seen in the hepatopancreas, congestion of the gill filaments and muscle lysis. However, the morphology of the causative dinoflagellate of BCD is distinctly different from that reported here. Meyers *et al.* (1987) have noted two distinct flagellate spore forms with associated trichocysts, and a vegetative form which lacks trichocysts. Although the possibility still exists that the organism infecting *N. norvegicus* does produce a flagellate spore stage, as evidenced by the presence of flagellar hair vesicles, the fact that the spore stages of the BCD organism are prominent within infections suggests different affinities for the two organisms.

In addition to revealing the probable causative agent of this condition in *N. norvegicus*, the initial histological and physiological investigations reported here have revealed much about the nature of the symptoms apparent in infected animals and the effects of infection on *N. norvegicus*. Although there is not yet unequivocal evidence that this condition is fatal to infected animals (see Chapter 7 for a full discussion of this point), the widespread systemic nature of the infection and its wide variety of effects upon the host suggest that severe debility and death probably result in many, if not all, cases. The possible causes of such disease-related deaths are thus many and varied.

Although the gross appearance of abdominal muscle indicates at least some lysis or damage in infected animals, analysis showed no increase in the water content of these muscles. This could imply that there was no loss of tissue from these muscles that resulted in replacement by water. However, microscopical investigations revealed that at least some lysis of abdominal deep musculature was occurring in infected animals. Moreover, this lysis was apparently more severe at the outer edges of muscle bundles, and this could account for the apparently normal water content even in the presence of lysis and tissue loss. If the lysis, and possible tissue loss, is occurring from the peripheral areas of muscles, this would not be detected by the measurement

methods used, as they involved removal of the muscle from surrounding cuticle, and hence areas where water was replacing tissue would not have been measured.

Further effects of this condition on muscle were detected in the analysis of energy metabolites. ATP concentrations in muscle were shown to be reduced in infected animals, whilst glucose concentrations were elevated. This raises the possibility that energy metabolism is affected by the dinoflagellate. This has been shown in other decapod haemocoelic infections. Most notable is gaffkaemia, a bacterial septicaemia of homarid lobsters caused by *Aerococcus viridans* (var.) *homari*. In this infection, ATP was also shown to be reduced in several tissues, including the hepatopancreas and abdominal muscle (Stewart & Arie, 1973). Hyperglycaemia was also observed in gaffkaemic lobsters, but Stewart *et al.* (1969a) attributed this to stress effects. This may also be true of the hyperglycaemia shown by infected *N. norvegicus*, although this may not be in response to handling alone, as this was controlled against. Internal stresses, brought about by the infection, may also contribute to this hyperglycaemia. In gaffkaemic lobsters (*Homarus americanus*), the ability of the haemocyanin to bind and carry oxygen is reduced according to Rittenburg *et al.* (1979). It has been postulated that the reduction in ATP in tissues associated with this disease is a result of the depletion of oxygen supplies to the affected tissues (Rittenburg *et al.*, 1979), or to direct nutrient depletion by the bacterial cells (Stewart *et al.*, 1969a). Depletion of oxygen supply to the tissues has been variously attributed to the reduction of carrying capacity of the haemolymph, to competition for available oxygen by the invading bacteria present within the haemocoel, or to disruption of blood flow by host haemocyte aggregations in response to phagocytosis of bacterial cells (Maynard, 1960; Rittenburg *et al*, 1979).

A reduction in the haemolymph oxygen carrying capacity is evident in the dinoflagellate infection of *N. norvegicus* reported here, and this may have a

similar role in the reduction of ATP in muscle tissues. Tissues other than the abdominal muscles may exhibit alterations of their energy metabolism, as seen in gaffkaemia (Stewart & Arie, 1973), and this may account for the general lethargy of infected animals.

Oxygen deprivation of tissues in infected *N. norvegicus* is possibly also occasioned, as in gaffkaemia, by competition for available oxygen by the myriads of proliferating parasite cells. Another salient feature of the presence of so many invading cells within the haemocoel is the mechanical disruption they cause to blood circulation. Apart from the viscosity-altering affects of this overburden of circulating cells, clogging of blood vessels and sinuses may also affect blood flow. This will be particularly so in areas of restricted diameter, such as the smaller capillaries and sinuses, and the haemal spaces within organs. Restriction of blood flow is likely to be further exacerbated within areas where large numbers of vermiform parasite cells are attached to host tissues. Clogging may also result from the formation of haemocyte aggregations as part of a host response to infection, as seen in the gill filaments of *N. norvegicus* (Figures 6.34 & 6.35), and postulated by Rittenburg *et al.* (1979) to contribute to tissue hypoxia in gaffkaemic lobsters. The aggregation of haemocytes, and the formation of acellular haemolymph clots in blue crabs (*Callinectes sapidus*) in response to bacterial infections have also been implicated in the impedance of blood flow through the gills (Johnson, 1976).

The afore-mentioned effects of infection on the haemolymph oxygen carrying capacity are also of interest. The oxygen carrying capacity of the haemolymph of infected *N. norvegicus* is significantly reduced. However, unlike the assertions of Rittenburg *et al.* (1979) for gaffkaemic lobsters, this is not due to an alteration of the 'oxygen binding capacity' of an unchanged haemocyanin pool by a 'subtle change in the molecule's conformation' by the bacteria, 'leading to a reduction in the number of active binding sites'. In *N. norvegicus*, the reduction in haemolymph oxygen carrying capacity is the result of a

reduction in the concentration of circulating copper, and thus haemocyanin, with no actual alteration of the ability of individual molecules of haemocyanin to bind and carry oxygen. The explanation of Rittenburg *et al.* (1979) of the mode of reduction of haemolymph oxygen carrying capacity, if correct, would be the first example of the alteration of the absolute capacity of haemocyanin to bind and carry oxygen when completely saturated (A.C.Taylor, pers. comm.). This explanation is, however, based upon the assumption that the measured difference in haemocyanin absorption spectra at 335 nm (i.e. the degree of oxygenation of the haemocyanin) between infected and uninfected lobsters is based on the presence of similar amounts of haemocyanin in both sets of samples. Since this measurement of haemocyanin was based only on measurements of total haemolymph protein content (by absorption spectra at 275 nm) and the assumption that approximately 98% of serum protein was haemocyanin in both sets of samples, and not on direct copper measurements, this explanation cannot be regarded as unequivocal. It seems much more likely that a similar situation pertains in gaffkaemic lobsters as in dinoflagellate-infected *N. norvegicus*, that reduction in carrying capacity is due to a reduction in haemocyanin concentration.

Haemolymph haemocyanin concentrations in *N. norvegicus* have been reported to vary with ambient oxygen tension. Under prolonged exposure to moderate environmental hypoxia due to eutrophication, haemocyanin concentrations in *N. norvegicus* in the Kattegat have been shown to increase, whilst severe hypoxia induced a reduction (Baden *et al.*, 1990). The hepatopancreas (=midgut gland) is heavily implicated in haemocyanin synthesis in decapods and isopods (Mangum, 1983), and it seems likely that in animals with reduced concentrations of haemocyanin in their blood the proteinaceous constituents of the molecule are broken down and the copper is resorbed into the hepatopancreas. Indeed, the hepatopancreata of animals suffering severe hypoxia, and thus much reduced haemolymph haemocyanin levels, have been

observed to have an abnormal bright green colouration (S.P.Baden. pers. comm.), indicative of the presence of large amounts of copper. This, however, is still to be quantitatively verified.

A similar phenomenon has been observed in dinoflagellate-infected *N. norvegicus* which had not been subjected to any kind of environmental oxygen stress. Animals sampled in the laboratory were kept under normoxic conditions, and those sampled in the field appear not to have been under any environmental oxygen stress, as shown by the results of the dissolved oxygen concentration survey (see Chapter 7). This, together with the changes evident in hepatopancreatic cells in infected animals, lends support to the hypothesis that reduced haemocyanin concentrations in infected *N. norvegicus* are due to some form of interference with haemocyanin synthesis or metabolism by the parasite, rather than to a reduced uptake of copper or some other constituent.

Apart from the effects apparent in the energy metabolism and respiration of infected animals, changes in tissues associated with digestion and assimilation are also evident. This raises the possibility that alterations in energy metabolism associated with the abdominal muscles are due in part to an altered supply of nutrients as well as oxygen. Casual observations indicate that the guts of a high proportion of infected animals contain food, implying that they had fed recently, probably within the previous 24 hours, since gut transport time in *N. norvegicus* is around 20-24h (depending on temperature) from feeding to near total gut evacuation (N.Bailey. pers. comm.). A degree of starvation could result from the destruction evident in both the hepatopancreas and the midgut. Damage to the epithelial cells of the hepatopancreatic tubular system in infected animals is likely to interrupt the production and secretion of digestive enzymes, and, since much digestion is thought to occur within the tubule system (Johnson, 1980), to disrupt digestion itself. Likewise, the midgut has been ascribed both absorptive (Barker & Gibson, 1977) and osmoregulatory (Talbot *et al.*, 1972) functions, and the large scale invasion and disruption of the muscular tissues and haemal

spaces of this organ may well have far-reaching implications in dinoflagellate-infected N. norvegicus.

It is also possible that starvation, as a result of impairment of digestion and absorption, could result in the observed reduction in haemocyanin concentration in the haemolymph of N. norvegicus, by virtue of a reduction in the availability of amino-acids for protein synthesis. Reductions in haemocyanin concentration induced by starvation have been reported for Carcinus maenas (Uglow, 1969) and Crangon vulgaris (Djangmah, 1970). Whilst Dall (1974) attributes this to the additional intracellular space and consequent haemal dilution brought about by fasting, it is possible that in N. norvegicus haemocyanin concentration reduction is a real phenomenon, since muscle water content appears unchanged. However, as stated earlier, this lack of muscle water change may be artefactual. If so, the wasted appearance of abdominal muscle may be due to starvation-induced tissue resorption (Dall, 1974), although haemocyanin concentration reduction may not be attributed entirely to the dilution artefact, as indicated by the supporting evidence detailed above. Furthermore, starvation, albeit infection-induced, is unlikely to cause the many other symptoms exhibited by infected animals, as N. norvegicus are known to be able to withstand starvation for at least three months without serious detriment, save for muscle water increases and a decrease in oxygen consumption (N.Bailey, pers. comm.). Thus starvation as a result of infection may contribute to the symptoms exhibited by N. norvegicus, although the dinoflagellate is likely to have other direct physiological effects.

The widespread systemic nature and effects of the dinoflagellate infection of N. norvegicus are typical of those reported for haemocoelic infections of decapods by both protistans and bacteria of several groups. The general features of tissue disruption of the digestive and excretory organs and of some form of interference with respiratory exchange or metabolism are typical of such haemocoelic infections caused by bacteria (Johnson, 1976), dinoflagellates

(Meyers et al., 1987; Meyers, 1990; Maclean & Ruddell, 1978.), Chlamydia-like organisms (Sparks et al., 1985) and ciliates (Sparks et al., 1982). However, many protistan and bacterial diseases, like gaffkaemia, and the presently reported dinoflagellate infection, may have notable effects on the acellular fractions of the haemolymph, as well as on the physiologies and metabolisms of their hosts. These aspects remain largely uninvestigated. More information is, however, available upon the histopathology of infected hosts (see above) and on the effects of infection on the tissues of the haemocoel, notably the circulating haemocytes and their progenitor, the haemopoietic tissue.

The investigations reported here into the effects of infection upon the cells within the haemolymph have not provided a definitive description of the situation. Total haemolymph cell counts have shown that with an apparent increase in severity, the total number of circulating cells (host haemocytes and parasite cells) also increases. The exact nature of this increase (seen in stage III cases - see Table 6.3) is still not entirely clear; it may be due to an increase in parasite cells, host haemocytes, or a combination of both. It has not been possible to ascertain this from the cell counts made, as smears were made before any method of designating severity had been developed, and no differential counts were made on fresh haemolymph from animals of designated severity. It seems likely, however, that this increase can be attributed to the proliferation of parasite cells within the haemocoel. This assertion is based on the observation that the majority of infected animals sampled for blood smears were by necessity the more severely affected animals (because they were chosen on the basis of body colour alone) and displayed high parasite cell numbers compared to host cells. Any change in host haemocyte numbers cannot be verified until quantitative differential counts can be made on haemolymph from infected animals of known severity. The apparent increase in the activity of the haemopoietic tissue does suggest an increase in haemocyte production in infected animals but, within most of the haemal spaces seen in sectioned tissue,

haemocytes appear absent except for those present in aggregations.

Haemocytopoenia, the reduction in the number of haemocytes, has been noted as a symptom of many haemocoelic infections caused by both bacteria and protists. Large scale disappearance of haemocytes from circulation in the haemolymph has been noted in gaffkaemia of *Homarus americanus* (Stewart *et al.*, 1969a; Johnson *et al.*, 1981), *Paranophrys* (a ciliate) infection of dungeness crabs, *Cancer magister* (Sparks *et al.*, 1982), *Paramoeba perniciosa* infections (Johnson, 1977a) and bacterial infections (Johnson, 1976) of blue crabs, *Callinectes sapidus*. Sparks *et al.* (1982) attribute the haemocytopoenia in dungeness crabs infected with ciliates to the direct action of the invading cells by consumption of haemocytes. However, reduction in haemocyte numbers in *Paramoeba* infections was reported to be the result of host defence reactions, the lysis of host cells after phagocytosing parasite cells, and possible disruption of haemopoietic tissue function (Johnson, 1977a). Similarly, in gaffkaemic lobsters, haemocyte aggregations are widely reported in response to the invading bacteria (Johnson *et al.*, 1981; Rittenburg *et al.*, 1979). They are accompanied by an increase in haemopoietic activity, suggesting the initiation of a widespread host reaction to infection. It is therefore possible that a similar situation occurs in *N. norvegicus* infected with dinoflagellates, and that the observed activity in the haemopoietic tissue and the presence of haemocyte aggregations are indications of the initiation of a host reaction to infection. The haemocytes may then be removed from circulation by the formation of aggregations around dinoflagellate cells.

Although the histopathological survey reported here has revealed the presence of infecting dinoflagellates associated with the condition affecting *N. norvegicus*, the results of the microbiological analysis also undertaken show some interesting features. Attempts were made to remove all samples for this analysis under total asepsis, by using sterilised instruments and equipment, and the thorough sterilisation of the cuticle of the subject animal with 70% alcohol.

If this was achieved, the results indicate that the haemolymph at least, and possibly internal organs of *N. norvegicus* apparently infected with dinoflagellates, are not sterile. This could indicate the presence of secondary infections, as a result of the weakening of the host by the primary dinoflagellate infection, or may indicate the presence of background bacterial load within the animal, which may infect most apparently healthy animals as well (this would support the findings of Colwell *et al.*, 1975 (in *Callinectes sapidus*) and Lightner, 1977 (in penaeid shrimps)). The fact that the bacterial loads found in cultures made from tissue and haemolymph samples did not represent particularly high initial levels (T.H.Birkbeck. pers. comm.) would suggest that the bacterial presence (if not an artefact of a failure to achieve complete asepsis) was indeed merely a background load.

CHAPTER 7. SURVEYS ASSESSING THE DISTRIBUTION AND PREVALENCE OF, AND MORTALITIES DUE TO, THE DINOFLAGELLATE INFECTION OF *Nephrops norvegicus* (L.) ON THE WEST COAST OF SCOTLAND.

7.1 INTRODUCTION

The increasing prevalence of apparently diseased *N. norvegicus* in samples caught on grounds in the Clyde Sea Area during the mid 1980's prompted a survey of west coast fishing grounds both in the Clyde and in the South Minch to assess the seasonality, prevalence and population effects of this condition. Concurrent with this, histopathological studies revealed the cause of this condition to be infection by a parasitic dinoflagellate (Chapter 6). This chapter presents the results of the surveys undertaken on the west coast between 1987 and 1991, in conjunction with the results of survival experiments undertaken to assess the effects of the condition on infected individuals. These results are discussed in terms of the possible effects of infection on the fishery and the possible life history of the infecting organism.

7.2 METHODS

7.2.1 Diagnosis. Infected animals were initially identified by an assessment of body colour alone but, in later investigations assessment was by body colour in conjunction with pleopod examination. Pleopod examination enabled assessment of moult stage, by the method of Aiken (1980), and severity of infection by use of the arbitrary scale detailed in Chapter 6, discriminating four stages of infection (from light - stage I, to severe - stage IV) and apparently healthy (stage 0).

7.2.2 Estimates of host mortality. During two sampling cruises aboard the RV *Aora* in the Clyde Sea Area in March and April 1991, a total of 202 undamaged *N. norvegicus* were collected by trawling. These animals, which comprised both healthy and infected individuals, were maintained under laboratory conditions in order to obtain estimates of mortality due to the infection. Immediately after capture, records were made of the carapace length, moult stage and sex of each animal together with its state and severity of infection. For the latter measurements only the tip of a pleopod was removed from each animal, in order to minimise stress. Animals were then assigned a numbered claw tag for individual identification. After immobilising their chelipeds with elastic bands to prevent fighting, the animals were maintained in running seawater tanks on board ship and later transferred to the aquarium in the Zoology Department, Glasgow University. The tanks were checked regularly and any dead animals were removed. Their tag numbers, moult stages, state of infection and date of death were then recorded.

7.2.3 Distribution and prevalence. From May 1987 to May 1989 small mesh trawl (SOAFD Type BT 126D) samples were taken periodically during routine cruises aboard the RV *Aora*, at three locations in the Firth of Clyde. The locations were south of Ailsa Craig (mean depth 60m), north of Arran (mean depth 89m) and in the Cumbrae-Bute Channel (mean depth 77m) (Figure 7.1, sites 14, 4 and 1 respectively). The prevalence of infection (assessed

FIGURE 7.1. Map of Scotland (inset) showing locations of west coast sampling stations during cruises of RV *Aora* between May 1987 and April 1991. Sites numbered as in the text and Tables 7.3 and 7.5.
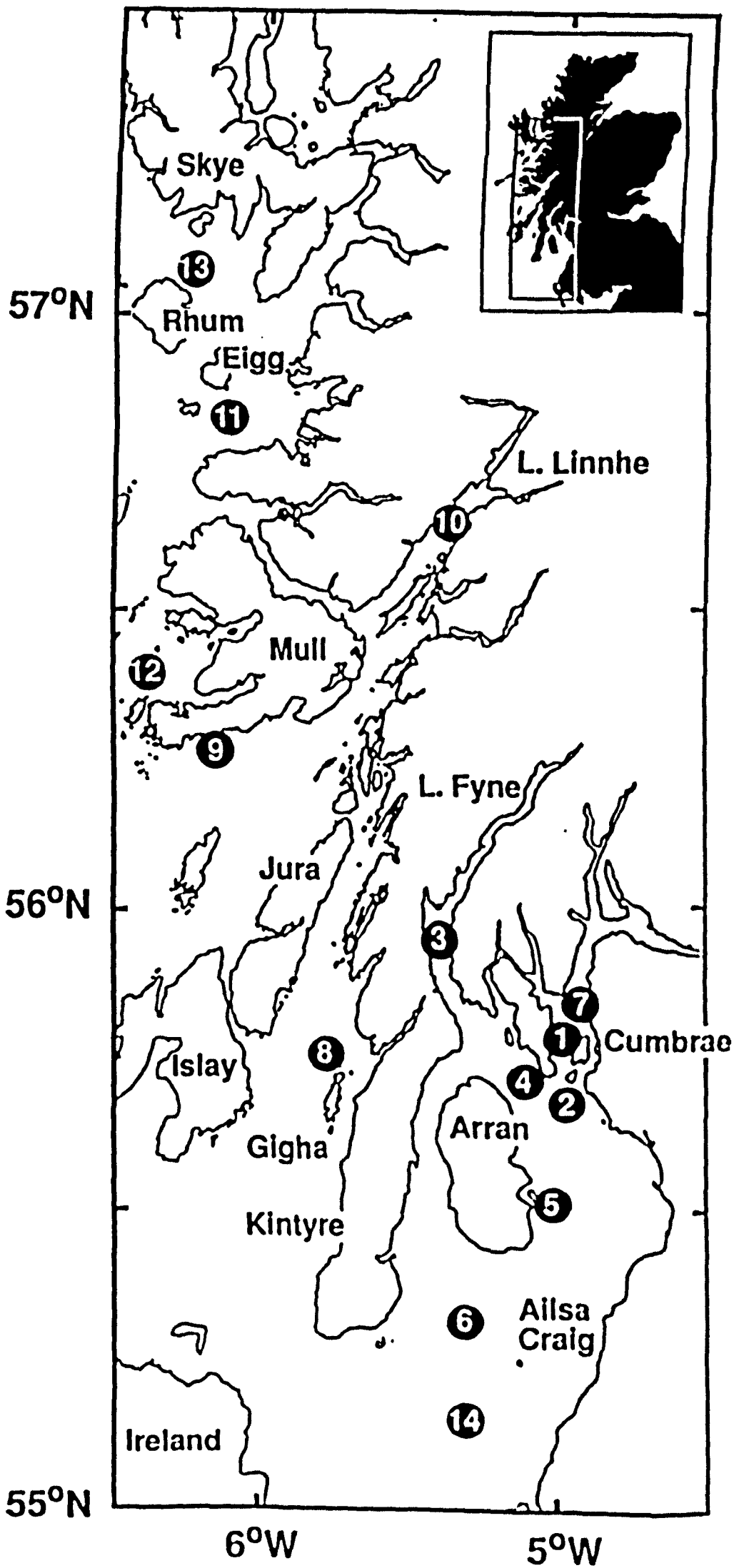
mainly by colour only) was recorded in relation to size and sex of *N. norvegicus* at these three sites.

In 1990, work was confined to the period March-April but, in addition to the Clyde, sampling was extended to other sites around the west coast of Scotland (Figure 7.1, see Table 7.3). Again, samples were taken using a small mesh trawl from the RV *Aora*, and bottom water samples were taken at each site, using an N10 reversing water bottle, for measurement of dissolved oxygen content (using the Winkler method), salinity (using an Autosal 8400 salinometer) and temperature. The prevalence and severity of infection was assessed at each site, by both colour and pleopod examination, in relation to sex, size and moult stage.

A similar sampling programme was undertaken during March and April 1991 at a range of sites in the Clyde and around the west coast of Scotland (Figure 7.1, see Table 7.5). Infection prevalence and severity, sex, size and moult stage data were recorded as in the 1990 survey. Additional trawl samples from the Clyde grounds close to the Isle of Cumbrae (Station 2, Figure 7.1) were obtained on 2 May, 12 June, 3 July and 22 August 1991.

7.3 RESULTS

7.3.1 Estimates of host mortality. Table 7.1 shows the results of survival experiments initiated on 11 March and 8 April 1991, involving (in each case) 50 dinoflagellate infected *N. norvegicus* and 51 apparently healthy control animals. These experiments were terminated on the 7 April and 22 June 1991, respectively. Mortality after 27 and 75 days was 86% and 100% in infected animals compared with 47% and 68.6% in control animals, respectively. In both survival experiments, the majority of deaths occurred within the first few days after capture, mortality being greater in the infected animals. No animals were observed to recover from infection but, more significantly, none were observed to suffer an increase in severity in condition before death. Also, no animals which were healthy upon capture developed the infection under laboratory conditions. The median survival times (in days) of all animals for which exact times of death were known, showed no significant variation in relation to severity of infection (P>0.5, Kruskal Wallis one-way ANOVA). Furthermore, these results indicate that the majority of deaths occurred soon after capture, the median survival time being approximately 4 days. Although survival time showed no variation in relation to severity of infection, percent mortality increased in this respect (Table 7.2). Mortality in healthy animals was 21% whilst that for infected animals increased from 50% for Stage I infection to 84.6% for Stage IV. Note that percentage mortalities presented in Table 7.2 are based only on animals for which the exact time of, and condition at, death were known, and are consequently lower than those shown in Table 7.1.

7.3.2 Distribution and prevalence. The prevalences of infected *N. norvegicus* (assessed by body colour alone) in trawl catches from three Clyde locations (Figure 7.1, Stations 14, 4 and 1) during the period May 1987 to May 1989 are shown in Figure 7.2 A,B & C respectively. The prevalence of infection showed a marked seasonal pattern in all three locations. Presence of infected animals was confined to the period between February and June, with evidence

TABLE 7.1 Cumulative percent mortality within groups of healthy (n = 51) and dinoflagellate infected (n = 50) *N. norvegicus* maintained in the laboratory from 11 March to 7 April (A), and 8 April to 22 June (B) 1991.

Number of Days	Cumulative mortality (%)			
	Healthy		Infected	
	A	B	A	B
1	0	3.9	18.0	8.0
2	3.9	3.9	28.0	10.0
3	3.9	3.9	32.0	20.0
4	9.8	7.8	44.0	24.0
5	19.6	11.7	52.0	26.0
6	23.5	11.7	58.0	30.0
7	23.5	-	60.0	-
8	-	13.7	-	32.0
9	-	13.7	-	38.0
10	-	13.7	-	46.0
11	23.5	-	66.0	-
12	23.5	13.7	68.0	48.0
-	-	-	-	-
16	23.5	-	70.0	-
-	-	-	-	-
22	23.5	-	72.0	-
-	-	-	-	-
27	47	-	86.0	-
75	-	68.6	-	100.0

TABLE 7.2 Percent mortality of healthy and dinoflagellate infected *N. norvegicus* in relation to severity of infection, assessed by pleopod staging on an arbitrary scale from I to IV (pooled from the two survival experiments).

	Healthy	Infected			
		I	II	III	IV
Number of individuals included in experiment	100	36	31	21	13
Number of individuals recorded as dead at termination of experiment	21	18	20	14	11
% mortality	21.0	50.0	64.5	67.0	84.6

FIGURE 7.2. Seasonal variation in the prevalence (%) of infected *N. norvegicus* based on small mesh trawl samples taken at three locations in 1987-1989. Sites A, B and C are labelled 14, 4 and 1 respectively in Figure 7.1. Note change in ordinal scale between A, B and C. Data prior to October 1988 were made available by SOAFD. Breaks in horizontal axes show gaps in monthly sampling programme.

A. Ailsa Craig
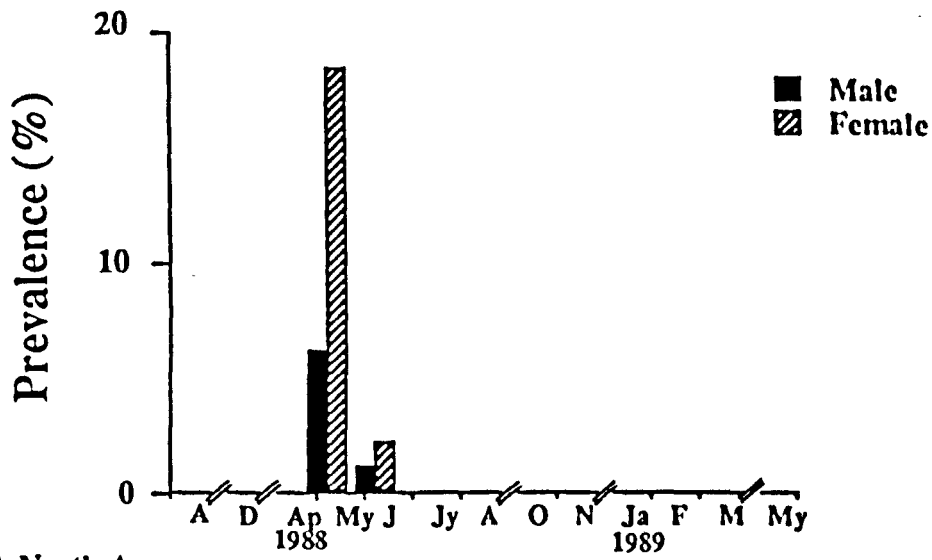
B. North Arran
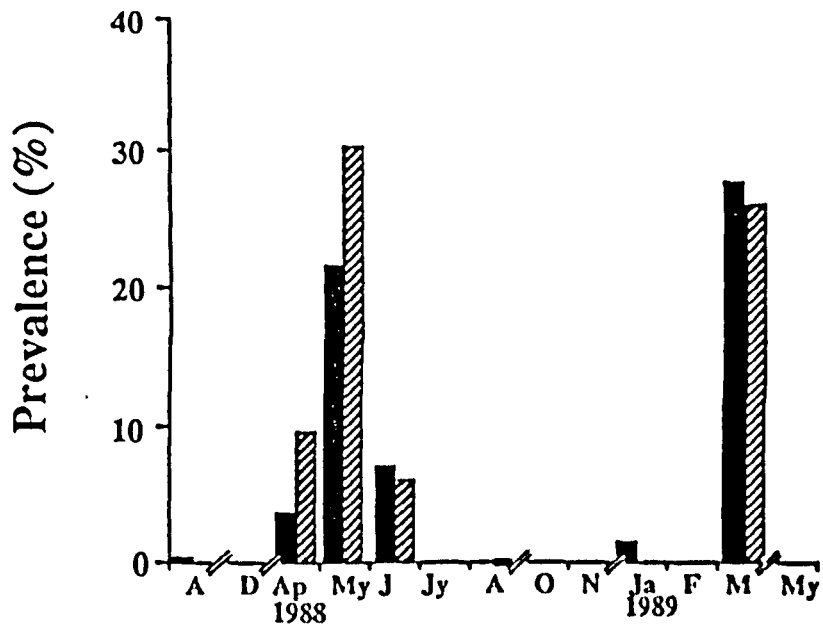
C. Cumbrae Channel
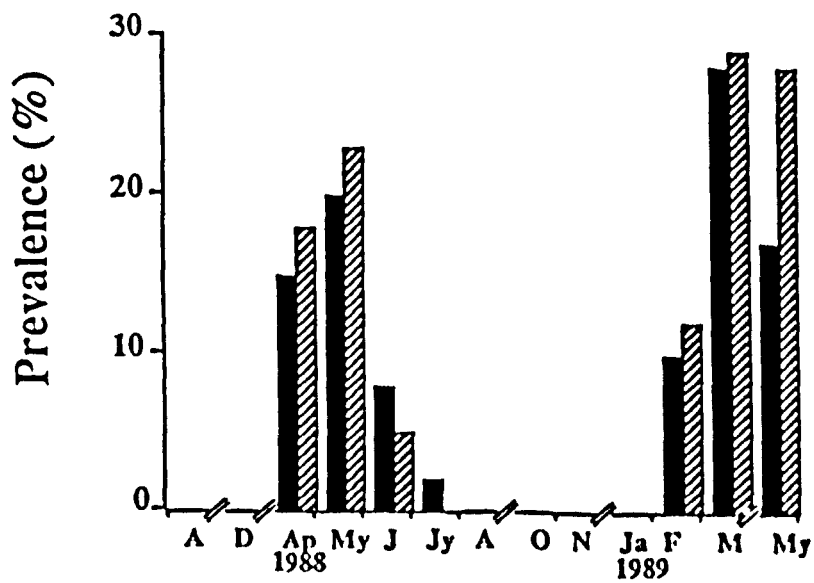
of peak numbers occurring in May, 1988 and March, 1989. At peak times, approximately 30% of trawled *N. norvegicus* taken from the North Arran and Cumbrae Channel sites were affected but there was a lower prevalence of infection at the Ailsa Craig site. Females generally revealed a higher prevalence of infection than males.

The results of sampling in the Clyde and at sites around the west coast of Scotland in 1990 (Figure 7.1) are summarised in Table 7.3. Prevalences of infection were assessed by both body colour and pleopod examination. Pleopod examination generally revealed a higher prevalence of infection because it more readily distinguished less severe cases. Trawl catches in the northern Clyde during early March showed that over 70% of *N. norvegicus* were infected, though sample numbers were small, especially for females. Clyde catches in April were also very poor, when the proportion of affected animals appeared to have decreased to 20% in males and 50% in females. On grounds outside the Firth of Clyde, in the Sound of Jura, Loch Linnhe, and off the Isles of Mull and Rhum, the overall prevalence of infection (14%) in April was less than that seen in the Clyde.

The examination of pleopods also allowed an assessment of the prevalence of the disease in relation to stage in the moult cycle. In March, infection on Clyde grounds was equally prevalent in animals in intermoult and those in premoult stages. In April 1990, however, intermoult animals taken at sites in the South Minch (except Loch Linnhe) showed higher infection rates than premoult animals taken at the same sites. Dissolved oxygen content, salinity and temperature at all sites surveyed in 1990 (Table 7.4) were considered normal for spring and early summer in these areas.

The results of the sampling programme undertaken in 1991 (Figure 7.1) are summarised in Table 7.5. In early March, approximately half of all trawled *N. norvegicus* in the northern Clyde grounds showed signs of infection, though sample sizes were small. By 11-13 March, and in April, the prevalence of the

TABLE 7.3 Prevalence (%) of infected *N. norvegicus* in small mesh trawl samples during March-April 1990 in the Clyde and South Minch assessed by A) body colour and B) pleopods. Sample sites are numbered as in Figure 7.1.. M = males; F = females; M&F = males and females combined; P = premoult; I = intermoult.

Area	Site	Date	Sample No.	A			B				
				M	F	M&F	M	F	M&F	P	I
Clyde	1. Cumbrae Channel	13/14 03	154	–	–	–	56.6	68.0	58.4	66.7	60.5
	2. S of Little Cumbrae	14 03	136	–	–	–	81.7	93.8	84.6	82.4	84.7
	4. N of Arran	15 03	121	–	–	–	77.9	62.8	73.6	77.7	72.2
	OVERALL		411	–	–	–	70.5	75.0	71.5	77.1	75.8
S Minch	9. Ross of Mull	06 04	606	8.1	15.1	11.2	18.0	15.5	16.9	11.7	17.8
	13. North Rhum	07 04	582	8.4	10.5	9.3	11.4	12.1	11.7	9.8	12.1
	10. Loch Linnhe	08 04	558	10.4	11.9	11.1	14.2	20.9	17.5	17.5	17.5
	8. NW Gigha	09 04	526	7.3	7.6	7.4	7.6	11.3	9.3	6.5	10.2
	OVERALL		2272	8.5	11.4	9.8	12.9	15.2	14.0	11.1	14.5
Clyde	1. Cumbrae Channel 2. S of Little Cumbrae 3. Loch Fyne 4. N of Arran 5. Off Holy Isle	10-13 04	256	17.9	43.6	23.4	20.0	50.0	22.7	–	–

TABLE 7.4 Environmental parameters of bottom water samples taken at sites in the Clyde and South Minch during March and April 1990.

Area	Site	Date	Dissolved O2 ml l^{-1}	Salinity ‰	Temperature °C
Clyde	1. Cumbrae Channel	13 03	6.60	35.3	9.1
		"	6.81	34.1	10.5
		"	7.02	32.7	10.6
		14 03	8.88	34.2	10.2
		12 04	6.78	33.7	11.5
	2. S of Little Cumbrae	14 03	7.11	33.7	11.2
	5. Off Holy Isle	14 03	7.21	33.5	10.8
	4. N of Arran	15 03	7.43	34.1	10.4
		"	7.91	34.9	11.2
		"	6.98	34.3	10.6
	3. Loch Fyne	10 04	6.86	34.2	10.8
S Minch	9. Ross of Mull	06 04	7.11	36.1	11.5
	13. N Rhum	07 04	7.00	35.5	10.7
	10. L Linnhe	08 04	6.87	33.7	10.8
	8. N W Gigha	09 04	6.95	34.7	11.1

TABLE 7.5 Prevalence (%) of infected *N. norvegicus* in trawl samples during March-April 1991 in the Clyde, Sound of Jura and South Minch assessed by A) body colour and B) pleopods. Sample sites are numbered as in Figure 7.1. M = males; F = females; M&F = males and females combined; P = premoult; I = intermoult.

Area	Site	Date	Sample No.	A			B				
				M	F	M&F	M	F	M&F	P	I
Clyde	1. Cumbrae Channel	04 03	8	20.0	33.3	25.0	-	-	-	-	-
	2. S of Little Cumbrae	04 03	34	28.0	77.8	41.2	-	-	-	-	-
	3. Loch Fyne	05 03	31	30.4	50.0	35.5	27.3	62.5	36.7	20.0	40.0
	4. North of Arran	05 03	154	32.0	42.9	35.7	46.3	66.7	54.0	-	-
	5. Off Holy Isle	05 03	7	33.3	-	28.6	-	-	-	-	-
	OVERALL		234	30.8	46.2	35.9	42.7	66.2	51.1	20.0	40.0
Sound of	8. NW Gigha	06 03	1339	18.2	16.7	17.8	18.5	-	-	-	-
Jura/	9. Ross of Mull	07 03	1724	20.5	18.5	19.9	24.9	-	-	-	-
S Minch	10. Loch Linnhe	08 03	1017	23.6	19.9	21.9	28.6	-	-	-	-
	11. Maxwell Bank	09 03	774	6.4	10.6	7.9	11.4	21.4	14.9	6.7	15.9
	12. South of Staffa	10 03	307	9.3	19.8	12.0	11.6	30.9	16.7	6.3	18.7
	OVERALL		5161	17.5	17.4	17.5	19.2	25.5	15.8	6.4	17.3
Clyde	6. South of Arran	11 03	267	8.7	18.9	12.4	14.5	28.1	19.4	5.2	22.7
	5. Off Holy Isle	11 03	43	5.9	22.2	9.3	-	-	-	-	-
	2. S of Little Cumbrae	11-13 03	257	14.1	24.2	16.7	26.9	31.0	27.7	-	-
	1. Cumbrae Channel	12 03	750	14.1	20.8	16.8	22.2	33.0	26.5	17.0	28.7
	4. North of Arran	12 03	474	21.7	26.3	23.2	29.8	39.0	32.8	6.7	38.9
	3. Loch Fyne	13 03	127	8.4	28.1	13.4	13.7	42.4	21.1	4.0	25.2
	OVERALL		1918	14.7	22.5	17.4	21.8	33.7	25.6	9.0	29.0
Clyde	1. Cumbrae Channel	08 04	390	18.4	37.3	22.0	23.3	43.6	27.6	6.3	30.5
	3. Loch Fyne	09 04	137	18.0	42.3	22.6	18.8	63.0	27.3	16.7	29.6
	7. Skelmorlie	10 04	213	19.1	39.3	24.9	-	-	-	-	-
	OVERALL		740	18.5	38.9	23.0	21.7	50.0	27.5	10.7	30.2
Clyde	Sites around Isle	02 05	334	41.7	75.4	54.2	-	-	-	-	-
	of Cumbrae	12 06	230	40.7	64.2	50.4	-	-	-	-	-
		03 07	-	-	-	19.0	-	-	-	-	-
		03 07	-	-	-	21.0	-	-	-	-	-
		22 08	-	-	-	1.0	-	-	-	-	-

condition had decreased to around 30%. Again a lower prevalence of infection (16-17%) was noted on grounds outside the Clyde, in the Sound of Jura and the South Minch.

Samples taken in 1991 confirmed the trend indicated by samples in April 1990, showing a significantly higher prevalence of infection in intermoult animals than in those in premoult, both on Clyde grounds and at other sites around the west coast (P<0.05, Wilcoxon's matched pairs test). Furthermore, as also indicated by 1990 samples, infection was significantly more prevalent (P<0.05, Wilcoxon's matched pairs test) in females than in males, in samples from all west coast grounds. Further trawl samples taken in May-August, assessed by body colour only, not only indicated an increase in the proportion of infected animals on grounds close to the Isle of Cumbrae (Station 2, Figure 7.1; over 50% in May and June), but also an apparent extension of the seasonal occurrence of this condition into July (with prevalence as high as 21%).

The March and April Clyde samples of 1991 were further analysed to compare infection levels in different sized *N. norvegicus* (Figure 7.3) and to provide an assessment of the severity of the disease based upon pleopod stages (Figure 7.4). Figure 7.3 shows that the infection was most prevalent in individuals of 25-35mm carapace length. Figure 7.4 suggests that the majority of affected *N. norvegicus* were suffering from light infections during March and April 1991.

FIGURE 7.3. Histogram showing the proportion of infected *N. norvegicus* in different 5mm carapace length size categories. Based on Clyde samples taken in March-April 1991.

FIGURE 7.4. Histogram showing the proportion of infected *N. norvegicus* in relation to the degree of severity of infection, assessed by pleopod staging on an arbitrary scale from I to IV (see Figure 6.1 & Table 6.3). Animals were collected from the Firth of Clyde in March and April 1991.
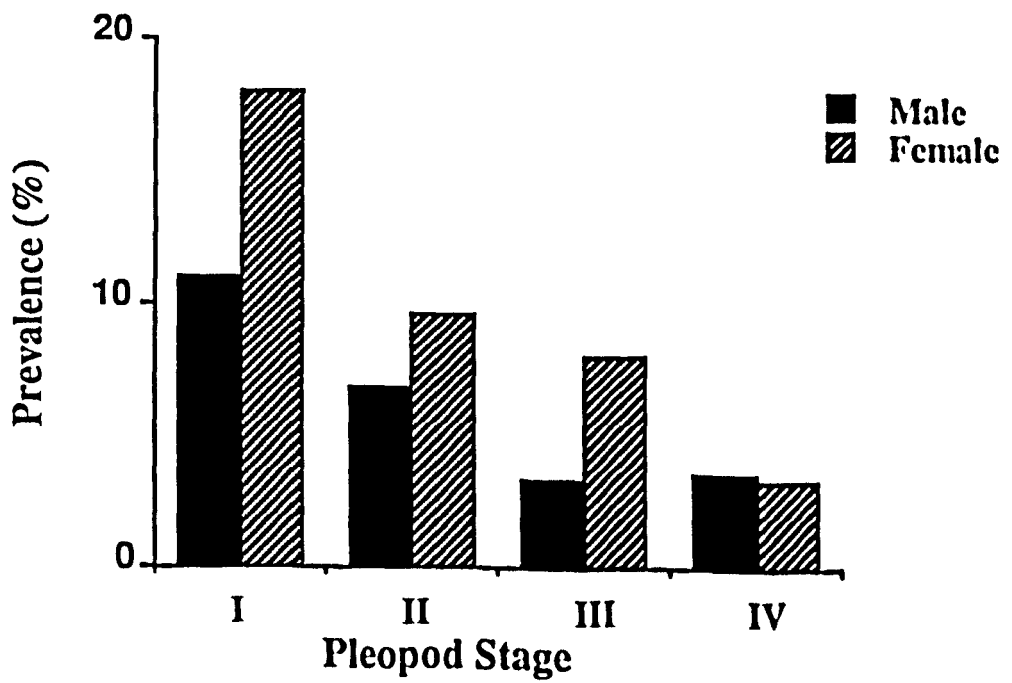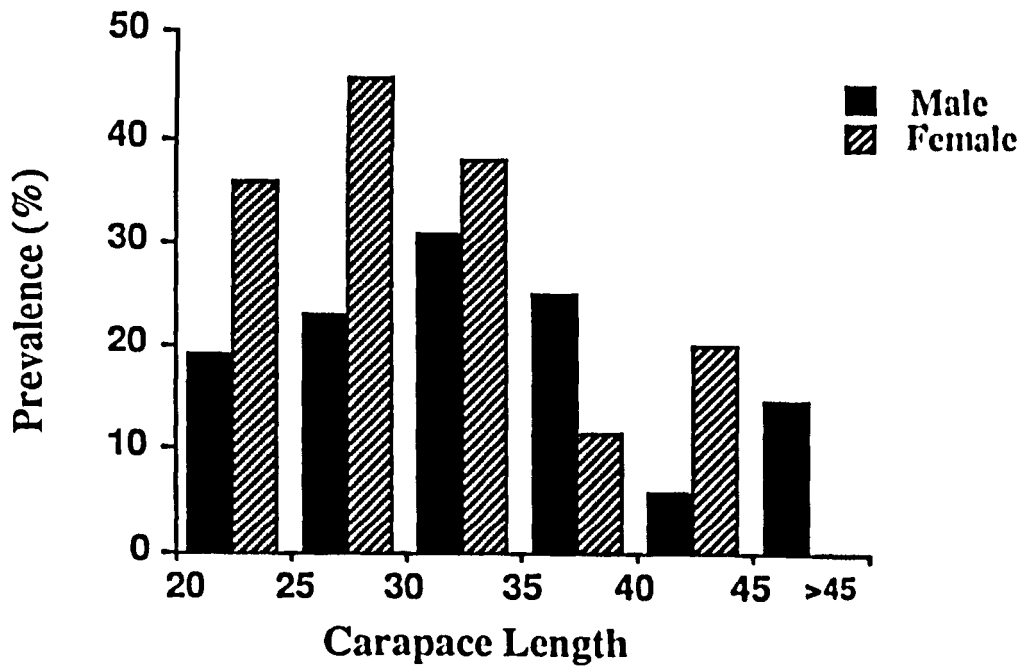
7.4 DISCUSSION

Survival experiments revealed higher mortality in infected *N. norvegicus*, compared with healthy animals. However, the majority of deaths in both infected and control groups occurred soon after capture, which may suggest that mortality was compounded by the additional stress of trawl capture. Median post-capture survival time was constant irrespective of the severity of infection, suggesting that death was not due to infection alone. Nevertheless, it seems likely that this condition causes significant mortalities in *N. norvegicus*, as numbers of deaths were much greater in infected animals. Also, Bitter Crab Disease (BCD) (Meyers, 1990) and *Hematodinium* sp. (Newman & Johnson, 1975) infections have similar effects on their hosts, and are implicated in causing death. Indeed, blue crabs are known to die from experimental infections of *Hematodinium perezi* (Newman, 1988) and virtually 100% mortality occurs in tanner crabs naturally infected with the BCD organism (Meyers, 1990).

The most striking similarity between the disease in *N. norvegicus* and BCD is the high prevalence of infection. Prevalence of *Hematodinium* sp. infections are generally low, but high levels of infection have been noted in *Necora puber* and *Cancer pagurus* (up to 87%) in coastal waters of north west France (Wilhelm & Boulo, 1988; Latrouite *et al.*, 1988). BCD has been reported to infect up to 95% of tanner crabs on some grounds, and infection levels as high as 70% have been seen in *N. norvegicus*. If the infection is fatal, then high levels of infection may have important implications for *N. norvegicus* populations and the fisheries they support. Meyers *et al.* (1987) reported significant reductions in experimental catch sizes of tanner crabs associated with high disease prevalence. Also, infections of benthic amphipods by two types of *Hematodinium*-like dinoflagellate have been implicated in population regulation (Johnson, 1986). Furthermore, as in BCD, meat quality of infected *N. norvegicus* is poor (see Chapter 6) which may have consequences for their

marketability. At least one catch containing diseased *N. norvegicus* from the Clyde has been refused at market in 1991 on quality grounds.

The occurrence of this condition is markedly seasonal, and coincides with the main annual moulting period of *N. norvegicus*. The relationship between prevalence of infection and moulting is not at present clear, but is probably important. A seasonal occurrence of *Hematodinium perezi* has been reported by Newman & Johnson (1975) in blue crabs (*Callinectes sapidus*) on the east coast of the United States, with the peak being in the autumn. These authors also note that the parasite was not found in animals collected from waters with salinities of less than 11‰. Similar seasonality and a relation to moult cycle have been noted in BCD in tanner crabs (Eaton *et al.*, 1991). In *N. norvegicus* there is a difference in the prevalence of infection between animals in intermoult and those in premoult, and this may be indicative of increased mortality in infected premoult animals. However, it is more likely that the temporal relationship of infection with moulting may reflect the susceptibility of recently moulted animals to infection, and/or the seasonal production by the parasite of infective stages, possibly, as yet unknown spore forms.

The moult-staging technique used is effective at identifying animals in various stages of premoult, but animals in postmoult cannot be distinguished from those in intermoult. This means that the high prevalence of infection in samples of intermoult animals may be due mainly to recently moulted postmoult animals infected shortly after ecdysis. This has been suggested by Eaton *et al.* (1991) as a possible mode of transmission of the causative agent of BCD. Their results indicate that infection prevalence is higher in crabs with newer, softer shells (i.e. more recently moulted), than in those with old shells. The highest prevalences in both old and new shell tanner crabs occur in summer, shortly after the peak moulting period of the populations concerned. In common with *N. norvegicus*, this may indicate that more recently moulted animals may be more susceptible to invasion. This explanation, however, does

not account for the presence of infection in animals in premoult or with old, hard shells. This situation is seen in both *N. norvegicus* and *Chionoecetes bairdi*. In addition, the putative transmissive stages of the BCD organism, the spores, are produced too late in the summer to account for the early summer peak of infection by invasion at, or just after, ecdysis (Meyers *et al.*, 1991). It seems likely, therefore, that infection of tanner crabs occurs through a number of routes, and Meyers *et al.* (1990) suggest transmission of vegetative stages also. No spore stage has been isolated for *Hematodinium*, or for the dinoflagellate infecting *N. norvegicus*, and so infection via the gut, or breaching of the cuticle (particularly just after ecdysis when numerous cuticular lesions occur) by vegetative stages may also be possible. The heavy involvement of the midgut wall in infections of *N. norvegicus* might implicate this area as a portal of entry to infective vegetative cells.

Further indications of a link between infection and moulting can be seen in the differences in prevalences of infection between males and females, and the higher prevalences in smaller animals. The results of the surveys conducted between 1987 and 1991 show a general trend of higher prevalences of infection in females than in males. This difference may be due to differences in moulting times of males and females. Female *N. norvegicus* moult before males, in order that they have soft cuticles to allow mating with hard shell males. In west coast populations, almost all female moulting occurs in spring, whilst small males moult in late spring to early summer, and larger males in autumn (C.J.Chapman. pers. comm.). This means that the majority of females are moulting at the time of peak disease prevalence, whereas fewer males are moulting at this time. The sex difference in prevalence may therefore indicate that infection occurs, or is greatly facilitated by, moulting. This hypothesis is further supported by the fact that infection is far more prevalent in smaller individuals (of both sexes), since smaller animals moult more frequently, and only small males moult during the spring moulting period. The implication of

moulting in the facilitation of infection may be due not only to the greater ease with which soft postmoult cuticle might be breached by an infective stage, but may also be due to the behavioural changes brought about by moulting. For a short period before, and until the cuticle has hardened after moulting, animals remain in their burrows in the sediment and may therefore expose themselves to a greater risk of infection by infective stages liberated into the sediment from the carcasses of recently dead infected animals. This would, however, only be true if animals were to die within their burrows. If not, then animals would be equally exposed to infection in burrows or on the sediment surface, and infection through the intestinal tract may occur *via* the eating of infected carcasses.

However, the presence of a spore stage (or stages) in the life cycle of the dinoflagellate infecting *N. norvegicus* cannot be discounted. The marked similarities between this organism and that causing BCD, for which such stages have been recognised, suggest that similar cells may be present. In addition, the presence in vegetative cells of structures resembling flagellar hair vesicles may either suggest the presence of a flagellate stage, or indicate the taxonomic affiliations of the organism (see Chapter 6).

The survey results in 1990 and 1991 suggest there may be a background level of infection over much of the west coast of Scotland, amounting to about 10-15% of the fishable population. On grounds in the North Clyde Sea Area (Figure 1, Stations 1-4) the proportion of infected animals is much higher and appears to have increased in recent years from 30% in 1988/89 to over 50% in 1990/91. It must be remembered that these figures are derived from small mesh trawl catches which may not be representative of the *N. norvegicus* population as a whole. Whether infected animals are more or less susceptible to capture by trawl is at present unknown.

The entirely subtidal nature of *N. norvegicus* populations, and the attendant problems of sampling such populations makes an accurate

assessment of the possible population effects of disease very difficult. Sampling by small mesh trawl is effective in capturing that portion of the population which is active on the sediment surface (see chapter 1), which enables assessment of the prevalence of disease within these animals. However, that proportion of the population which remains within burrows in the sediment, for whatever reason, or does not respond to fishing gear with the normal tail-flip escape response, will not be sampled. This is well demonstrated by the seasonal variations in the sex ratio in trawl catches of *N. norvegicus*, corresponding to different stages in the life cycle (Bailey, 1984).

In addition to these 'natural' fluctuations in activity, and hence capture rate, it is also possible that disease may alter the activity of *N. norvegicus*. It has already been noted that *N. norvegicus* infected with the dinoflagellate discussed here appear to be lethargic and weak, and this may prejudice their foraging and escape activities. Furthermore, behaviour may be more directly affected by invasion of, or damage to, neural tissues of infected animals. In these ways, infected individuals may be more, or less exposed to trawling by alteration of the extent to which they forage on the sediment surface, and their responses to fishing gear. In order to assess the degree of bias in trawl sampling as a measure of infection prevalence, and to gain a more accurate picture of the real effects of dinoflagellate infection upon Clyde *N. norvegicus* populations, other sampling methods must be used. Direct observational methods, such as the use of underwater video equipment, and sampling of the sediment with some form of grab, would enable evaluations to be made of the presence and numbers of dead animals, and of the reactions of infected animals to fishing gear. In addition, further laboratory examinations of the behaviour and survival of infected animals are needed in order to assess the effects of the disease on population dynamics. However, this will require the development of procedures to artificially induce infection, in order to discount capture stresses, which are apparently important (see above).

CHAPTER 8. CONCLUSIONS AND PROSPECTS.

During the course of this study, the true nature of the so-called 'post moult trauma' has been elucidated. *N. norvegicus* exhibiting the symptoms of this condition have been found to be sustaining haemocoelic infection by large numbers of protistan parasites. These parasites have been identified as parasitic dinoflagellates probably belonging to the botanical order Syndiniales and resembling members of the genus *Hematodinium*. Identification as parasitic dinoflagellates was made on the basis of the presence of the characteristic amphiesmal structure of the cell surface, the presence of tubulocristate mitochondria, abundant trichocysts, and prominent chromosomes in mitotic figures.

The exact affiliations of the infecting organism are still uncertain, with its inclusion in the Syndiniales resting upon the demonstration of the presence of paired centrioles within a pocket of the nuclear envelope (Professor K. Vickerman, pers. comm.). The parasite cells do, however, closely resemble those identified by several authors as belonging to the genus *Hematodinium*, and causing similar symptoms in several species of crab (Chatton & Poisson, 1931; Newman & Johnson, 1975; Maclean & Ruddell, 1978; Latrouite *et al.*, 1988; Wilhelm & Boulo, 1988;) and benthic amphipods (Johnson, 1986). Marked similarities are also shown between the condition reported here and that caused by a similar organism in Tanner crabs (BCD)(Meyers *et al.*, 1987, 1990; Eaton *et al.*, 1991).

The results of the histopathological survey presented in Chapter 6 have shown the widespread and systemic nature of the infection, involving most of the major organs and tissues so far examined. These results, in conjunction with those of the physiological investigations undertaken, have indicated a number of serious metabolic and physiological perturbations in infected animals, which may result in death. The issue of mortality remains unresolved by these results and by those of the survival experiments presented in Chapter 7, although the implications are that death results from infection. In addition to the direct

observational methods for the assessment of mortality specified in Chapter 7, further investigations of the apparently abnormal haemocyanin and muscle metabolisms and histopathology of infected animals are required. Furthermore, more definitive experiments are required to assess the survival of infected animals. In order to achieve this, it is necessary to induce infection in healthy animals, to circumvent the bias caused in survival experiments by the additional stress of trawling. This fulfils one of Koch's postulates, which are an essential part of any further investigation based upon the results presented in this thesis. Fulfilment of these conditions are essential in the explanation of the seasonality and sex and moult differences in infection prevalence reported here.

Future work should also focus on the route of transmission of infective parasite stages, which may be important in the planning of fisheries management. In the best documented case of dinoflagellate infection of a decapod, BCD in Tanner crabs, high infection prevalences, coupled with deleterious effects on crab meat, have resulted in significant economic losses and the closure of some grounds (Meyers *et al.*, 1987, 1990; Eaton *et al.*, 1991). Their investigations of the distribution of infection, both spatial and temporal, and the aetiology of the agent have led to the introduction of management practices controlling the spread of the parasite to uninfected areas, and the reduction of market losses through poor meat quality. In the light of the similarities between BCD and the condition affecting *N. norvegicus*, similar investigations are warranted to assess its possible implications upon west coast populations and the fisheries they support.

In addition to the threat this infection may pose to *N. norvegicus* populations and fisheries, the effects of parasitism upon meat quality may also affect the fishery. The apparent lysis of abdominal muscle makes meat processing more difficult as well as appearing unattractive to both fishermen and consumer, therefore increasing losses. If, as postulated for the causative agent of BCD, the effects upon meat quality and taste are at least in part due to

substances produced by the parasite, there may be implications for public health, and this aspect should be investigated with some urgency.

As suggested in Part 1 of this thesis, many factors are important in the production of the tail-flip escape reaction of *N. norvegicus*, not least the health of the animal. Since tail-flipping is an integral part of the capture of this animal by trawling, and the condition apparently causes a degree of lethargy, it may have a significant effect upon the success of trawling. This suggests that the swimming performance of infected animals, and other aspects of their behaviour should be investigated, to determine the presence and importance of parasite induced behavioural changes which may affect the success of either trawling or creeling operations and, more importantly, to assess the degree of bias in sampling prevalences in populations.

LITERATURE CITED

Aiken, D. E. (1980). Moulting and growth. In: Cobb, J. S. & Phillips, B. F. (eds.) The Biology and Management of Lobsters. Vol.1. Acedemic Press, New York. p. 91-163

Alderman, D. J. & Polglase, J. L. (1988). Pathogens, parasites and commensals. In: Holdich, D. M. & Lowery, R. S. (eds.) Freshwater Crayfish. Biology, Management and Exploitation. Croom Helm, London. p. 167-212

Armstrong, D. A., Burreson, E. M. & Sparks, A. K. (1981). A ciliate infection (*Paranophrys* sp.) in laboratory-held dungeness crabs, *Cancer magister*. *J. Invertebr. Pathol.* 37: 201-209

Atkinson, R. J. A. & Naylor, E. (1976). An endogenous activity rhythm and the rhythmicity of catches of *Nephrops norvegicus* (L.). *J. exp. mar. Biol. Ecol.* 25: 95-108

Baden, S. P., Pihl, L. & Rosenberg, R. (1990). Effects of oxygen depletion on the ecology, blood physiology and fishery of the Norway lobster *Nephrops norvegicus*. *Mar. Ecol. Prog. Ser.* 67: 141-155

Bailey, N. (1984). Some aspects of reproduction in *Nephrops. I.C.E.S.* C.M. 1984/K:33 (mimeo)

Bang, F. B. (1970). Disease mechanisms in crustacean and marine arthropods. In: Snieszko, S. F. (ed.) A Symposium on Diseases of Fishes and Shellfishes. American Fisheries Society, Washington, D.C. p. 383-404

Bang, F. B., Audouin, J. & Leglise, M. (1972). Ciliate infection of the blood of the edible crab, *Cancer pagurus*, in holding tanks in Brittany, France. *J. Invertebr. Pathol.* 20: 226-227

Barkate, J. A., Laramore, C. R., Hirnon, Y. & Persyn, H. (1974). Some marine microorganisms related to shrimp diseases. *Proc. Wld Maricult. Soc.* 5: 267-282

Barker, P. L. & Gibson, R. (1977). Observations on the feeding mechanism, structure of the gut, and digestive physiology of the European lobster *Homarus gammarus* (L.) (Decapoda: Nephropidae). *J. exp. mar. Biol. Ecol.* 26: 297-324

Barnes, R. D. (1974). The crustaceans. In: Invertebrate Zoology. 3rd edn. W.B.Saunders, Philadelphia. p. 510-618

Baross, J. A., Tester, P. A. & Morita, R. Y. (1978). Incidence, microscopy and etiology of exoskeleton lesions in the tanner crab *Chionoecetes tanneri*. *J. Fish. Res. Bd. Can.* 35: 1141-1149

Booth, C. E. & McMahon, B. R. (1985). Lactate dynamics during locomotor activity in the blue crab, *Callinectes sapidus*. *J. exp. Biol.* 118: 461-465

Bowser, P. R., Rosemark, R. & Reiner, C. R. (1981). A preliminary report of vibriosis in cultured American lobsters, *Homarus americanus*. *J. Invertebr. Pathol.* 37: 80-85

Brock, J.A. & Lightner, D. V. (1990). Diseases of Crustacea - Diseases caused by microorganisms. In: Kinne, O. (ed.) Diseases of Marine Animals. Vol. 3. Biologische Anstalt Helgoland, Hamburg. p. 245-349

Bridges, C. R., Bicudo, J. E. P. W. & Lykkeboe, G. (1979). Oxygen content measurement in blood containing haemocyanin. *Comp. Bioch. Physiol.* 62A: 457-462

Brooke, M. H. & Kaiser, K. K. (1970). Three 'myosin adenosine triphosphatase' systems: the nature of their pH lability and sulfhydryl dependence. *J. Histochem. Cytochem.* 18: 670-672

Bulnheim, H.-P. (1975). Microsporidian infections of amphipods with special reference to host-parasite relationships: A review. *Mar. Fish. Rev.* 37: 39-45

Cachon, J. & Cachon, M. (1987). Parasitic dinoflagellates. In: Taylor, F. J. R. (ed.) The Biology of Dinoflagellates. Blackwell Scientific Publications, Oxford. p. 571-610

Cattaert, D., Clarac, F. & Neil, D. M. (1988). Anatomical and physiological organization of the swimmeret system of the spiny lobster *Jasus lalandii* as adaptive components of the tail-flick. *J. Comp. Physiol.* A162: 187-200

Chapman, C. J., Johnstone, A. D. F. & Rice, A. L. (1975). The behaviour and ecology of the Norway lobster, *Nephrops norvegicus* (L.). *Proc. 9th Europ. Mar. Biol. Symp:* 59-74

Chatton, E. (1952). Classe des Dinoflagellés ou Péridiniens. In: Grassé, P. P. (ed.) Traité de Zoologie. Vol.1. Masson, Paris. p. 309-390

Chatton, E. & Poisson, R. (1931). Sur l'existence, dans le sang des crabs, de peridiniens parasites *Hematodinium perezi* n.g., n.sp. (Syndinidae). *C. r. séanc. Soc. biol.* 105: 553-557

Chiba, A., Hamaguchi, M., Kosaka, M., Asai, T., Tokuno, T. & Chichibu, S. (1989). Phosphorus nuclear magnetic resonance studies on energy metabolism during escape reflex in crayfish. *Jpn. J. Physiol.* 39 Supple: S150

Colwell, R. R., Wicks, T. C. & Tubiash, H. S. (1975). A comparative study of the bacterial flora of the haemolymph of *Callinectes sapidus. Mar. Fish. Rev.* 37: 29-33

Cooke, I. R. C. & MacMillan, D. L. (1985). Further studies of crayfish escape behaviour. I. The role of the appendages and the nature of non-giant escape swimming. *J. exp. Biol.* 118: 351-365

Cornick, J. W. & Stewart, J. E. (1968). Pathogenicity of *Gaffkya homari* for the crab *Cancer irroratus. J. Fish. Res. Bd. Can.* 25: 795-799

Cornick, J. W. & Stewart, J. E. (1975). Red crab (*Geryon quinquedens*) and snow crab (*Chionoecetes opilio*) resistance to infection by the lobster pathogen *Aerococcus viridans* var. *Homari. J. Fish. Res. Bd. Can.* 32: 702-706

Couch, J. A. (1983). Diseases caused by Protozoa. In: Bliss, D. E. (ed.-in-chief) The Biology of Crustacea. Vol. 6. Provenzano, A. J. (ed.) Pathobiology. Academic Press, New York. p. 79-111

Couch, J. A. & Courtney, L. (1977). Interaction of chemical pollutants and virus in a crustacean: a novel bioassay system. *Ann. N. Y. Acad. Sci.* 298: 497-504

Cromarty, S. I., Cobb, J. S. & Kass-Simon, G. (1991). Behavioral analysis of the escape response in the juvenile lobster *Homarus americanus* over the molt cycle. *J. exp. Biol.* 158: 565-581

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

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

Dodge, J. D. (1987). Dinoflagellate ultrastructure and complex organelles: A. General ultrastructure. In: Taylor, F. J. R. (ed.) The Biology of Dinoflagellates. Blackwell Scientific Publications, Oxford. p. 93-118

Drach, P. (1939). Mue et cycle d'intermue chez les Crustacés Décapodes. *Ann. Inst. Océanogr.* 19: 103-391

Drury, R. A. B. & Wallington, E.A. (1967). Carleton's Histological Technique. 4th edn. Oxford University Press, London.

Eaton, W. D., Love, D. C., Botelho, C., Meyers, T. R., Imamura, K. & Koeneman, T. (1991). Preliminary results on the seasonality and life cycle of the parasitic dinoflagellate causing bitter crab disease in Alaskan tanner crabs (*Chionoecetes bairdi*). *J. Invertebr. Pathol.* 57: 426-434

Engel, P. C. & Jones, J. B. (1978). Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD+ in alkaline hydrazine buffers: improved conditions for the assay of L-glutamate, L-lactate and other metabolites. *Anal. Biochem.* 88: 475-484

England, W. R. & Baldwin, J. (1983). Anaerobic energy metabolism in the tail musculature of the Australian yabby *Cherax destructor* (Crustacea, Decapoda, Parastacidae): role of phosphagens and anaerobic glycolysis during escape behaviour. *Physiol. Zool.* 56: 614-622

England, W. R. & Baldwin, J. (1985). Anaerobic energy metabolism in the tail musculature of the Australian yabby *Cherax destructor* (Crustacea, Decapoda, Parastacidae): regulation of anaerobic glycolysis. *Comp. Biochem. Physiol.* 80B: 327-335

Figueredo, M. J. de. & Thomas, II. J. (1967). On the biology of the Norway lobster, *Nephrops norvegicus* (L.). *J. Cons. perm. int. Explor. Mer.* 31: 89-101

Findley, A. M., Blackeney, E. W. & Weidner, E. II. (1981). *Ameson michaelis* (Microsporida) in the blue crab, *Callinectes sapidus*: parasite-induced alterations in the biochemical composition of host tissues. *Biol. Bull.* 161: 115-125

Fisher, W. S. & Wickham, D. E. (1976). Mortalities and epibiotic fouling of eggs from wild populations of the dungeness crab, *Cancer magister. Fish. Bull.* 74: 201-207

Fisher, W. S. & Wickham, D. E. (1977). Egg mortalities in wild populations of the dungeness crab in central and northern California. *Fish. Bull.* 75: 235-237

Fisher, W. S., Rosemark, T. R. & Nilson, E. II. (1976). The susceptibility of cultured American lobsters to a chitinolytic bacterium. *Proc. Wld Maricult. Soc.* 7: 511-520

Fowler, W. S. (1990). Histochemical satining of lobster muscle demonstrates no ATPase pH reversal. *J. Physiol. (Lond.).* 420: 117

Gäde, G. (1984). Effects of oxygen deprivation during anoxia and muscular work on the energy metabolism of the crayfish, *Orconectes limosus. Comp. Biochem. Physiol.* 77A: 495-502

Gäde, G. & Meinardus-Hager, G. (1987). Anaerobic energy metabolism in Crustacea, Xiphosura and Mollusca: Lactate fermentation versus multiple fermentation products. *Zool. Beitr. N. F.* 30: 187-203

Gallagher, M. L., Rittenberg, J. II., Bayer, R. C. & Leavitt, D. F. (1979). Incidence of *Aerococcus viridans* (var.) *homari* in natural crab (*Cancer irroratus, Cancer borealis*) populations from Maine coastal waters. *Crustaceana.* 37: 316-317

Giles, I. G., Poat, P. C. & Munday, K. A. (1975). Regulation of pyruvate kinase from the hepatopancreas of the crab *Carcinus maenas. Biochem. Soc. Trans.* 3: 400-402

Gopalan, U. K. & Young, J. S. (1975). Incidence of shell disease in shrimp in the New York Bight. *Mar. Poll. Bull.* 6: 149-153

Govind, C. K. & Kent, K. S. (1982). Transformation of fast fibres to slow prevented by lack of activity in developing lobster muscle. *Nature.* 298: 755-757

Govind, C. K., Budd, T. W. & Atwood, II. L. (1981). Fiber composition and innervation patterns of the limb closer muscle in the lobster *Homarus americanus. Biol. Bull.* 160: 69-79

Green, H. J., Reichmann, G. & Pette, D. (1982). A comparison of two ATP-ase based schemes for histochemical muscle fibre typing in various mammals. *Histochemistry.* 76: 21-31

Gutmann, I. A. & Wahlefeld, W. (1974). L-(+)-Lactate determination with lactate dehydrogenase and NAD. In: Bergmeyer, H. U. (ed.) Methods of enzymatic analysis. Academic Press, New York. p. 1464-1468

Hamilton, N. M. & Houlihan, D. F. (1992). Respiratory and circulatory adjustments during aquatic treadmill exercise in the European shore crab *Carcinus maenas. J. exp. Biol.* 162: 37-54

Head, G. & Baldwin, J. (1986). Energy metabolism and the fate of lactate during recovery from exercise in the Australian freshwater crayfish *Cherax destructor. Aust. J. Mar. Freshwater Res.* 37: 641-646

Hibbits, J., Hughes, G. C. & Sparks, A. K. (1981). *Trichomaris invadens* gen. et sp. nov., an ascomycete parasite of the tanner crab (*Chionoecetes bairdi* Rathbun Crustacea, Brachyura.). *Can. J. Bot.* **59**: 2121-2128

Hill, A. D. (1989). The anaerobic metabolism of the common shore crab, *Carcinus maenas* (L.). PhD. Thesis, University of Glasgow.

Hill, A. D., Taylor, A. C. & Strang, R. II. C. (1991). Physiological and metabolic responses of the shore crab *Carcinus maenas* (L.) during environmental anoxia and subsequent recovery. *J. Exp. Mar. Biol. Ecol.* **150**: 31-50

Hinde, R. A. (1970). Behavioural habituation. In: Horn, G. & Hinde, R. A. (eds.) Short-term changes in neural activity and behaviour. Cambridge University Press, Cambridge. p. 3-40

Holdich, D. M. & Reeve, I. D. (1988). Functional morphology and anatomy. In: Holdich, D. M. & Lowery, R. S. (eds.) Freshwater Crayfish. Croom Helm, London. p. 11-51

Jahromi, S. S. & Atwood, II. L. (1969). Correlation of structure, speed of contraction and total tension in fast and slow abdominal muscle fibres of the lobster (*Homarus americanus*). *J. exp. Zool.* **171**: 25-38

Johnson, P. T. (1976). Bacterial infection in the blue crab, *Callinectes sapidus*: course of infection and histopathology. *J. Invertebr. Pathol.* **28**: 25-36

Johnson, P. T. (1977a). Paramoebiasis in the blue crab *Callinectes sapidus*. *J. Invertebr. Pathol.* **29**: 308-320

Johnson, P. T. (1977b). Gray crab disease. In: Sinderman, C. J. (ed.) Disease Diagnosis and Control in North American Marine Aquaculture. 1st edn. Elsevier, Amsterdam p. 122-125

Johnson, P. T. (1980). Histology of the blue crab, *Callinectes sapidus*. A model for the Decapoda. Praeger, New York

Johnson, P. T. (1983). Pathogens: viral, rickettsial, bacterial, fungal. In: Bliss, D. E. (ed.-in-chief) The Biology of Crustacea. Vol. 6. Provenzano, A. J. (ed.) Pathobiology. Academic Press, New York. p. 2-78

Johnson, P. T. (1986). Parasites of benthic amphipods: dinoflagellates (Duboscquodinida: Syndinidae). *Fish. Bull.* **84**: 605-614

Johnson, P. T. (1988). Gray crab disease. In: Sindermann, C. J. & Lightner, D. V. (eds.) Disease Diagnosis and Control in North American Marine Aquaculture. 2nd edn. Elsevier, New York.

Johnson, P. T., Stewart, J. E. & Arie, B. (1981). Histopathology of *Aerococcus viridans* var, *homari* infection (Gaffkemia) in the lobster, *Homarus americanus*, and a comparison with histological reactions to a Gram-negative species *Pseudomonas perolens*. *J. Invertebr. Pathol.* **38**: 127-148

Johnston, M. A., Elder, II. Y. & Davies, P. S. (1973). Cytology of *Carcinus* haemocytes and their function in carbohydrate metabolism. *Comp. Biochem. Physiol.* **46A**: 569-581

Kent, K. S. & Govind, C. K. (1981). Two types of tonic fibers in lobster muscle based on enzyme histochemistry. *J. exp. Zool.* **215**: 113-116

Keppler, D. & Decker, K. (1974). Glycogen determination with amylglucosidase. In: Bergemeyer, H. U. (ed.) Methods of Enzymatic Analysis. 2nd edn. Academic Press, New York. p. 1127-1131

Klutymans, J. H., Veenhof, P. R. & de Zwaan, A. (1975). Anaerobic production of volatile fatty acids in the sea mussel, *Mytilus edulis* (L.). *J. Comp. Physiol.* 104: 71-78

Knox, P. C. & Neil, D. M. (1991). The coordinated action of abdominal postural and swimmeret motor systems in relation to body tilt in the pitch plane in the Norway lobster *Nephrops norvegicus. J. exp. Biol.* 155: 605-627

Krasne, F. B. & Woodsmall, K. S. (1969). Waning of the crayfish escape response as a result of repeated stimulation. *Anim. Behav.* 17: 416-424

Lamprecht, W. & Trautschold, I. (1974). ATP, Bestimmung mit Hexokinase und Glucose-6-phosphate dehydrogenase. In: Bergmeyer, H. U. (ed.) Methoden der Enzymatischen Analyse, Bd.2. Verlag Chemie, Weinheim. p. 2151-2159.

Latrouite, D., Morizur, Y., Noël, P., Chagot, D. & Wilhelm, G. (1988). Mortalité du tourteau *Cancer pagurus* provoquée par le dinoflagelle parasite: *Hematodinium* sp. *I.C.E.S.* C.M. 1988/K:32 (mimeo)

Lee, J. S. & Pfeiffer, D. K. (1975). Microbiological characteristics of dungeness crab *Cancer magister. Appl. Microbiol.* 30: 72-78

Lewis, D. H. (1979). Serology of shrimp pathogenic vibrios. In: Lewis, D. H. & Leong, J. K. (eds.) Proceedings of the Second Biennial Crustacean Health Workshop. Sea Grant College Program, Texas A&M University, College Station, Texas. p. 133-136

Lightner, D. V. (1977). Shrimp diseases. In: Sindermann, C. J. (ed.) Disease Diagnosis and Control in North American Marine Aquaculture. 1st edn. Elsevier, Amsterdam. p. 10-77

Lightner, D. V. (1988). Diseases of cultured penaeid shrimp and prawns. In: Sindermann, C. J. & Lightner, D. V. (eds.) Disease Diagnosis and Control in North American Marine Aquaculture. 2nd edn. Elsevier, New York. p. 8-127

Lightner, D. V. & Lewis, D. H. (1975). A septicemic bacterial disease syndrome of penaeid shrimp. *Mar. Fish. Rev.* 37: 25-28

Lojda, Z., Gossrau, R. & Scheibler, T. H. (1976). Enzymhistochemische Methoden. Springer, Berlin. p. 233-259

Mabuchi, K. & Sréter, F. A. (1980). Actomyosin ATPase. II. Fiber typing by histochemical ATPase reaction. *Muscle & Nerve.* 3: 233-239

Maclean, S. A. & Ruddell, C. L. (1978). Three new crustacean hosts for the parasitic dinoflagellate *Hematodinium perezi* (Dinoflagellata: Syndinidae). *J. Parasitol.* 64: 158-160

Main, J. & Sangster, G. I. (1982). A study of separating fish from *Nephrops norvegicus* (L.) in a bottom trawl. *Scott. Fish. Rep.* 26

Main, J. & Sangster, G. I. (1985). The behaviour of the Norway lobster, *Nephrops norvegicus* (L.) during trawling. *Scott. Fish. Rep.* 34

Maier, L., Rathmayer, W. & Pette, D. (1984). pH lability of myosin ATPase activity permits discrimination of different muscle fibre types in crustaceans. *Histochemistry.* 81: 75-77

Mangum, C. (1983). Oxygen transport in the blood. In: Bliss, D. E. (ed-in-chief) The Biology of Crustacea. Vol. 5. Mantel, L. H. (ed.) Internal anatomy and physiological regulation. Academic Press, New York. p. 373-429

Mantel, L. H. & Farmer, L. L. (1981). Osmotic and ionic regulation. In: Bliss, D. E. (ed-in-chief) The Biology of Crustacea. Vol. 5. Mantel, L. H. (ed.) Internal anatomy and physiological regulation. Academic Press, New York. p. 54-161

Maynard, D. M. (1960). Circulation and heart function. In: Waterman, T. H. (ed.) The Physiology of Crustacea. Vol. 1. Metabolism and growth. Academic Press, New York. p. 161-226

Melzack, R. (1961). On the survival of mallard ducks after 'habituation' to the hawk-shaped figure. *Behaviour.* 17: 9-16

Meyers, T. R. (1990). Diseases of Crustacea - Diseases caused by protistans and metazoans. In: Kinne, O. (ed.) Diseases of Marine Animals. Vol.3. Biologische Anstalt Helgoland, Hamburg. p. 350-389

Meyers, T. R., Koeneman, T. M., Botelho, C. & Short, S. (1987). Bitter crab disease: a fatal dinoflagellate infection and marketing problem for Alaskan tanner crabs *Chionoectes bairdi*. *Dis. aquat. Org.* 3: 195-216

Meyers, T. R., Botelho, C., Eaton, W., Koeneman, T. M., Short, S. & Imamura, K. (1990). Distribution of bitter crab dinoflagellate syndrome in Southeast Alaskan tanner crabs, *Chionoectes bairdi*. *Dis. aquat. Org.* 9: 37-43

Miyan, J. A. (1984). A method for the neurophysiological study of reflexes elicited by natural statocyst stimulation in lobsters. *J. exp. Biol.* 108: 465-469

Morris, S. & Greenaway, P. (1989). Adaptations to a terrestrial existence in the robber crab, *Birgus latro* (L.), IV. L-lactate dehydrogenase function and L-lactate accumulation during exercise. *Comp. Biochem. Physiol.* 94B: 59-64

Munday, K. A. & Poat, P. C. (1971). Respiration and energy metabolism in Crustacea. In: Florkin, M. & Scheer, B. T. (eds.) Chemical Zoology. Vol. 6. Academic Press, New York. p. 191-211

Newman, M. W. (1988). Hematodinium disease of blue crabs. In: Sindermann, C. J. & Lightner, D. V. (eds.) Disease Diagnosis and Control in North American Marine Aquaculture. 2nd edn. Elsevier, New York.

Newman, M. W. & Johnson, C. A. (1975). A disease of blue crabs (*Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp. *J. Parasitol.* 61: 554-557

Newland, P. L. (1985). The control of escape behaviour in the Norway lobster, *Nephrops norvegicus* (L.). PhD. Thesis, University of Glasgow.

Newland, P. L. & Chapman, C. J. (1989). The swimming and orientation behaviour of the Norway lobster, *Nephrops norvegicus* (L.), in relation to trawling. *Fish. Res.* 8: 63-80

Newland, P. L. & Neil, D. M. (1987). Functional plasticity in a stereotyoed motor act: Redirection of the tail flick in the Norway lobster, *Nephrops norvegicus*, by asymmetrical stimuli. *Neurosci. Lett.* Suppl. 29: S124

Newland, P. L. & Neil, D. M. (1990a). The tail-flip of the Norway lobster, *Nephrops norvegicus*. I. Giant fibre activation in relation to swimming trajectories. *J. Comp. Physiol.* A166: 517-527

Newland, P. L. & Neil, D. M. (1990b). The tail-flip of the Norway lobster, *Nephrops norvegicus*. II. Dynamic righting reactions induced by body tilt. *J. Comp. Physiol.* A166: 529-536

Newland, P. L., Chapman, C. J. & Neil, D. M. (1988). Swimming performance and endurance of the Norway lobster *Nephrops norvegicus*. *Mar. Biol.* 98: 345-350

Newland, P. L., Cattaert, D., Neil, D. M. & Clarac, F. (1992a). Steering reactions as adaptive components of the tail-flip in the spiny lobster *Jasus lalandii*. (In press).

Newland, P. L., Neil, D. M. & Chapman, C. J. (1992b). Escape swimming in the Norway lobster. (In press).

Ogonowski, M. M. & Lang, F. (1979). Histochemical evidence for enzyme differences in crustacean fast and slow muscle. *J. exp. Zool.* 207: 143-151

Onnen, T. & Zebe, E. (1983). Energy metabolism in the tail muscles of the shrimp *Crangon crangon* during work and subsequent recovery. *Comp. Biochem. Physiol.* 74A: 833-838

Overstreet, R. M. (1973). Parasites of some penaeid shrimps with emphasis on reared hosts. *Aquaculture.* 2: 105-140

Parsons, D. W. & Mosse, P. R. L. (1982). The leg flexor muscle of *Carcinus*, II. Distribution of muscle fiber types. *J. exp. Zool.* 224: 169-175

Parry, G. (1960). Excretion. In: Waterman, T. H. (ed.) The Physiology of Crustacea. Vol. 1. Metabolism and Growth. Academic Press, New York. p. 341-366

Paterson, N. F. (1968). The anatomy of the Cape rock lobster, *Jasus lalandii* (H. Milne-Edwards). *Ann. S. Afr. Mus.* 51: 1-232

Pauley, G. B., Newman, M. W. & Gould, E. (1975). Serum changes in the blue crab, *Callinectes sapidus*, associated with *Paramoeba perniciosa*, the causative agent of gray crab disease. *Mar. Fish. Rev.* 37: 34-38

Phillips, B. F., Cobb, J. S. & George, R. W. (1980). General biology. In: Cobb, J. S. & Phillips, B. F. (eds.) The Biology and Management of Lobsters. Vol. 1. Academic Press, New York. p. 2-82

Phillips, J. W., McKinney, R. J. W., Hird, F. J. R. & Macmillan, D. L. (1977). Lactic acid formation in crustaceans and the liver function of the midgut gland questioned. *Comp. Biochem. Physiol.* 56B: 427-433

Pilgrim, R. L. C. & Wiersma, C. A. G. (1963). Observations on the skeleton and somatic musculature of the abdomen and thorax of *Procambarus clarkii* (Girard), with notes on the thorax of *Panulirus interruptus* (Randall) and *Astacus. J. Morph.* 113: 453-487

Rabin, H. & Hughes, J. T. (1968). Studies on host-parasite relationships in gaffkemia. *J. Invertebr. Pathol.* 10: 335-344

Raffin, J. P., Thébault, M. T. & LeGall, J. Y. (1988). Changes in phosphometabolites and intracellular pH in the tail muscle of the prawn *Palaemon serratus* as shown by *in vivo* 31P NMR. *J. Comp. Physiol.* B158: 223-228

Reichert, H. & Wine, J. J. (1983). Coordination of lateral giant and non-giant systems in crayfish escape behaviour. *J. Comp. Physiol.* A153: 3-15

Rittenburg, J. H., Gallagher, M. L., Bayer, R. C. & Leavitt, D. F. (1979). The effect of *Aerococcus viridans* (var.) *homari* on the oxygen binding capacity of hemocyanin in the American lobster (*Homarus americanus*). *Trans. Am. Fish. Soc.* 108: 172-177

Rogers, W. L., Melzack, R. & Segal, J. R. (1963). 'Tail flip response' in goldfish. *J. comp. physiol. Psychol.* 56: 917-923

Sawyer, T. K., Cox, R. & Higginbottom, M. (1970). Hemocyte values in healthy blue crabs *Callinectes sapidus* and crabs infected with the amoeba *Paramoeba perniciosa. J. Invertebr. Pathol.* 15: 440-446

Scottish Sea Fisheries Statistical Tables, 1987. (1988). Fisheries Economics and Statistics Unit, Department of Agriculture and Fisheries for Scotland, Edinburgh.

Silverman, H. & Charlton, M. P. (1980). A fast-oxidative crustacean muscle: histochemical comparison with other crustacean muscle. *J. exp. Zool.* 211: 267-273

Sindermann, C. J. (1977). (ed.) Disease diagnosis and Control in North American Marine Aquaculture. 1st edn. Elsevier, Amsterdam.

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

Slein, M. W. (1965). D-Glucose: Determination with hexokinase and glucose-6-phosphate dehydrogenase. In: Bergmeyer, H. U. (ed.) Methods of enzymatic analysis. 2nd edn. Academic Press, New York. p. 117-123

Sparks, A. K. & Hibbits, J. (1979). Black may syndrome, an invasive mycotic disease of the tanner crab, *Chionoecetes bairdi. J. Invertebr. Pathol.* 34: 184-191

Sparks, A. K. & Morado, J. F. (1985). A preliminary report on the diseases of Alaska king crabs. In: Proceedings of the International King Crab Symposium. Alaska Sea Grant College Program, University of Alaska, Fairbanks. p. 333-339

Sparks, A. K., Hibbits, J. & Fegley, J. C. (1982). Oservations on the histopathology of a systemic ciliate (*Paranophrys* sp.?) disease in the dungeness crab, *Cancer magister. J. Invertebr. Pathol.* 39: 219-228

Sparks, A. K., Morado, J. F. & Hawkes, J. W. (1985). A systemic microbial disease in the dungeness crab *Cancer magister*, caused by a *Chlamydia*-like organism. *J. Invertebr. Pathol.* 45: 204-217

Snow, D. H., Billeter, R., Mascarello, F., Carpene, E., Rowlerson, A. & Jenny, E. (1982). No classical type IIB fibres in dog skeletal muscle. *Histochemistry.* 75: 53-65

Sprague, V. (1970). Some protozoan parasites and hyperparasites in marine decapod Crustacea. In: Snieszko, S. F. (ed.) A Symposium on Diseases of Fishes and Shellfishes. American Fisheries Society, Washington, D.C. p. 416-430

Stewart, J. E. & Arie, B. (1973). Depletion of glycogen and adenosine triphosphate as major factors in the death of lobsters (*Homarus americanus*) infected with *Gaffkya homari. Can. J. Microbiol.* 19: 1103-1110

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

Stewart, J. E., Arie, B., Zwicker, B. M. & Dingle, J. R. (1969a). Gaffkemia, a bacterial disease of the lobster, *Homarus americanus*: effects of the pathogen, *Gaffkya homari*, on the physiology of the host. *Can. J. Microbiol.* 15: 925-932

Stewart, J. E., Cornick, J. W. & Zwicker, B. M. (1969b). Influence of temperature on Gaffkemia, a bacterial disease of the lobster, *Homarus americanus. J. Fish. Res. Bd. Can.* 26: 2503-2510

Talbot, P., Clark, W. H. & Lawrence, A. L. (1972). Fine structure of the midgut epithelium in the developing brown shrimp, *Penaeus aztecus. J. Morph.* 138: 467-486

Telford, M. (1968). The effects of stress on blood sugar composition of the lobster, *Homarus americanus. Can. J. Zool.* 46: 819-826

Thébault, M. T. & Rafffin, J. P. (1989). Use of 31P NMR in *in vivo* ecophysiological studies: evaluation of the energetic state in crustacean tail muscles. *Océanis* 15: 489-498

Thébault, M. T. & Raffin, J. P. (1991). Seasonal variations in *Palaemon serratus* abdominal muscle metabolism and performance during exercise, as studied by 31P NMR. *Mar. Ecol. Prog. Ser.* 74: 175-183

Thébault, M. T., Raffin, J. P. & LeGall, J. Y. (1987). *In vivo* 31P NMR in crustacean muscles: fatigue and recovery in the tail musculature from the prawn *Palaemon elegans. Biochem. Biophys. Res. Comm.* 145: 453-459

Thompson, R. F. & Spencer, W. A. (1966). Habituation: A model phenomenon for the study of neuronal substrates of behavior. *Psychol. Rev.* 173: 16-43

Trendall, J. T. & Prescott, J. (1989). Severe physiological stress associated with the annual breeding emigration of *Panulirus ornatus* in the Torres Strait. *Mar. Ecol. Prog. Ser.* 58: 29-39

Tucker, V. A. (1967). Method for oxygen content and dissociation curves on microliter blood samples. *J. Appl. Physiol.* 23: 410-414

Uglow, R. F. (1969). Haemolymph protein concentrations in portunid crabs - II. The effects of imposed fasting on *Carcinus maenas. Comp. Biochem. Physiol.* 31: 959-967

Unestam, T. (1972). On the host range and origin of the crayfish plague fungus. *Rept. Inst. Freshwater Res., Drottningholm.* 52: 192-198

Unestam, T. & Weiss, D. W. (1970). The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by a susceptible and a resistant species. *J. gen. Microbiol.* 60: 77-90

Vey, A., Boemare, N. & Vago, C. (1974). Recherches sur les maladies bacteriennes de l'ecrevisse *Austrpotamobius pallipes* Lereboullet. *Freshwater Crayfish.* 2: 287-297

Vivarès, C. P. & Cuq, J-L. (1981). Physiological and metabolic variations in *Carcinus mediterraneus* (Crustacea: Decapoda) parasitised by *Thelohania maenais* (Microspora: Microsporida): an ecophysiopathological approach. *J. Invertebr. Pathol.* 37: 38-46

Wiersma, C. A. G. (1947). Giant nerve fiber system of the crayfish. A contribution to the comparativephysiology of synapse. *J. Neurophysiol.* 10: 23-38

Wilhelm, G. & Boulo, V. (1988). Infection de l'étrille *Liocarcinus puber* (L.) par un dinoflagelle parasite de type *Hematodinium* sp. *I.C.E.S.* C.M. 1988/K:41 (mimeo)

Wine, J. J. & Krasne, F. B. (1972). The organisation of escape behaviour in the crayfish. *J. exp. Biol.* 56: 1-18

Wine, J. J. & Krasne, F. B. (1982). The cellular organisation of crayfish escape behaviour. In: Bliss, D. E. (ed.-in-chief). The Biology of Crustacea. Vol. 4. Sandeman, D. C. & Atwood, H. L. (eds.) Neural Integration and Behaviour. Academic Press, New York. p.

Wine, J. J., Krasne, F. B. & Chen, L. (1975). Habituation and inhibition of the crayfish lateral giant fibre escape response. *J. exp. Biol.* 62: 771-782

Young, J. S. & Pearce, J. B. (1975). Shell disease in crabs and lobsters from New York Bight. *Mar. Poll. Bull.* 6: 101-105

Zucker, R. S. (1972). Crayfish escape behavior and central synapses II. Physiological mechanisms underlying behavioral habituation. *J. Neurophysiol.* 35: 621-637