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Post capture physiology and pathology of the Norway lobster, *Nephrops norvegicus.*

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Abstract

This thesis describes a study into two post capture conditions, idiopathic muscle necrosis (IMN) and a post capture opportunistic bacterial infection, affecting the Norway lobster, *Nephrops norvegicus*, and a comprehensive analysis of the stressors involved in the capture and post capture periods of the fishery process.

A multivariate approach was used to study progression in the post capture condition of trawl-caught *N. norvegicus* for the live transport market. Prolonged periods of aerial exposure resulted in large disruptions to the carbohydrate profile, increases in haemolymph L-lactate and crustacean hyperglycaemic hormone concentrations, and corresponding fluctuations in haemolymph pH. These disruptions increased with the increasing temperature of aerial exposure, which impacted the immunocompetence of the lobsters: circulating haemocytes and phenoloxidase levels were significantly reduced and the degree of bacteraemia increased.

The health status of *N. norvegicus* captured during trials in spring and autumn by means of short trawl (1 h duration), long trawl (5 h duration) and creeling was assessed using a range of physiological (haemolymph L-lactate, crustacean hyperglycaemic hormone (CHH), abdominal muscle glycogen concentrations), immunological (Total haemocyte counts (THC)) and physical (mortality, damage indices) parameters. Increased duration of trawl did not significantly alter physiological parameters, while time of landing did have a significant effect on L-lactate, where animals landed in the morning exhibited higher concentrations. Seasonal variations in abdominal muscle glycogen concentrations were also apparent. Irrespective of season individuals captured by short trawls in the morning

suffered lowest mortalities. Damage assessment data revealed that a greater proportion of individuals were categorised as heavily damaged following longer trawls conducted in spring.

The carbohydrate profile and immunocompetence of *N. norvegicus* was significantly affected following trawl capture and subsequent handling and aerial exposure post capture. The recovery period was investigated through a range of parameters (L-lactate, glycogen, glucose, THC) and the data suggested that animals should be submerged and unstressed for at least 24 h following capture and handling to avoid further alterations to the carbohydrate profile and reduce the window of opportunity for invading bacteria to cause meat spoilage.

The influence of air temperature on the condition of *N. norvegicus* caught for the live export market was assessed by correlating meteorological data with the percentage of catch accepted for live transport. Results illustrated a large degree of variability in the condition of animals on arrival at the processing plant. Air temperature was the only meteorological factor that had an impact on the morbidity and mortality of the catch. In one instance in particular, mean air temperature on the day of capture had a significant negative impact on the health of the catch.

The pathology and progression of IMN was further investigated through histopathology and assessment of possible aetiologies. Trawl capture, aerial exposure and handling readily initiated IMN, however the condition was also initiated through periods of aerial exposure in the absence of trawling. Current results expand on earlier studies and reaffirm that the condition observed in *N. norvegicus* most closely resembles IMN and that no pathogenic agents in the aetiology of the condition were evident.

During the course of this project a bacterial septicaemia of *N. norvegicus* was discovered. A range of techniques including molecular (16s rRNA gene sequencing), biochemical, and morphological criteria were used to characterise bacterial isolates from necrotic animals. Results suggested a multi-species bacterial infection, with *Vibrio* spp. being the predominant isolate. Histological analysis demonstrated extensive loss of abdominal muscle structure and electron micrographs revealed rod shaped gram negative bacteria in the abdominal muscle demonstrating evidence of tissue degradation. It is believed that this is an opportunistic bacterial infection connected to the stressful conditions of the fishery process. Immuno-suppression and physical damage, which have earlier been attributed to the fishery process, may provide the window of opportunity for invading bacteria to cause meat spoilage.

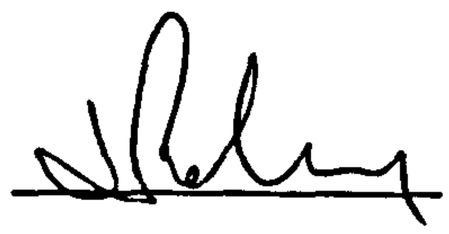
The extracellular products (ECP) secreted by two strains of gram-negative bacteria were investigated to further understand their role in the spoilage of host muscle tissue. ECP from isolate N8, a *Vibrio sp.*, demonstrated no proteolytic activity, while ECP from isolate N10, a *Pseudoalteromonas* sp., degraded a number of substrates, and proteolytic activity increased with temperature. Through the API ZYM system, both isolates demonstrated strong leucine arylamidase activity, with isolate N8 showing strong acid phosphatases activity. These enzymes have been identified as virulence factors in other bacterial species suggesting that isolate N10 is able to cause the pathological damage observed. It is likely that there is a complex pathway to the final condition, involving virulence factors of other species and the stresses involved in the fishery process.

The research undertaken in this study will help in the recognition of critical periods in the post capture period that promote poor stock condition and mortality.

Specifically an adequate period of recovery following trawl capture before re-handling and minimising aerial exposure at elevated temperatures are both critical in ensuring a high quality catch. The results of this research have been used to generate a Code of Practice for the capture, handling and transport of commercially exploited decapod crustaceans.

Candidate declaration

I declare that the work recorded in this thesis is my own, unless otherwise stated and that it is of my own composition. No part of this thesis has been submitted for any other degree.



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Chapter 1

1.0 GENERAL INTRODUCTION

This thesis describes a study into two post capture conditions, idiopathic muscle necrosis (IMN) and a post capture opportunistic bacterial infection, affecting the Norway lobster, *Nephrops norvegicus*, and a comprehensive analysis of the stressors involved in the capture and post capture periods of the fishery process.

1.1 The Norway lobster *Nephrops norvegicus*

The Norway lobster, *Nephrops norvegicus,* is a burrowing decapod belonging to the sub-phylum Crustacea, infraorder Astacidea and family Nephropidae. *Nephrops norvegicus* is found on substrates of fine soft mud to muddy sands, which are suitable for burrow construction (Figueiredo & Thomas, 1967; Chapman *et al.*, 1975). *Nephrops norvegicus* has a wide depth distribution, being found from 10 – 800m, though more often found in waters of 30 – 200m (Farmer, 1975). They demonstrate adaptability to temperature and salinity and are present in NE Atlantic waters, from Arctic waters down to the NW coast of Africa, as well as the Mediterranean (Farmer, 1975; Merella *et al.,* 1998). *N. norvegicus* is known to undertake small scale movements (metres), but no large scale migrations have been observed (kilometres) (Chapman, 1980; Chapman & Bailey, 1987).

N. norvegicus (Fig. 1.1) has two body main body parts: the cephlothorax and the abdomen and are generally pink/orange. *N. norvegicus* reacts to threatening objects by producing a series of rapid swimming movements, tail flips, which have been the subject of a number of studies (Newland & Neil, 1987; 1990; Newland *et al.*, 1988). The main function of this escape response is the avoidance of natural predators and other *N. norvegicus* (Chapman &

Rice, 1971), but similar behaviour occurs in response to fishing gear (Main & Sangster, 1985; Newland & Chapman, 1989). Normally they move by walking on their legs (pereiopods), and by paddling with their swimmerets (pleopods).

It is established that female *N. norvegicus* exhibit a seasonal pattern of moulting that varies with size. However, no seasonal pattern of moulting has been observed in males (Mouat, 2002). In the Clyde Sea Area, Bailey (1984) discovered that small females showed a peak of moulting in the early summer whereas larger females moulted later in the year, during the autumn. It was also reported that females in the size class 21-33 mm carapace length (CL) displayed peaks of moulting in both the spring and winter as females approached maturity. Sarda (1991) has also demonstrated that mature females are found, albeit in small numbers, all year round in the Catalan Sea. Mouat (2002) reported that during the September following moulting and copulation, female *N. norvegicus* generally remain in their burrows, incubating the eggs on the pleopods until the following spring when they are released as planktonic larvae. During this incubation period the females reside in their burrows and are unavailable for trawl capture, as they are less active in searching for food (Dunthorn, 1967), which leads to a strong bias in the sex ratio of the catch (Farmer, 1974; Chapman, 1980).

1.2 The *Nephrops* fishery

Scottish coastal waters support a major fishery for *N. norvegicus*. Until the 1950s they were considered to be a nuisance bycatch (O'Riordan, 1964) and were discarded in the Clyde (Bailey *et al.*, 1986) and adjacent waters (Briggs, 1997). However, since 1951 the demand for *N. norvegicus* has increased dramatically (O'Riordan, 1964) and the fishery has grown. With the decline of the whitefish catch it has become the most valuable species in the area (Bailey *et al.*, 1986; Fish Industry Yearbook, 2002). According to FAO

statistics released in February 1997, *N. norvegicus* trawling was the main activity for 18% of UK vessels over 10m in length. The total UK fishery landings in 1997 were valued at £492 million, with the *Nephrops* fishery the second most valuable at £67 million, with the Scottish fishery contributing over 76% (£48 million) (FRS, 1999). In Scotland the trawl is the most common means of capture of *N. norvegicus,* accounting for about 95% of the landings (Newland *et al.,* 1988) although a small creel fishery also exists. The animals are either tailed for 'scampi' or sold as 'whole fresh'; however there is an increasingly important market for live animals from trawl (and creel) catches. Animals suitable for live transport will generally achieve at least double the price of tailed 'scampi'.

Recent figures published in the Fish Industry Yearbook 2002 show that in the UK in 2001, 28,236 tonnes (£60,433,000) of *N. norvegicus* were caught, with 20,531 tonnes (£47,700,000) coming from the Scottish fishery. For 2000, the value of the exports was reported to be worth £69,710,000, up 42.2 % since 1999 (Fish Industry Yearbook, 2002).

Traditionally, larger animals are creel-caught and often exported live to continental Europe. However, after the continuing growth of the fishery, quotas have been reduced in recent years (EU Fisheries Council, 2000). Where lobster catches are regulated, increasing the proportion of the catch that is transported live and improving the quality of the catch are important means of adding value to the product (Taylor *et al.,* 1997). Creel capture leaves the animal in better condition; however, there is now an increasing number of vivier-transported *N. norvegicus* coming from trawl capture instead of creel capture. Therefore conditions and treatment post capture will be even more crucial in ensuring that a high quality product is delivered to market. In some parts of the UK, notably the waters of Northern Ireland, it is too deep to deploy creels and there is no other option but to export trawled animals. To ensure their sale, larger animals must be in good condition on arrival

at market. In recent times there has been increasing demand for quality seafood, which can have a guarantee of quality attributed to it. Research into the post capture treatment of *N. norvegicus* will lead to better quality meat reaching the consumers.

Following trawl capture the animals are exposed on deck while they are sorted. As the catch is always mixed, sorting may take up to 5 h (Bergmann *et al.*, 2001). The animals are then generally inserted into "Prawn tubes" (commercial transport cartons which allow the storage of a large number of lobsters, separately in close proximity) and taken to processing plants where they are further stressed during re-handling and aerial exposure.

The capture process can be expected to cause stress and exhaustion since trawl durations in the Clyde may last up to 4-5 h (Bergmann *et al.*, 2001). During this period, *N. norvegicus* is likely to endure physiological stress, as a result of increased swimming and tail flip activity in attempting to escape the trawl. Also, damage can occur in the trawl, due to crushing and abrasive action of other species (Lancaster & Fridd, 2002) and objects in the cod-end, or due to the pressure differences experienced in hauling (ICES, 1994). Ulmestrand *et al.* (1998) observed that the survival of *N. norvegicus* that escaped from trawls was significantly higher than that of animals discarded after exposure on deck, indicating the importance of the post capture period on mortalities.

The seriousness of such stress to crustaceans is increased by periods of on-deck exposure to temperature changes (Zainal *et al.*, 1992), increased light intensity (Chapman *et al.*, 2000) and air (Patterson & Spanoghe, 1997). Bergmann *et al.* (2001) reported that sorting times on Clyde Sea *Nephrops* trawlers ranged from 45 – 300 minutes. A number of studies have shown that these periods induce hypoxia (Schmitt & Uglow 1997; Taylor & Waldron, 1997). When the animal is exposed to air the support seawater provides to the gill structure is lost reducing the diffusion capacity of the gills. This causes an inadequate supply of

oxygen across the gills leading to internal hypoxia. As well as physiological damage, Zainal *et al.* (1992) reported that the squat lobster, *Munida rugosa,* became torpid after periods of aerial exposure and elevated temperatures which reduce its acceptability for the live market. It has also been shown in a number of studies that the immuno-competence of crustaceans is affected by periods of stress, making the animal more susceptible to pathogens (e.g. Snieszko, 1974; Direkbusarakom & Danayadol, 1998; Le Moullac *et al.*, 1998; Lorenzon *et al.*, 2001).

Although it is recognised that lobsters are stressed by handling, limited information is available on the post-harvest physiology of lobsters. Several regional codes of practice describe common industry handling regimes for other crustaceans (Beard & McGregor, 1991; Harvie, 1993), but some recommendations are not supported by extensive physiological research (Taylor *et al.*, 1997).

1.3 Stress indicators of marine crustaceans

Lobster health is influenced by a range of factors, of which stress is one of the most important (Evans, 1999). A stress response occurs when a regulated physiological system is extended beyond normal bounds by one or more physical factors or "stressors" acting upon it (Barton & Iwama, 1991). Physical factors, such as post capture handling, lead to short- and long-term changes in cardiovascular and respiratory function, energy metabolism, fluid and ionic imbalance, acid-base balance and immunity (Selye, 1973; Barton & Iwama, 1991; Thompson *et al.*, 1993; Paterson & Spanoghe, 1997; Hall & van Ham, 1998). A temporary disturbance is likely if the stressor is of limited duration and mild; however, if the stressor is extreme the outcome may be a reduced resistance to disease, and reduced survival (Wood *et al.*, 1983; Pickering & Pottinger, 1989; Lee & Wilkins, 1992). Stress responses may be evaluated subjectively (by observations of

behaviour, vigour, or simple postural tests), or expressed quantitatively by measured changes in physiological variables (Taylor *et al.*, 1997).

Taylor *et al.* (1997) concluded in a report on the live transport of lobsters that more comprehensive data are required on stress associated with the fishery process and on the effects of stress on the quality and survival of lobsters. These data would facilitate the estimation of recovery rates and would allow the industry to make the process changes needed to maximise the return on the fishery resource by improving meat quality and increasing survival of exported lobsters.

It is well documented that despite being unaccustomed to air, lobsters may demonstrate effective compensation for the acidosis which occurs during periods of aerial exposure provided that they are undisturbed (Taylor *et al.*, 1997). However, as Taylor *et al.* (1997) reported, the extent of such compensation varies greatly among species and with temperature. At 15°C, *Homarus gammarus* avoids major pH shifts for 14 h, whereas *Janus edwardsii* at 17°C shows similar compensation for only 4-8 h, and *Panulirus argus* for less than 1 h at 25°C. Such observations demonstrate the need for detailed data on emersion stress for each species, fishery and post capture stage. Increases in oxygen uptake during prolonged air exposure of *Janus edwardsii* (Taylor & Waldron, 1997) and other crustaceans (Taylor & Wheatly 1981, Innes *et al.*, 1986) suggest that as their gills dry out, their diffusion capacity, and thus their oxygen uptake increases markedly. If these increases in the oxygen uptake can offset the added accumulation of L-lactate from increased metabolic rates due to temperature, it may explain the apparent plateauing out of L-lactate concentrations. Tyler & Wheatly (1981) exposed *Austropotamobius pallipes* in air for 24 h (at 15°C) and observed both abdominal muscle and haemolymph L-lactate returning to concentrations observed in submerged animal after the early increases. They

discussed the fate of this L-lactate and suggested that there was no evidence for the re-oxidation of L-lactate during aerial exposure, since oxygen consumption in air is maintained at a rate similar to that measured in settled, submerged crayfish. They concluded that the L-lactate may be sequestered into tissues other than abdominal muscles and that it is redistributed after emersion. Recent research by Jackson *et al.* (2001) demonstrated L-lactate sequestration in the carapace of the crayfish, *Austropotamobius pallipes*, during exposure in air and concluded that the exoskeleton appears to provide a reserve of buffering capacity and a sink for L-lactate during anaerobic metabolism. A similar mechanism has been identified in pond turtles (Jackson, 1997).

It has been observed that lobsters do not have the ability to regulate haemolymph osmotic pressure independently of the environment (Dall, 1974), so the loss of the ability of the lobster to osmoregulate cannot be used as an indicator of stress (Paterson & Spanoghe, 1997). However, physiologically important ions in the haemolymph can be regulated, a process typically disrupted by moulting or stress. It has been demonstrated by Dall (1974) that the potassium ion concentration is closely regulated in the haemolymph of crustaceans and the calcium ion concentration changes during buffering of acidic conditions in the haemolymph and during moulting as part of the process of calcification (Mercaldo-Allen, 1991). Paradoxically, increasing or decreasing the salinity of the water in which Western rock lobsters, *Panulirus cygnus,* are held causes an elevation of the magnesium ion concentration in the haemolymph, suggesting that the role of the antennal gland in eliminating excess fluid from the body at low salinity outstrips the capacity of the gland to regulate magnesium concentration (Dall, 1974). Increases of haemolymph magnesium ion concentration occur during moulting or during periods of hypoxia in lobsters and crabs (Albert & Ellington, 1985; Mercaldo-Allen, 1991; Whiteley & Taylor, 1992).

Stressed crustaceans exhibit high gill ventilation rates (e.g. Jouve-Duhamel & Truchot, 1985), but despite this behaviour the increased oxygen demands mean that even a small reduction in oxygen concentration in the water limits the rate of oxygen uptake but many species do regulate oxygen consumption (Nimura & Inoue, 1969). Following environmentally-induced hypoxia (aerial exposure) haemolymph oxygen concentration decreases and impacts on the ability of the respiratory pigment, haemocyanin, to function (Taylor & Whiteley, 1989; Burnett, 1992; Mangum, 1992). During such periods of hypoxia the haemolymph may become colourless (losing the characteristic blue-grey of oxygenated haemocyanin) indicating that it is no longer saturated and changes in the rate of oxygen delivery to the tissues during stress will also influence the entry of other potential stress indicators such as metabolite waste, into the haemolymph (Paterson & Spanoghe, 1997).

Glucose concentration in crustacean haemolymph generally increases in response to stress (Dall, 1974; Spindler-Barth, 1976; Santos & Keller, 1993b; Hall & van Ham, 1998). It has been observed that during moulting the glucose concentration of the haemolymph fluctuates, but no generalisations can be made from the species studied so far (Telford, 1968; Lacerda & Sawaya, 1986; Chan *et al.,* 1988; Mercaldo-Allen, 1991).

In normoxic conditions, crustacean tissue will oxidise metabolic substrates completely to carbon dioxide during respiration. However during periods of hypoxia L-lactate and carbon dioxide accumulate in the blood (Johnson & Uglow, 1985; DeFur *et al.,* 1988; Whiteley *et al.,* 1990). Since Teal & Carey (1967) observed the expected correlation between the utilisation of glucosyl units and the accumulation of L-lactate ratio of 1:2, it has been concluded that the anaerobic metabolism of decapod crustaceans is almost totally reliant on the production of L-lactate through glycolysis (Bridges & Brand, 1980; Taylor &

Spicer, 1986; Hill *et al.*, 1991a). There are a few exceptions, in the isopod *Saduria entomon* and the mud shrimps *Upogebia pugettensis* and *Neotrypaea californiensis* ATP may also be produced via the metabolic pathway leading to the formation of alanine (Zebe, 1982; Hagerman & Szaniawska, 1990). Respiring crustacean tissues oxidise metabolic substrates completely to carbon dioxide when oxygen is plentiful, and incompletely to L-lactate when it is not (Albert & Ellington, 1985). The resulting acidosis must be balanced principally by buffering in the haemolymph and then following re-submersion by carbon dioxide and ionic exchanges at the gills (Wheatley & Henry, 1992). The L-lactate itself is not normally excreted (Gäde *et al.*, 1986), but is instead converted back to other metabolic intermediates and is either oxidised further to carbon dioxide or used to re-synthesize storage carbohydrates such as glycogen when sufficient oxygen is available (Gäde *et al.*, 1986; Hill *et al.*, 1991b). Recent research by Jackson *et al.* (2001) demonstrated L-lactate sequestration in the carapace of the crayfish, *Austropotamobius pallipes*, during exposure in air and concluded that the exoskeleton appears to provide a reserve of buffering capacity and a sink for L-lactate during anaerobic metabolism. A similar mechanism has been identified in pond turtles (Jackson, 1997).

The physiological status of crustaceans is regulated by various peptide, amine and steroid hormones that circulate in the haemolymph (Fingerman, 1995). Hormones, such as the crustacean hyperglycaemic hormone (CHH), have already been implicated in stress or handling reactions (Chang *et al.*, 1998). CHH is an eyestalk-derived factor, which increases the haemolymph glucose concentration in response to stress (handling and aerial exposure), though the mechanism of this response is still being elucidated (Santos & Keller, 1993b). Wilkens *et al.* (1995) have reported that other neurohormones apparently contribute to the prolonged periods of elevated heart rate and gill ventilation following handling or experimental surgery. Regnault (1987) proposed that by influencing the

dynamics of tissue perfusion and exchange of materials at the gill, these hormones may influence the rate of waste excretion and possibly even the concentrations of other haemolymph components in circulation.

The nitrogen derived from protein and amino acid catabolism is excreted primarily through the gills as ammonia in aquatic crustaceans, and variations in the extent of this excretion have been observed to vary in response to stress (handling and hypoxia) and moulting (Spaargaren *et al.*, 1982; Hagerman *et al.*, 1990; Hunter & Uglow, 1993; Chen *et al.*, 1994). When the excretion rate across the gills increases during periods of stress, the ammonia concentration of the blood decreases (Regnault & Lagardere, 1983; Regnault, 1987).

A reduction of the protein concentration in the haemolymph has been observed to occur during moulting (Magnum, 1992; Mercaldo-Allen, 1991), during emersion (Schmitt & Uglow, 1997) and during starvation (Stewart *et al.*, 1967; Dall, 1975). Elevated haemolymph volume and reduced protein concentrations were reported by Dall (1975) in recently captured western rock lobsters, *Panulirus longipes*. Chen *et al.* (1994) and Chen & Cheng (1995) suggested that protein catabolism was responsible for the reduced protein concentrations observed in *Penaeus monodon* exposed to high concentrations of ammonia and nitrate. However, an increase in haemolymph volume is possible if the haemolymph osmotic pressure falls in these cases.

Acid base regulation is broadly controlled by the haemolymph proteins and the carbonate bicarbonate system (Cameron, 1989; Wheatley & Henry, 1992; Truchot, 1994). Paterson & Spanoghe (1997) reported that the majority of the protein fraction is involved in the transport of oxygen and host defence reactions. Enzymes also occur in the haemolymph

(Najafabadi *et al.*, 1992), and the activity of one of these, acid phosphatase, has already been linked to stress related lysis in shrimp (Dillon & Fisher, 1983).

Haemocyanin, the oxygen binding molecule in crustaceans, makes up the largest fraction of the haemolymph protein (Depledge & Bjerregaard, 1989). Stress impacts on the function of haemocyanin through haemolymph factors that change in response to stress, such as pH and concentrations of inorganic ions (particularly calcium) and organic ions (e.g. L-lactate and urate) (Truchot, 1980; Morris *et al.*, 1985; Lallier & Truchot, 1989; Burnett, 1992). Haemocyanin is disabled by some stressors, such as extreme oxygen deprivation; however, compensatory synthesis of additional haemocyanin in response to hypoxia has also been reported only in response to long-term hypoxia (Hagerman & Baden, 1988; Hagerman *et al.*, 1990). More recent work by Spicer & Baden (2001) has revealed that the response of *N. norvegicus* haemolymph haemocyanin concentrations to stress is dependant upon initial haemocyanin concentrations in the individual, but there is also large inter-animal variation, which further complicates the matter.

Taylor *et al.* (1997) concluded that: "almost anything that can be measured in crustacean haemolymph may be a stress indicator of some kind". They also criticised the fact that stress in lobsters has been considered, in the past, from the relatively narrow perspective of a particular discipline or a small set of parameters. It was also suggested more progress could be made by a collaborative study combining the diverse approaches of haemolymph chemistry, tissue biochemistry and histology.

An integral part of this study was to identify which internal parameters would be suitable for detecting the initial phases of stress and to record the long-term alterations to the health status of *N. norvegicus*.

1.4 Stress and disease

Evans (1999) stated that stress is one of the most important factors in lobster health, and detailed the sequence of events that occurs in a stress response (Fig. 1.2). If the stressor is mild only temporary disturbances occur; however, if the stressor is more severe detrimental long-term effects can occur. A number of researchers have described these detrimental outcomes such as reduced growth, reduced resistance to disease and reduced survival (Pickering & Pottinger, 1989; Lee & Wilkins, 1992; Iwama *et al.,* 1997). During the capture and post capture periods of the *N. norvegicus* fishery, lobsters are exposed to both mild and extreme stressors.

1.5 Crustacean immune system

In the habitat of *Nephrops norvegicus* there is a diverse range of parasites and pathogens, against which the exoskeleton provides the first level of defence, and gives protection from physical injuries and a barrier against pathogen entry. A second level of defence is provided by the immune system.

The immune system of invertebrates evolved under selective pressure imposed by infectious microorganisms (Medzhitov & Janeway, 1997). Invertebrate animals lack true antibodies and, hence, an adaptive immune response. Although they have proteins with immunoglobulin domains (Mendoza & Faye, 1996), they have to rely solely on innate immune mechanisms (Söderhall & Cerenius, 1998). A non-self recognition system operates on the recognition of a broad range of factors present on the surfaces of microorganisms, such as lipo-polysaccharide (LPS) that is shared by all gram-negative bacteria. LPS, and other non-self substances such as peptidoglycans from bacteria or mannans from fungi, are recognised by proteins called pattern recognition proteins (PRP).

The crustacean PRPs will initiate the prophenoloxidase activating system (ProPO system), a defence system in arthropods (for reviews see Söderhall, 1982; Ratcliffe *et al.*, 1985; Söderhall *et al.*, 1985; 1994; Ashida & Yamazaki, 1990; Sritunyalucksana & Söderhall, 2000). Phenoloxidase is the enzyme involved in the melanistaion process, and is the terminal enzyme of the ProPO system. Prophenoloxidase is converted to its active form, phenoloxidase, by a proteolytic cleavage caused by an endogenous serine proteinase (Söderhall, 1992), that is under the control of inhibitors (Hergenhahn & Söderhall, 1985) (See Fig. 1.3). As this is one of the main defence reactions, several authors have suggested that prophenoloxidase activity may be a sensitive indicator of environmentally-induced immuno-suppression in marine crustaceans (Hauton *et al.*, 1997; Jussila *et al.*, 1997).

When a large number of microorganisms invade the body tissues of a crustacean they are trapped by the process of nodule formation: cells clump about the non-self bodies and form several layers of haemocytes around the infected area, phenoloxidase activity by the host then leads to the encapsulation of the infected area (Söderhall, 1982).

Host-defence proteins in crustacean haemolymph include a protein associated with coagulation and putative antibacterial agents such as bactericidans and agglutinins (Söderhall & Cerenius, 1992). Stress may leave crustaceans more vulnerable to bacterial infection (Sugita & Deguchi, 1994), and once infection occurs, this can result in disturbances in the total protein concentration, the concentrations of individual protein components in the haemolymph, and the ability of the haemolymph to clot (Johnson, 1976; Spindler-Barth, 1976; Henke, 1985; Evans, 1999).

Invertebrate blood cells may also commence cytotoxic reactions (Söderhall & Ajaxon, 1982) against non-self particles. Another defensive cell is the phagocyte. Phagocytes are

mainly fixed cells which perform clearance through specialised pinocytosis that removes foreign bodies from the haemolymph (Cooper-Willis, 1979; McCumber *et al.*, 1977).

Crustaceans have a largely 'open' circulatory system (McMahon & Burnett, 1990), and the haemolymph is the prime mediator in homeostasis. The plasma contains respiratory pigments, hormones and other complex regulatory molecules, while haemocytes function in a variety of processes for example, transport and storage of materials, pigment synthesis, clotting, wound repair, deposition of the cuticle, tanning and, importantly, protection and against microbial or parasitic infection (Bauchau & Plaquet, 1973; Bauchau, 1981; Smith & Chisholm, 1992). The haemolymph contains three types of blood cell, hyaline cells, semi-granular cells, and granular cells (Hose & Martin, 1989; Hose *et al.*, 1990). The circulating haemocytes play central roles in phagocytosis, capsule formation and killing of bacteria either intra or extra-cellularly (Smith, 1991; Chisholm & Smith, 1992; Smith & Chisholm, 1992). Certain types of haemocytes have a molecular mechanism associating several proteins. Three morphologically different types of crustacean haemocytes can be identified. The specific functional activity associated with each haemocyte type, such as prophenoloxidase activation (Lanz *et al.*, 1993; Söderhall & Cerenius, 1998), phagocytosis, encapsulation, coagulation, release of agglutinins, and synthesis of melanin, may result in different responses by these cells when various abnormal environmental factors induce stress in the western rock lobster (Jussila *et al.*, 1997). A way of evaluating sub-acute disturbance of the homeostatic integrity of crustaceans is through total haemocyte counts.

In mammalian phagocytic cells, the oxygen-dependant defence mechanism consists of the generation of reactive oxygen intermediates (ROIs) with powerful micobicidal activity (Babior, 1984). The phenomenon known as the 'respiratory burst' can be elicited, upon

suitable stimulation, by soluble components such as LPS or by particulate phagocytic stimuli such as zymosan (yeast cell wall). This stimulation leads to an increased consumption of oxygen, the reduction of which, catalysed by a membrane bound enzyme, NAD (P)H-oxidase, gives rise to superoxide (O_2^-). Starting from superoxide, a number of reactions lead to the production of hydrogen peroxide, singlet oxygen, hydoxyl radical and numerous other reactive products (Finkel & Holbrook, 2000).

Bell & Smith (1983) demonstrated the production of superoxide radicals by haemocytes, a respiratory burst, in the common shore crab, *Carcinus maenas.* They showed that hyaline cells are responsible for this activity, whereas semi-granular and granular haemocytes are involved in the production of ROIs.

The respiratory burst is a parameter encompassing several immunological activities, recognition, phagocytosis and cytotoxity. The functional capability of the haemocytes to generate a respiratory burst upon stimulation with a standard non-self stimulus can be assessed using the method of Munoz *et al.* (2000).

Through measuring immunological parameters critical periods of the fishery process responsible for the initiation of immuno-suppression may be detected. These periods affect the short-term immuno-competence of the animal and may allow pathogens to invade and damage tissue.

1.6 Crustacean diseases

Crustacean diseases can be categorised as infectious and non-infectious diseases. Non-infectious diseases are those in which no pathogen has been implicated in the aetiology. Infectious diseases are those caused by a pathogen that can be passed through horizontal (animal-to-animal or via another vector) or vertical (generation-to-generation) pathways.

1.6.1 Non-infectious diseases of crustaceans

Idiopathic muscle necrosis (IMN)

A number of studies have described muscle wastage in crustaceans but its aetiology is unknown (Lindqvist & Mikkola, 1978). Idiopathic muscle necrosis (IMN), also referred to in the literature as spontaneous muscle necrosis, muscle opacity, tail rot, or muscle necrosis, is believed to be a host-induced process. The cause of the condition is believed to be environmental or physiological stresses, such as high salinity, temperature, overcrowding, or hyperactivity, and it has been reported to occur in both the wild and in prawns subjected to aquaculture stress (Sindermann, 1977). The condition was first reported by Rigdon & Baxter (1970) in the brown shrimp, *Crangon crangon*, due to host metabolic reactions caused by temperature and hypoxic stress. Since then it has been reported in the freshwater crayfish, *Cherax tenuimanus*, in which it is thought to be due to air exposure (Evans *et al.*, 1999); in commercial penaeid shrimps where it was concluded that handling, brief exposure to air, or changes in temperature or salinity could result in the development of necrosis. Attempts to transmit the condition by feeding diseased shrimp tissue have been carried out by Venkataramaiah, (1971), with kuruma shrimp, *Penaeus japonicus*; by Sandifer *et al.* (1975) and Akiyama *et al.* (1982) with the freshwater prawn, *Macrobrachium rosenbergii*. However these attempts proved unsuccessful.

More recently, IMN was reported by Stentiford & Neil (2000) in *N. norvegicus* from the west coast of Scotland. Its occurrence appeared to be associated with the stress of the post capture process, and was implicated in heavy economic losses through mortality during the transport and storage in live export process.

The gross clinical signs of this condition are that the animal becomes lethargic and the body muscles become white. Stentiford & Neil (2000) reported that, in *N. norvegicus*, necrosis of the abdominal muscles was evident within h of capture and progressed to complete opacity of the abdominal musculature within 48 h. Electron microscopy revealed that affected tissue displayed a progressive disruption of sarcomere organisation, loss of Z-line material, condensation of myofibrils and infiltration of the necrotic lesions by granulocytes. It was also concluded during this study that there was no evidence for any pathogenic agents within the abdominal musculature, and no evidence for bacterial involvement in the observed pathology.

The precise cause of IMN has not been ascertained. Stentiford & Neil (2000) suggested that breaches in the integument might provide foci for initiation of necrosis, and its progression is exacerbated by subsequently imposed stressors. It has been reported that exertional myopathies in mammals are initiated by intensive or exhaustive activity primarily involving strong glycolytic fibres (Hulland, 1985). Extreme exertion by these strongly glycolytic muscles causes rapid utilisation of glycogen, generating heat and L-lactate, both of which induce degenerative changes in these and adjacent muscle fibres (Hulland, 1985). Stentiford & Neil (2000) suggested that repetitive tail flipping by *N. norvegicus* during capture could create these conditions in the deep abdominal flexor muscle, and the condition would be further progressed during the post capture stresses.

A finding of notable significance is that of Johnson (1974) who reported that the treatment of penaeid shrimps with the anaesthetic Quinaldine could induce spontaneous necrosis. Quinaldine has been used for many years as an anaesthetic for fish to reduce the chance of injury during handling. It was proposed for use in the shrimp industry to reduce physical damage during handling, but during trials it was observed that Quinaldine induced

hyperactivity in the shrimp, leading to necrosis. This led Johnson (1974) to suggest that the chemical should not be used for routine handling of shrimp. Incidentally, Kumlu & Yanar (1999) and Yanar & Kumlu (2001) when treating sea bass, *Dicentrarchus labrax*, with Quinaldine, reported mortalities due to excitation of the animals by the chemical.

It has also been suggested that the rapid progression of the necrosis may be linked to haemocytic infiltration of the muscle and subsequent production of superoxide radicals (Fridovich, 1978; Thronqvist & Söderhall, 1997) which cause peroxidation of membrane lipids and damage to protein molecules leading to cellular injury in muscle (Hulland, 1985). Interestingly, nutritional myopathy syndrome (NMS) of fish is known to be associated with free radicals. Fish food contains poly-unsaturated lipids, which contain the essential fatty acids for fish; however these have a tendency to auto-oxidise and to produce lipid peroxides (LPO). Free radicals (peroxyl radical, hydroxyl radical), derived from hydroperoxides of LPO, attack muscle fibres, hepatocytes, lipid cells, and all other cells (Miyazaki, 1995).

Sindermann (1977) reported that the only treatment for IMN in crustaceans is to reduce the stress-inducing environmental factors quickly when the first signs of it appear. Indeed, it has been noted that in *Penaeus aztecus* the necrosis can be reversed if the environmental stressors are removed soon after the onset of the condition (Rigdon & Baxter, 1970; Lakshmi *et al.*, 1978).

Stentiford & Neil (2000) observed IMN in all segments of the abdomen, but with a tendency for the condition to occur in the segments adjacent to the cephlothorax. A similar pattern was observed in IMN in *P. japonicus* (Momoyama & Matsuzato, 1987) and *Cherax tenuimanus* (Evans *et al.*, 1999), while in *P. aztecus* (Rigdon & Baxter, 1970; Lakshmi *et*

al., 1978) and in *M. rosenbergii* (Akiyama *et al.*, 1982; Nash *et al.*, 1987) it was associated with the more distal segments of the abdomen.

Interestingly, the loss of Z-line material, a prominent feature of IMN in *N. norvegicus* (Stentiford & Neil, 2000), represents an early step in pre-moult atrophy in crustaceans (Mykles & Skinner, 1990a). In the chelae of the Bermuda land crab (*Gecarcinus lateralis*) approximately 30-60% of the muscle is lost during proecdysis. Atrophy facilitates the withdrawal at ecdysis of the very large muscle mass contained in the propodus through the narrow aperture of the old exoskeleton, and the trigger for this is likely to be hormonal (Skinner, 1985). Studies of land crab chela muscle have indicated the importance of calcium-dependent proteases (CDPs), localised in the sarcoplasm, in mediating muscle protein degradation (Mykles, 1990). Mykles & Skinner (1990b) reported that CDPs degrade myofilaments directly and are also involved in the removal of myofilaments from myofibrils prior to degradation (Mykles, 1990). Mykles & Skinner (1990b) also concluded that compounds that elevate intracellular Ca^{2+} concentrations, such as caffeine, stimulate proteolysis. Interestingly, the Ca^{2+} ion concentration changes during buffering of acidosis (Mercaldo-Allen, 1991). Stentiford & Neil (2000) stated that it is conceivable that IMN in *N. norvegicus* involves activation of these proteases, leading to the initial breakdown of Z-line material.

1.6.2 Bacterial infections of crustaceans

There has been considerable debate about the sterility of crustacean haemolymph. Bang (1970), Lee & Pfeiffer (1975) and Johnson (1976) reported that bacteria are not present in the haemolymph of healthy crustaceans. However, this is refuted by Collwell *et al.* (1975) and by Lightner (1977) who reported the isolation of bacteria from the haemolymph of apparently healthy crustaceans, indicating that the haemolymph is not sterile environment

and that the host immune system is able to control low level bacterial infection (Brock & Lightner, 1990).

The common bacteria recovered from disease outbreaks in Crustacea have been identified as mainly gram-negative *Vibrio* spp. (Lightner & Lewis, 1975), but other bacterial groups have been reported. One of the most widespread and economically damaging pathological conditions in marine organisms is bacterial haemorrhagic septicaemia, in which a multiplicity of bacterial agents have been reported as causal agents (Conroy, 1984). Of these, Vibriosis and Furunculosis are the most typical examples.

In the past decade there has been increased interest in the *Vibrionaceae,* and the taxonomy of the genera and species has been improved. In particular, *Vibrio* has been distinguished from other genera such as *Aeromonas, Photobacterium* and *Plesiomonas* (Montero, 1998).

A typical example of a *Vibrio* outbreak is that associated with the "bolitas" syndrome in the Ecuadorian shrimp farms (Morales, 1992). The clinical signs were anorexia and absence of food in the intestinal tract. Light microscopy revealed desquamation of epithelial cells of the hepatopancreas and intestine. The sloughed epithelial cells of hepatopancreas tubules give the appearance of small balls or "bolitas". Contractile movement of the gut ceased and death occurred shortly after (Morales, 1992; Zherdmant, 1996). *Vibrio harveyi* was found in all diseased animals but it remains to be proven that it was the causative agent and not an invader of already damaged animals. The way in which *V. harveyi* causes diseases is largely unknown. Many reports suggest that *Vibrio* spp. are opportunistic rather than primary pathogens (Song *et al.,* 1993; Chou *et al.,* 1995). Many cultured shrimp species have suffered outbreaks of Vibriosis (*Penaeus monodon, P. merguiensis, P. japonicus*) and these have been attributed to stressful conditions often associated with poor water quality and aquaculture management (Lightner, 1996).

Vibrio splendidus was reported to be associated with summer mortalities of juvenile oysters (*Crassostrea gigas*) in the Bay of Morlaix (Brittany, France). Though preliminary studies could isolate only non-pathogenic opportunistic bacteria, successive studies challenging stressed animals, as indicated by the noradrenaline concentration of the haemolymph, with *V. splendidus,* induced similar symptoms to those observed in naturally infected oysters. Along with findings that *V. splendidus* was present in diseased oysters and scarce or absent in healthy oysters, this suggests that *V. splendidus* is a probable causative agent of the observed mortalities (Lacoste *et al.,* 2001).

Vibriosis has also been reported in the American lobster, *Homarus americanus* (Bowser *et al.,* 1981), and the blue crab, *Callinectes sapidus,* (Johnson, 1976). Diggles *et al.* (2000) also reported luminous vibriosis in phyllosoma larvae of the rock lobster (*Janus verreauxi*) reared in an experimental culture facility. The evidence again suggested that *Vibrio harveyi* was the causative agent. To date, with the exception of an experimental infection with *V. harveyi* (Montero, 1998), there have been no reports of bacterial infections in *N. norvegicus.*

Diagnosis is usually carried out on a number of levels. Lightner & Lewis (1975) summarised the clinical symptoms of vibriosis. The first recognisable sign is a gradual change in the appearance of the abdominal musculature, from colourless to opaque. Melanisation of the gill filaments and ventrolateral edges of the carapace and cuticular lesions develop in some individuals. Expansion of integumental erythrophores produces a reddening of the pleopods of moribund or recently-dead shrimp. Moribund animals also exhibit a characteristic dorsal flexion of the abdomen. As disease progresses, swimming is retarded and disorientated, and near-moribund animals become lethargic and rest on the bottom, often upright (Sparks, 1985). Histological observations demonstrate significant

necrosis and inflammation of the internal organs and often the presence of rod shaped bacteria (usually slightly curved) within the tissues (Lightner, 1996). Most pathogenic *Vibrio* spp. from the tissue or haemolymph of affected animals will grow on the medium Thiosulphate Citrate Bile Salts Agar (TCBS) (Lightner, 1983). Detailed procedures and culture characteristics of several species of *Vibrio* have been described by Lightner (1996).

Vibrio spp. are pathogenic bacteria capable of overcoming the host immune system and then multiplying in the body fluids and internal organs. Once a virulent strain of bacterium has gained access into the host tissue, then disease and death are normally imminent. Virulance factors, such as cytolytic and haemolytic toxins, have been described for *V. damsela* (Kreger, 1984; Kreger *et al.*, 1987; Toranzo *et al.*, 1993).

Sparks (1985) reported that bactereamias of penaeid shrimp have been attributed to bacteria from the genus *Vibrio;* however there is evidence that an *Aeromonas* spp. may also be implicated. Cheng & Chen (1998) also reported a bacterial necrosis in *M. rosenbergii* associated with poor culture facilities, and identified a gram-positive *Enterococcus*-like bacterium as the causative pathogen.

'Red tail' (gaffkaemia) is a fatal disease of the lobster (*Homarus americanus*) caused by the bacterium *Aerococcus viridans*. A lobster will contract the disease only if there is a wound break in the shell through which bacteria can pass, since these bacteria lack exoenzymes, and thus intrinsic invasive powers (Stewart, 1975). Problems with the disease are magnified in the post capture process of the fishery. Outbreaks of the infection have occurred in European waters in recent years (Alderman, 1996). As the disease progresses, a reduction in the number of circulating haemocytes occurs (Johnson *et al.*, 1971), while the bacterium is resistant to destruction within the haemocyte phagosomes, possibly due to its acidic polysaccharide capsule (Johnson *et al.*, 1981).

In addition to internal infections, bacteria have also been associated with shell disease in Crustacea. Chitonolytic shell disease, 'rust disease', or 'black spot' has been reported in crustaceans inhabiting environmental conditions such as aquaculture systems (Delves-Broughton & Poupard, 1976; Prince *et al.*, 1993) or polluted locations (Young & Pearce, 1975; Sawyer, 1991). The disease commences with the loss of the epicuticle layer of the crustacean exoskeleton, and may occur by proteolytic microbial action, predatory or cannibalistic attack, chemical attack, or the abrasive action of the sediment and/or articulated body parts (Vogan *et al.*, 2000). Following the exposure of the chitin-containing procuticle, shell degradation is attributed to chitinolytic bacteria. Getchell (1989) stated that bacteria belonging to the genera *Vibrio, Aeromonas, Pseudomonas, Alteromonas, Flavobacterium* have all been reported as probable agents involved in the disease syndrome.

1.6.3 Fungal diseases of crustaceans

Fungal infections are commonly associated with shell disease, often in conjunction with bacteria-induced lesions. The fungus *Psorospermium* has been implicated in the mortalities of cultured freshwater crayfish in Australia and Europe. However, its pathogenicity is debatable as concomitant infections are usual in such cases (Edgerton *et al.*, 1995). Black matt disease (BMD) caused by the ascomycete, *Trichomaris invadens,* has been identified in the tanner crab, *Chionoecetes bairdi* (Sparks, 1982). There have been numerous reports of fungi infecting crustaceans for example, *Lagenidium callinectes* infecting the blue crab *Callinectes sapidus* (Sindermann, 1990), *Cancer magister* larvae (Armstrong *et al.*, 1976) and on the eggs of the shrimp *Palaemon macrodactylus* (Fisher, 1983), but probably the most important is the European crayfish 'plague'. This condition is caused by the phymocete, *Aphanomyces astaci*, and was first reported as infecting *Astacus astacus* in

1865, and has since spread rapidly throughout European populations, also infecting *A. leptodactylus* and *Austropotamobius pallipes* (Johnson, 1983).

1.6.4 Viral diseases of crustaceans

The first viral disease of Crustacea was reported in the swimming crab *Liocarcinus depurator* by Vago (1996). Since then over 30 species of viruses have been identified in wild and culture conditions (Brock & Lightner, 1990). In the penaeid shrimp industry, the viral diseases *Baculovirus penaei* (BP), infectious haematopoietic necrosis virus (IHHV) and more recently Taura syndrome have had large economic impacts. Viral diseases have also been reported in the blue crab, *Callinectes sapidus*; in members of the genera *Carcinus* and *Paralithodes* (Johnson, 1983; Sindermann,1990). The first report of a viral disease was in the freshwater crayfish, *Cherax quadricarinatus* (Anderson & Prior, 1992), since then other non-occluded baculoviruses have been discovered in freshwater crayfish *Pacifastacus leniusculus* in the US (Hendrick *et al.,* 1995), and in *Astacus astacus* in Finland (Edgerton *et al.,* 1996). The first report of a lobster being the host for a viral disease was in the Caribbean Spiny lobster, *Panulirus argus,* by Behringer *et al.* (2001) who identified a herpes-like DNA virus. The presence of a virus does not necessarily mean disease. Many viruses remain dormant for much of the crustacean life cycle, however disease often manifests during stressful periods, such as the fishery process.

Microsporidian infections are the most common and most pathogenic diseases of crustaceans (Sindermann, 1971). More than 140 species have been described and parasitize virtually all Orders of the Crustacea. Microsporidian-infected prawns are known as "cotton shrimp" or "chalky white" prawns. These diseases are highly pathogenic and cause epizootics in wild crustacean populations (Iverson & Manning, 1959; Overstreet, 1973; Lightner & Lewis, 1975; Sindermann, 1990; Ramasamy *et al.,* 2000). Ramasamy *et al.*

(2000) reported cotton shrimp disease of *Penaeus indicus*, and observed that infected prawns appeared sluggish, partially paralysed and gradually debilitated, mainly as a result of the muscle degeneration. Ultrastructural examination reveals that during a microsporidian infection the muscle fibres slowly degenerate, to be eventually replaced by spores. However, the mechanism of muscle fibre degeneration and replacement by microsporidians remains unknown. Generally, the survival rates of infected animals are poor and, moreover, these animals are discarded resulting in economic losses.

1.6.5 Other conditions affecting *Nephrops norvegicus*

There have been few studies into pathological conditions associated with *Nephrops norvegicus,* and most of this research has centred on the parasitic dinoflagellate, *Hematodinium*.

In the late 1980s there was an increasing number of reports from fishermen and food processors of moribund animals, with an opaque musculature, milky haemolymph and dull orange colouration of the carapace and appendages. Further research confirmed that the non-motile dinoflagellate parasite *Hematodinium* spp. was the causative pathogen (Field *et al.,* 1992), further research identified it as *Hematodinium perezi*. The parasite demonstrates a seasonal pattern of abundance with peak numbers of infected animals occurring between March and May (Field *et al.,* 1992; Appleton *et al.,* 1997; Field *et al.,* 1998). In the Clyde Sea area, the proportion of the catch infected was observed to be as high as 80% (Field *et al.,* 1992), with females exhibiting a greater prevalence of infection (Field *et al.,* 1992; Stentiford *et al.*, 2001a).

Nephrops norvegicus infected with *Hematodinium* exhibit a reduced swimming performance, and a large alteration of the carbohydrate profile, thought to be induced by

parasitic cells depleting haemolymph glucose reserves (Stentiford *et al.*, 2000a; Stentiford *et al.*, 2001b). Histologically, the infection causes alterations in the sarcolemmal structure and disruption of myofibrillar bundles (Stentiford *et al.*, 2000b). The severity of the infection can be identified using the pleopod method, in which the pleopod is examined under low power microscopy, and assigned to one of 5 categories (Field & Appleton, 1995). A more sensitive diagnostic method has been recently been developed utilising an enzyme linked immunosorbent assay (ELISA) (Small *et al.*, 2002).

Anderson & Conroy (1968) reported the infestation of *N. norvegicus* larvae by ciliated *Zoothamnium* spp. in which the body surface becomes completely covered by the sessile protozoan, and death was thought to have resulted from trauma and interference with respiration. Recently, Small (2004) reported the infection of *N. norvegicus* by a parasitic ciliate belonging to the genus *Mesanophrys* which caused extensive damage to the heart tissue in affected lobsters.

Cunningham (1887) observed the trematode (*Stichocotyle nephropis*) in the hind-gut of *N. norvegicus*. The gregarine, *Porospora nephopis,* has also been reported from the gut of *N. norvegicus* (Léger & Duboseq, 1915). There is also a report of the epibiotic polycheate, *Histriobdella homari,* loosely attached to the pleopod setae of an adult female *N. norvegicus* (Briggs *et al.*, 1997).

1.7 Aims and objectives of the project

The current study has used a multivariate approach in the progression of the post capture condition of trawl-caught *N. norvegicus* destined for the live continental market. Physiological, endocrinological, immunological, microbiological and pathological measures of condition were used. It is hoped that data of this kind will help to identify critical periods in the post capture period that promote poor stock condition and mortality. Such data may be used to generate an internationally accepted Code of Practice for the capture, handling and transport of commercially exploited decapod crustaceans.

In this chapter the stress impacting on the physiological and immunological health status of a crustacean such as *N. norvegicus* has been discussed. During the fishery process *N. norvegicus* are subjected to a variety of stressors. It is the intention of this study to describe the impact of these stressors on the overall health status of the animal, describe the conditions associated with disturbances of physiological and immunological health of *N. norvegicus,* and provide guidance to the industry on how to minimize the degree of stress experienced by the animals during the fishery process.

For the reasons discussed above, the following specific aims were formulated:

- To investigate the effect of prolonged aerial exposure at different temperatures on the physiology, immunology, pathology and mortality of *N. norvegicus* (Chapter 2).
- To investigate the degree of stress, damage and mortality caused to *N. norvegicus* through trawls of different durations (Chapter 3).
- To identify the period of time necessary for the recovery of *N. norvegicus* from the dual stressors of the capture and post capture processes (Chapter 4).

- To investigate the affect of meteorological factors on the morbidity and mortality observed from industrial records (Chapter 5).
- To progress the understanding of idiopathic muscle necrosis (IMN) in *N. norvegicus*; pathology, progression and possible aetiology (Chapter 6).
- To discover characterise and undertake histological analysis of a post capture opportunistic bacterial infection of *N. norvegicus* (Chapter 7).
- To study further the opportunistic bacterial infection, possible routes of infection and proteolytic enzymes produced by bacteria isolated from necrotic *N. norvegicus* (Chapter 8).

Figure 1.1: The Norway Lobster *Nephrops norvegicus* (Scale bar 2cm) (from Huxley, 1879).

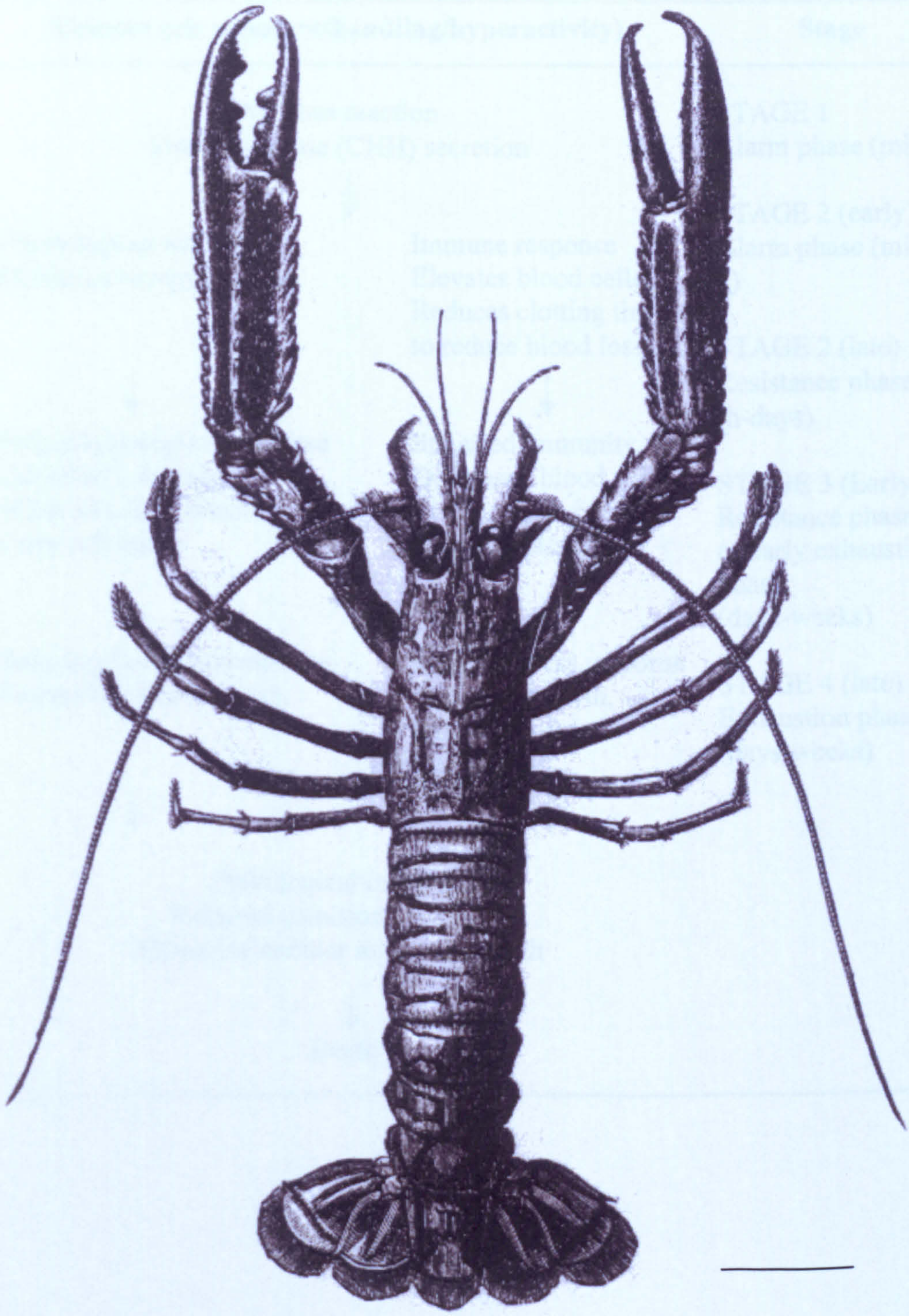


Figure 1.2: Classification of the stress responses in the lobster that may occur during the capture and post capture processes (from Evans, 1999).

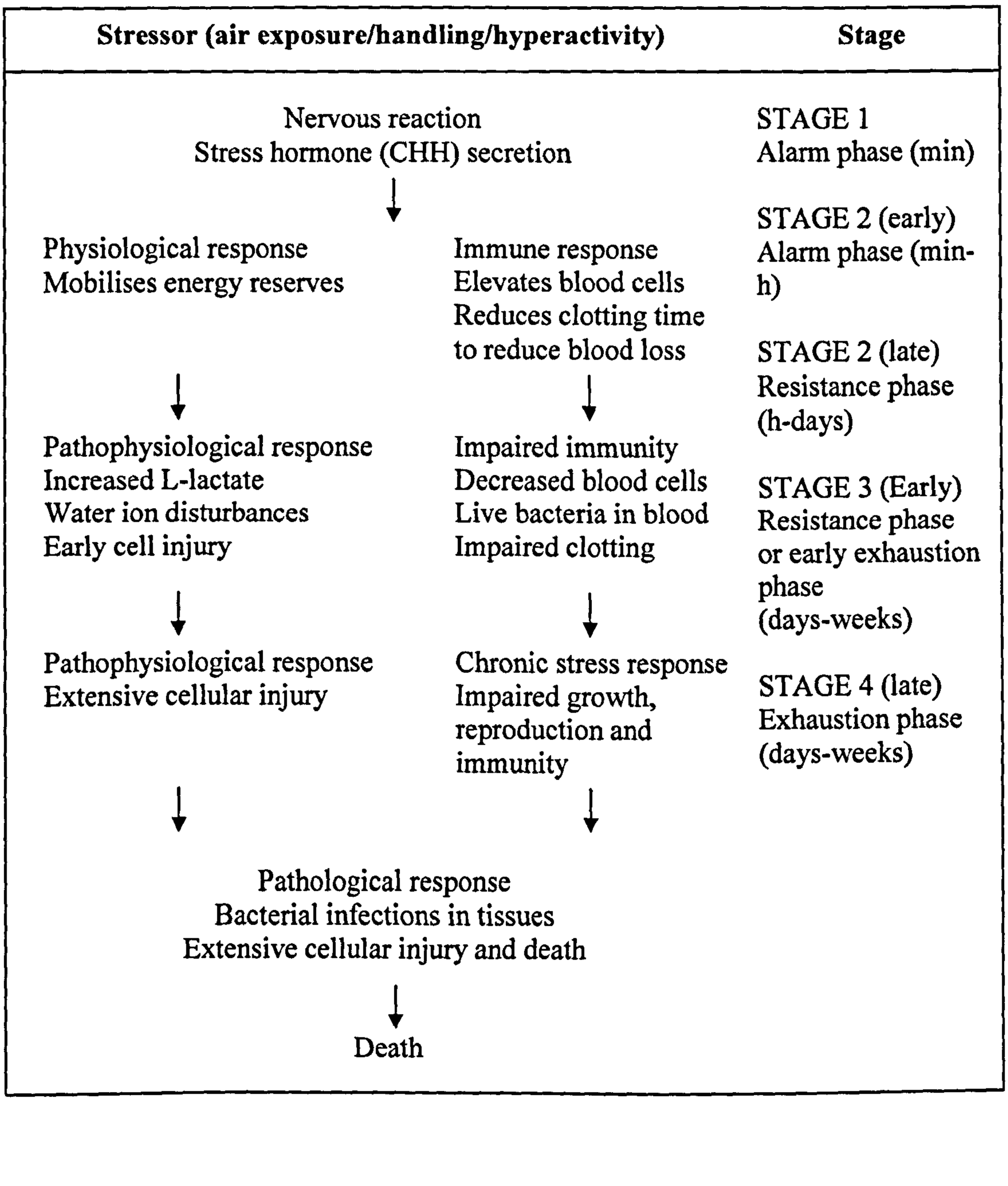
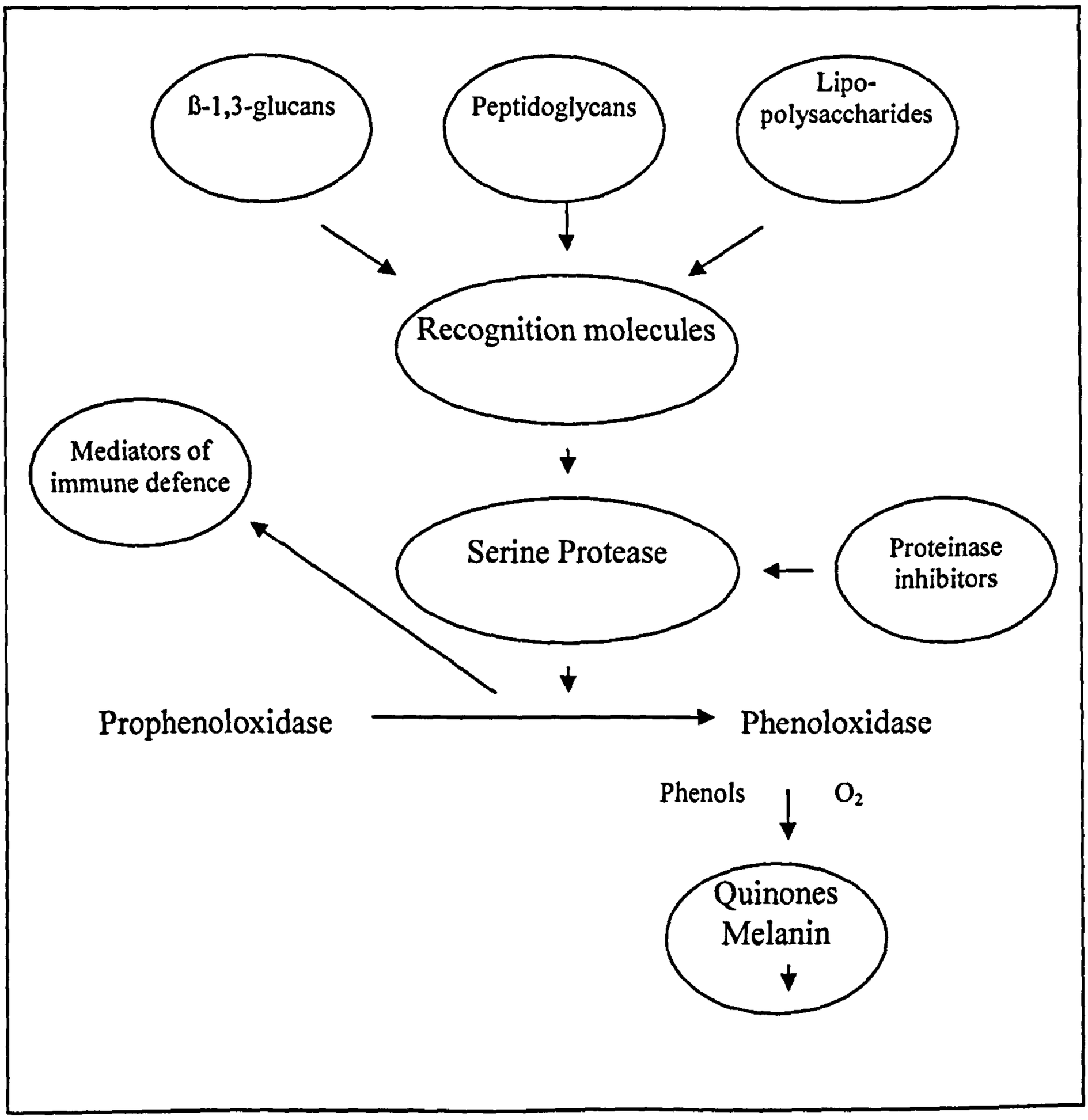


Figure 1.3: A scheme for the prophenoloxidase activation in arthropods (from Söderhall & Cerenius, 1998).



Chapter 2

Effects of the temperature of aerial exposure on the physiology, immunology and histopathology of *Nephrops norvegicus* (L.)

2.1 INTRODUCTION

During the capture and post capture period *Nephrops norvegicus* are exposed to air at several stages, sometimes for prolonged periods, up to 280 minutes (Bergmann *et al.*, 2001), and the seriousness of such trauma is increased by temperature changes during aerial exposure (Spicer *et al.*, 1990, *N. norvegicus*; Zainal *et al.*, 1992, *Munida rugosa, M. sarsi*). Such stresses make the animal too weak to enter the live export process. Failure of all or part of the integrated homeostatic response may lead to increasing physiological disturbance and ultimately death (Morris & Airriess, 1988).

Paterson & Spanoghe (1997) concluded that stress in rock lobsters and other commercially important decapods has been considered in the past from the relatively narrow perspective of a particular discipline or small set of parameters. They suggest that more progress could be made by a combined approach using the diverse approaches of haemolymph chemistry, tissue biochemistry and histology.

This approach has been taken in the present study, and has been further extended by including measures of microbiological and immunological parameters. These act as indicators of the stress experienced by *N. norvegicus* during aerial exposure, and of the effect air temperature have on the overall health status of the animal. This work can then be serve as a template for a model study of the assessment of health status of decapod crustaceans.

Taylor *et al.* (1997) reported the ability of lobsters to compensate for acidosis experienced during aerial exposure, although the extent of such compensation varied greatly among species and with temperature. They then identified the need for detailed data on emersion stress for each species, fishery and post capture stage.

There are several regional codes of practice that describe common industry handling regimes (Beard & McGregor, 1991; Estrella 2002; Harvie, 1993), but Taylor *et al.* (1997) suggested that some of the recommendations are not supported by extensive physiological research. It is hoped that this more comprehensive data set will allow the industry to make the process changes needed to minimise the mortalities of live *N. norvegicus* exported.

Several studies have examined the effect of aerial exposure on the carbohydrate dynamics of crustaceans. To summarise, as the animal becomes progressively more hypoxic, haemolymph L-lactate levels rise. This triggers the release of the crustacean hyperglycaemic hormone which stimulates glycogenolysis thus mobilising glucose reserves from intracellular glycogen reserves (Santos & Keller, 1993b). The liberated glucose either moves to the extracellular fraction or is converted to L-lactate via glycolysis (Santos & Keller, 1993a).

Tyler-Jones & Taylor (1988); Hagerman *et al.* (1990); Spicer *et al.* (1990); Paterson *et al.* (1997) and Schmitt & Uglow (1997) have all documented a rise in L-lactate concentration during emersion in *N. norvegicus* and other decapod crustaceans. This results in a decrease in the pH of the haemolymph, which becomes more acidic (Tyler-Jones & Taylor, 1988). Taylor & Whitely (1989); Spicer *et al.* (1990), Hall & Van Ham (1998) and Santos & Keller (1993a) have studied variations in haemolymph glucose following aerial exposure, and Telford (1968) and Paterson *et al.* (1997) following capture. CHH variations with aerial exposure have been studied in a number of crustaceans (Webster, 1996 (*Cancer*

pagurus); Chang *et al.,* 1998 (*Homarus americanus*)) but no research has been done on the effect of prolonged exposure (>4 h) of *N. norvegicus* to air.

A workshop report by Taylor *et al.* (1997) suggested that observations of high haemolymph L-lactate or low pH are not necessarily indicative of a lobster in poor condition, but may merely indicate that it is temporarily challenged, highlighting the importance of measuring a wide range of parameters in assessing stress.

A number of studies have looked at the effect of aerial exposure and subsequent hypoxia on the immune status of decapod crustaceans. Le Moullac *et al.* (1998), Gomez-Jimenez *et al.* (2000), Mikulski *et al.* (2000), Fotedar *et al.* (2001), and Cheng *et al.* (2002) all concluded that air exposure or subsequent hypoxia had a significant adverse effect on the immune system and hence on the health status of lobsters. Several authors have suggested that haematological parameters such as total haemocyte counts, phenoloxidase activity and bacteraemia might represent sensitive indicators of immuno-suppression in marine decapods, such as *N. norvegicus* (Smith & Johnson, 1992; Hauton *et al.,* 1995; Jussila *et al.,* 1997). However the effects of aerial exposure on the immuno capability of *N. norvegicus* have been virtually untouched.

Histopathological analysis aims to indicate the fate of the haemocytes, following a stressful period. Cornick & Stewart (1968) concluded that there is an increased phagocytosis and sequestration in the tissues during exposure to stress, though Lorenzon *et al.* (2001) suggested that it may be due to cell lysis, or degranulation, or to a generalised stress response as proposed by Smith & Johnson (1992) and Smith *et al.* (1995). Martin *et al.* (1998) reported that in the presence of bacteria the haemocytes of *Homarus americanus* rapidly formed nodules that move through the individual eventually being trapped in the gills.

Many previous studies have restricted their attention to one particular discipline. The aim of this chapter is to investigate the effect of prolonged aerial exposure at different temperatures on the physiology, immunology, pathology and mortality of *N. norvegicus*. Through measuring haemolymph pH and L-lactate concentrations the severity of internal hypoxia experienced by the individual can be assessed. The analysing of Haemolymph CHH and glucose concentrations, in addition to abdominal muscle glycogen concentrations, will provide information on the mobilisation of carbohydrate reserves and the stress response in *N. norvegicus*. Immunological parameters (haemocyte counts, prophenoloxidase activity, bacteraemia) will provide an assessment of the immune competence of the animal, if the stress of aerial exposure results in immuno-suppression, then associated pathology may be observed, such as increase in haemolymph bacteria concentrations. The objective of the Histological analysis is to provide suggestions of the fate of the haemocytes following the expected reduction in THC following prolonged aerial exposure.

2.2 MATERIALS AND METHODS

Collection and treatment of animals

Norway lobsters, *Nephrops norvegicus,* were obtained by an otter trawl (70 mm mesh size) from the Fairlie Channel, north of the Isle of Cumbrae in the Clyde Sea area, Scotland, UK (55.48° N, 04.56° W). Lobsters were transferred to the University of Glasgow in medium sized "Prawn-tubes" (commercial export cartons allowing the transportation and storage of a large number of *N. norvegicus* separately in a confined area) where they were maintained in aquaria (100 litre tanks with available shelter, 10°C, salinity 33ppt) for two weeks to allow the animals to recover from the stress effects of the capture and post capture period (see chapter 4 for information regarding the recovery period). Only male *N. norvegicus*

(size range of 30 – 40 mm carapace length) were used for the experiments. All the animals were in the intermoult stage as defined by Aiken (1980).

2.2.1 Experimental design

This investigation was carried out through a number of exposure trials, as not all variables could be measured at one time. In the first trial glucose, L-lactate, pH, bacterial concentration within the haemolymph, total haemocyte counts, and prophenoloxidase were measured. Four groups of 20 animals were exposed to air at each of 3 different temperature regimes (10°C, 15°C and 25°C) for up to 12 h. These exposures occurred in temperature-controlled aquaria at the University of Glasgow. At each of the time intervals (0, 4, 8, and 12 h), a group of 20 animals was sampled by removing haemolymph from the sinus behind the 5th pereiopod using a 1ml syringe and 25 gauge needle. The haemolymph samples were subdivided prior to analysis. After removal of haemolymph all animals were sacrificed, and in none of these experiments was sequential sampling carried out as too much haemolymph was removed for the individuals to survive the sampling procedure.

A second exposure trial using the same conditions was carried out to measure muscle glycogen concentrations.

For measurement of the crustacean hyperglycaemic hormone (CHH) in the haemolymph, a pilot trial was initially carried out using 12 groups of 3 animals per group. Following 0, 4, 8, and 12 h exposure to air at 10°C, 15°C, and 25°C, haemolymph was removed from the animals for CHH analysis. The haemolymph samples were frozen in liquid nitrogen, freeze-dried and dispatched to Bodego Bay Marine laboratory (Professor E. Chang) for the determination of the CHH titre using ELISA.

The CHH trial confirmed that the CHH titre of *N. norvegicus* was detectable and measured at an expected volume using HPLC-purified CHH from the crayfish *Orconectes limosus* (Kegel *et al.*, 1991) as previously done by Stentiford *et al.* (2000). The CHH titre increased with the duration of exposure, and this increase was greater the higher the temperature of aerial exposure.

Once it was established that this trial yielded measurable CHH titres, a larger trial was performed in which 15 groups of 12 animals per group were exposed to air for durations of 0, 1, 2, 4, and 10 h at 10°C, 15°C, and 25°C. Haemolymph samples were taken from all these animals (see above), and each was divided (50 µl for L-lactate analysis, 200 µl for CHH analysis) in order to allow L-lactate concentrations as well as CHH titres to be assayed. The haemolymph samples were frozen in liquid nitrogen, and stored at -80°C prior to analysis.

A final exposure trial was carried to provide tissue samples for histopathological analysis. For this trial the animals were exposed for up to 4 h at 25°C. 3 animals were sampled hourly and fixed for later analysis.

2.2.2 Determination of haemolymph metabolites

Glucose

Following extraction haemolymph (75 µl) was immediately mixed with an equal volume of ice cold 0.6 M perchloric acid (PCA) to deproteinize the sample, and frozen at – 20°C for later analysis. After thawing the solution was neutralised by the drop-wise addition of potassium bicarbonate (2 M) (the pH was checked using full range indicator paper). Haemolymph glucose concentration was measured using a glucose assay kit (Sigma GAGO-20). 150 µl of the sample was incubated for exactly 30 minutes at 37 °C with 300

µl of the glucose oxidase / peroxidase reagent. The reaction was stopped by the addition of 300 µl 12 N sulphuric acid. The absorbance of each sample was read at 540 nm on a spectrophotometer (Shimadzu, UV Mini 1240). The glucose concentration was calculated using a calibration curve produced at the same time using standards of known concentrations (see Bergmeyer & Brent, 1974; Southgate, 1976; Washko & Rice, 1961).

L-lactate

Following extraction haemolymph (50 µl) was immediately mixed with an equal volume of ice cold 0.6 M PCA to deproteinize the sample, and frozen at – 20°C for later analysis. To determine the concentrations of L-lactate, 50µl of the thawed sample was added to 50µl of NAD, 1000 µl of hydrazine hydrate buffer and 5µl of L-lactate dehydrogenase (LDH), and incubated for 2 h at 37°C. The absorbance of the sample was then measured at 340 nm on a spectrophotometer (Shimadzu, UV Mini 1240) and converted to L-lactate concentrations using a calibration curve constructed with standards of known L-lactate (lithium salt) concentrations (See Gutmann & Whalefeld, 1974; Engel & Jones, 1978; Hill, 1989).

Muscle glycogen

To obtain a sample of the deep abdominal flexor muscle (DAF), the muscle of the second abdominal segment was excised whole, and frozen at -20°C. The frozen muscle samples were then freeze-dried for 24 h and then ground using a pestle and mortar. For the determination of glycogen, 20 mg of the ground muscle sample was added to 400µl of 30% KOH, which was then boiled for 10 minutes. 700µl of absolute ethanol was added, and the sample was placed on ice for two h. The samples were then centrifuged at 14k rpm for 10 minutes, and the supernatant was discarded. Afterwards, 1ml of double distilled H_2O was added, the sample was sonicated, and then a 50µl volume was incubated at 95-

100°C for 10 minutes with 1ml of anthrone reagent. The absorbance of the sample was measured on a spectrophotometer at 600nm and converted to total glycogen (as glucose) concentrations (see Carroll *et al.*, 1956). The anthrone method measures total carbohydrate concentrations in the muscle, and as glycogen is the dominant carbohydrate, is used as an approximate measure of glycogen in terms of glucosyl units.

CHH

Chang *et al.* (1998) described the production of an antibody against the purified CHH-A from *H. americanus*, and previous studies have shown that *N. norvegicus* CHH titres can be detected and quantified using this antibody (Stentiford *et al.* 2001b). Haemolymph was freeze-dried immediately after a sample had been removed for concurrent L-lactate analysis. The freeze-dried samples were resuspended to their original volume with double distilled H_20 and assayed for CHH-A using the ELISA method of Chang *et al.* (1998). The standards were HPLC-purified CHH from the crayfish *Orconectes limosus* (Kegel *et al.*, 1991). Since purified *N. norvegicus* CHH was not available, it was not possible to quantify absolute concentrations of *N. norvegicus* CHH. Therefore the data are presented as *O. limosus* equivalents, which allow relative quantification.

pH

Fresh haemolymph was transferred into an microcentrifuge tube (Eppendorf) and the pH was measured immediately at ambient temperature using a precalibrated microelectrode (Thermo Russell) and pH meter (Corning ion Analyser 255).

2.2.3 Immunological measures

Total haemocyte counts (THC)

Following withdrawal, the haemolymph was immediately mixed (1:3 ratio) with 10% neutral buffered formalin (NBF) (100 ml formalin (40% aqueous solution of formaldehyde), 4g sodium dihydrogen orthophosphate (monohydrate), 6.5g disodium hydrogen orthophosphate (anhydrous), 900 ml distilled water). Counting of haemocytes was performed within 2 weeks of sampling (sequential measures from the same sample having established that values stayed constant over this period). Before the counting process, the solution was vortex-mixed to bring the cells into suspension and to stop coagulation. Total haemocyte counts were made using a haemocytometer (improved Neaubauer counting chamber) using standard procedures (Baker *et al.* 1966).

Prophenoloxidase

Untreated haemolymph samples (100 µl) were taken and added to an equal volume of ice cold CAC buffer (0.1 M sodium cacodylate; 0.45 M NaCl, 10 mM $CaCl_2.6H_2O$; 26 mM $MgCl_2.2H_2O$; pH 7.0). Cell integrity was maintained and clotting was prevented by diluting the haemolymph immediately after sampling with ice cold marine anticoagulant (0.1 M glucose; 30 mM trisodium citrate; 26 mM citric acid; 0.45 M NaCl; 10 mM EDTA; pH 4.6). Phenoloxidase activity in the whole haemolymph was estimated spectrophotomically at 490 nm using L-DOPA as substrate and trypsin as elicitor. A 200 µl volume of each sample was incubated with 0.1 % trypsin in CAC buffer at 20°C. After 30 minutes, 200 µl of L-DOPA (3 $mg.ml^{-1}$) was added, and prior to absorbance measurements, each reaction mixture was diluted with 600 µl of CAC buffer. Parallel tubes, in which CAC buffer was substituted for trypsin, were run as controls. Absorbance

measurements were made against a blank consisting of CAC buffer, L-DOPA and elicitor to control for spontaneous oxidation of the substrate alone. Enzyme activity for all assays was expressed in units that represented the change in absorbance per minute. For the whole haemolymph, enzyme activities were calculated as relative phenoloxidase activities, i.e. units per ml. This method was adapted from that of Smith & Söderhall (1991).

Bacterial counts

50 µl of haemolymph was removed and mixed immediately with 450 µl of sterile seawater. 100 µl of this solution was then plated out onto marine agar plates (DIFCO™ Marine Agar 2216). Each sample was duplicated. The remaining solution was diluted 10 fold and a further 100 µl of the solution was plated out in duplicate. These plates were then incubated at 20°C for 72 h. Colonies were counted by eye and the results expressed as colony forming units per ml of haemolymph ($cfu.ml^{-1}$).

2.2.4 Histology

Nephrops norvegicus were fixed whole using Davidson's seawater fixative (330 ml ethyl alcohol, 220 ml 100% formalin, 115 ml glacial acetic acid, and 335 ml distilled water (Mahoney, 1973)). Approximately 5 ml of the fixative was injected directly into the haemolymph to ensure rapid euthanasia and tissue fixation. Once injected, specimens were placed into the 250ml of the same fixative for 24 h (See Hopwood 1996; Bell & Lightner, 1988), before being transferred to 70% industrial methylated spirit (IMS) for storage and transportation. The various tissues (abdominal and claw muscle, gut, hepatopancreas, heart, gonad) were dissected from fixed specimens, dehydrated in ethanol and embedded in paraffin wax at the CEFAS Laboratory, Weymouth. Sections were cut (3-5 µm thickness) and stained with haemotoxylin and Eosin (H&E), and assessed using a Nikon Eclipse E800

microscope fitted with a DXM 2000 digital camera and the LuciaG image capture software (all Nikon, UK).

2.2.5 Mortality

A separate set of experiments was carried out to investigate the impact of the duration of aerial exposure at the three different temperatures on the mortality of *N. norvegicus*. After 0 (control) 4, 8 and 12 h aerial exposure at 10, 15 and 25°C, groups of 20 lobsters were returned to the aquarium tanks (water temperature 8°C) and their status was monitored over the following 5 days. Mortality was expressed as a percentage.

Data analysis

As long as the relevant criteria were met, the data were analysed using ANOVA or T tests, and further comparisons between the treatment means were made using Tukey's or Fisher's pairwise tests. Non-parametric data were analysed using the Kruskal and Wallis One way ANOVA test, with further pairwise comparisons being made using the Mann-Whitney test.

2.3 RESULTS

2.3.1 Mortality

The mortality data (Fig. 2.1) show a clear effect of temperature and the duration of aerial exposure on the mortality of *N. norvegicus*. At all three experimental temperatures, mortality rates increased as the duration of aerial exposure increased. This increase in mortality was more pronounced as the experimental temperature was increased.

At 10°C, no deaths were observed until the animals had been exposed for 8 h, and even after 12 h the mortality rate was only 20%. A similar mortality rate was observed at 15°C following short aerial exposures (≤4 h), but was far greater than at 10°C after more prolonged periods of aerial exposure (8 and 12 h). At 25°C, 80% of the *N. norvegicus* died following aerial exposure for 4 h and this number increased to 100% after 8 h, with very few animals surviving until the 8 hour point. Hence no samples were taken after 8 h at 25°C. Overall, the data demonstrate a large difference in the mortality rates of those animals exposed at 25°C, compared to those exposed at 10 and 15°C.

2.3.2 Haemolymph pH values

Aerial exposure of *Nephrops norvegicus* at all three experimental temperatures resulted in a significant decrease in the pH of the haemolymph as the duration of the exposure increased (see Table 2.1). At 10°C, the reduction in pH was most pronounced during the first 4 h of exposure, reaching values significantly lower than those of control animals maintained in water at the same temperature. However, as the duration of the period of exposure increased, the changes in pH were not significant (ANOVA F value 8.23, $p<0.001$, d.f. 68). A pronounced reduction in the pH of the haemolymph was observed when *N. norvegicus* were exposed for 4 h at 15°C but, again, any further reduction in pH was not significant (F value 37.33, $p<0.001$, d.f. 71).

The greatest decrease in the pH of the haemolymph was recorded in animals exposed at 25°C. After 4 h exposure, the pH had decreased significantly (t value 24.26, $p<0.001$, d.f. 30) to a mean value of 6.57 ± 0.22 compared with a mean of 7.90 ± 0.05 in the control animals. Clearly, these animals were highly stressed since no *N. norvegicus* survived aerial exposure until the 8 hour sampling point at this temperature.

2.3.3 Carbohydrate profile results

Haemolymph L-lactate concentrations

Aerial exposure of *Nephrops norvegicus* resulted in a progressive increase in the mean concentration of L-lactate in the haemolymph (Fig. 2.2), with the increase being greater in animals exposed to air at higher temperatures. At 10°C, the increase in L-lactate concentration was most pronounced following the first 4 h exposure. The L-lactate concentration increased steadily during the 12 h period of exposure, although the increase from 4 h to 8 h exposure was not significant (F value 18.11, $p<0.001$, d.f. 65). When *N. norvegicus* were exposed to air at 15°C, the increase in L-lactate concentration of the haemolymph was greater than at 10°C. The greatest increase in haemolymph L-lactate concentrations occurred at 25°C. After 4 h exposure, the L-lactate concentrations had increased significantly (t value = 20.27, $p <0.001$, d.f. 16) from 1.05 $mmol.l^{-1}$ in control animals to 16.8 $mmol.l^{-1}$.

Haemolymph glucose concentrations

The changes in the glucose concentration of the haemolymph during aerial exposure differed according to the temperature at which they were exposed (Fig. 2.3). The large inter-quartile variations indicated in Figure 3 reflect the large variation in haemolymph glucose concentrations between individual animals. At 10°C, median haemolymph glucose concentrations after prolonged periods of aerial exposure did not differ significantly from those observed in control animals (0.797 $mmol.l^{-1}$) (Kruskal-Wallis H value, 0.22, $p = 0.974$). A similar pattern was observed at 15°C for the first 8 h, although a significant decrease occurred after 12 h aerial exposure (H value 18.67, $p <0.001$), when haemolymph glucose concentrations decreased to a median of 0.186 $mmol.l^{-1}$, compared to 0.907

mmol.l^{-1} in control animals. At 25°C there was a significant reduction in glucose concentrations after only 4 h to a value of 0.347 mmol.l^{-1} from a median of 0.952 mmol.l^{-1} in control animals (Mann Whitney W value 391.0, $p < 0.001$).

Abdominal muscle glycogen concentrations

The concentration of glycogen in the abdominal muscle (Fig. 2.4) decreased sharply after 4 h exposure at each temperature, with the reduction in glycogen concentration being similar at each temperature. The mean abdominal muscle glycogen concentration decreased from 10.6 mg.g^{-1} in control animals to values 3.5 mg.g^{-1} after 8 h exposure and 1.8 mg.g^{-1} after 12 h exposure to air. The glycogen concentrations observed at all exposure durations were significantly different from the values obtained for control animals (F value 18.88, $p < 0.001$, d.f. 95), regardless of the temperature of aerial exposure. There were, however, no further significant reductions in abdominal muscle glycogen concentrations between 4 and 12 h.

Haemolymph CHH concentrations

In control animals the mean CHH concentration in the haemolymph was 3.1 fmol.ml^{-1}. The response of the haemolymph CHH concentrations to aerial exposure varied both with the temperature and with the duration of aerial exposure (Fig. 2.5). At all three experimental temperatures there was a steady increase in haemolymph CHH titre following the initial exposure (<2 h), by which time a significant increase from the control values had occurred.

At 10°C, there was an initial significant increase in CHH concentration after 2 h exposure (Kruskal-Wallis H value 28.36, $p < 0.001$) and, interestingly, CHH concentrations following this sampling period were not statistically different from those observed in control animals.

At the higher temperatures (15°C and 25°C), there was a progressive increase in CHH concentration as the duration of aerial exposure increased. This increase was more pronounced at higher temperatures. At 25°C, the mean CHH titre in the haemolymph increased to a peak of 421 fmol.ml^{-1} after 4 h exposure. However, the increases between each sampling period were not always statistically significant.

Haemolymph L-lactate concentrations taken concurrently with CHH

This second set of data for L-lactate was obtained because the exposure experiments to determine changes in CHH concentrations in the haemolymph of *N. norvegicus* were carried out at a different time of the year and using different exposure durations (Fig. 2.6). It was thought that obtaining a second set of data for L-lactate concurrently with the CHH data would allow this data set to be compared with the data from the previous larger exposure trials. This second set of exposure trials was required because sampling limitations meant that insufficient haemolymph could be withdrawn from a lobster to enable all measurements to be carried out at the same time. In addition, the collaboration with Professor Chang was not established until after the first exposure experiments had been completed.

The data obtained from the second experimental trial of the effect of prolonged aerial exposure on L-lactate concentrations closely matched those of the first trial. Again there was a progressive increase in mean haemolymph L-lactate concentration as the duration of aerial exposure was increased, and the increase was of a greater magnitude at the higher the temperatures (Fig. 2.6).

At 10 °C, L-lactate concentrations increased significantly (F value 15.63, $p < 0.001$) from a mean value of 0.55 mmol.l^{-1} for control animals, up to 11.14 mmol.l^{-1} after 10 h exposure.

Only between 2 h exposure and 4 h were these differences not significantly different from the values obtained for other exposure durations. After 4 h exposure at 10°C the mean L-lactate concentration was less than half that measured in *N. norvegicus* exposed to air at 15°C and 25°C for the same duration. At 15°C and 25°C significant increases occurred (F value 66.95, $p < 0.001$, d.f. 59 and F value 65.89 $p < 0.001$, d.f. 47 respectively) in mean L-lactate concentrations after each sampling period, though as already stated these differences were greater at 25°C.

2.3.3 Immunological results

Total haemocyte count

Total haemocyte count (THC), expressed as million haemocytes.ml^{-1}, showed a progressive decrease as the duration of exposure increased. This decrease was greater the higher the temperature of exposure (Fig. 2.7).

At 10°C, although there was a progressive decrease in the mean total haemocyte count (ANOVA F value 7.68, $p < 0.001$, d.f. 66), the THCs were not significantly reduced compared to control animals until after 8 h exposure, at which time they had decreased from 21.4 million haemocytes.ml^{-1} to 15.2 million haemocytes.ml^{-1}, respectively. After 12 h, no further significant reductions were observed. A similar pattern was observed at 15°C. However, in this instance a significant reduction in THC was observed after only 4 h (F value 7.25, $p < 0.001$) though, again, there were no further significant reductions after prolonged exposure.

The greatest decrease in THC was observed in animals exposed at 25°C. After 4 h exposure, the THC had decreased significantly (T value 21.24, $p < 0.001$) to a mean value

of 7.18 million haemocytes.ml^{-1}, compared with a mean of 21.24 million haemocytes.ml^{-1} in the control animals.

Haemolymph prophenoloxidase activity.

Prophenoloxidase activity was measured as the absorbance change per minute per millilitre of haemolymph. High variability in the prophenoloxidase activity of the haemolymph was observed among control groups (Fig. 2.8), although in preliminary experiments indicated prophenoloxidase activity determined in replicate blood samples from the same individual showed little variation. In general, the prophenoloxidase activity of the blood decreased with prolonged aerial exposure, and the extent of this decrease was greater at higher temperatures.

At 10°C and 15°C there was a significant reduction in prophenoloxidase activity (Kruskal-Wallis H value 12.32, p = 0.006 and ANOVA F value 3.65, p = 0.018, d.f. 59 respectively), although at both experimental temperatures significant reductions were not observed until after 8 h exposure. No further significant reductions were observed in the prophenoloxidase activity of the haemolymph when the duration of aerial exposure extended beyond 8h. In fact, after 12 h exposure at 15°C the observed prophenoloxidase activity was not significantly lower than that measured in control animals.

At 25 °C the picture is clearer, with a large significant reduction (F 25.04, p <0.001, d.f. 31) in prophenoloxidase activity from 0.31 Absorbance $\Delta.min^{-1}.ml^{-1}$ in control animals maintained in water to 0.13 Absorbance $\Delta.min^{-1}.ml^{-1}$ in the haemolymph of *N. norvegicus* exposed for 4 h.

Haemolymph bacterial concentrations

The bacterial concentrations in *N. norvegicus* subjected to prolonged exposure to air at 10°C did not vary significantly (Kruskal-Wallis H value 4.65, p = 0.200) over the 12 h period of aerial exposure (Fig. 2.9). Throughout the exposure period at 10°C, the bacterial concentrations of the haemolymph did not rise above 10^3 cfu.ml^{-1}.

At 15°C a significant increase in the bacterial concentration of the haemolymph was observed after 4 h aerial exposure, however no further significant increases (Kruskal-Wallis H value 20.86, p <0.001) were observed as the duration of the period of aerial exposure increased. After more prolonged periods of aerial exposure there was far greater variation in the bacterial numbers in the haemolymph of individual *N. norvegicus* (Fig, 8).

The data from the 25°C exposure period showed a significant increase in bacterial concentration of the haemolymph as the duration of aerial exposure increased (Kruskal-Wallis H value 18.05, p <0.001). Bacterial concentrations increased from a median of 725 cfu.ml^{-1} in control animals to 8600 cfu.ml^{-1} after 4 h exposure. Bacteria were also counted in the haemolymph in *N. norvegicus* exposed to air for 8 h at 25 °C. All animals in this group were dead at the time of sampling; therefore haemolymph counts (median value of 143200 cfu.ml^{-1}) were taken as an indication of muscle spoilage. The increase after each time period was significant.

2.5.4 Histology

The results of the histological analysis of the tissues of exposed animals provide answers as to the disappearance of haemocytes from the haemolymph. In Figure 2.10, taken from *N. norvegicus* exposed for 1 h at 25°C, the hepatopancreas appears to have few

haemocytes filling the haemal spaces between the tubules. There are some phagocytic cells lining the blood vessels, which form part of the host's defence system. After 3 h exposure, the haemocytes appear to have now aggregated into the tissues, and are filling the inter tubule spaces (Fig. 2.11). It should be noted that the difference in appearance between the hepatopancreas tubules in Figures 2.10 and 2.11 is due to differences in the cell structure in that particular area of the tubules.

Further infiltration of haemocytes into the hepatopancreas was observed in animals exposed for 4 h (Fig. 2.12), where haemocytes could be observed densely filling the inter-tubular spaces.

Apart from infiltrating the tissues, haemocytes were also observed to form nodules, possibly a reaction to the increase in bacterial numbers in the haemolymph. Figure 2.13 illustrates such an encapsulation response; the cells on the outside of the nodule are just beginning to flatten out to provide a barrier in an attempt to encapsulate a zone of foreign material, possibly bacteria.

A reduction in the number of haemocytes was observed as the temperature of aerial exposure increased. The histological studies have indicated that the haemocytes are lost due to two factors viewed in this study, the infiltration and aggregation in the tissues, and nodule formation.

Bacterial rods can be clearly seen in the area of the hepatopancreas (Fig. 2.14), and the tail muscle (Fig. 2.15) of an *N. norvegicus* exposed to air for 8 h at 25°C, indicating an adverse affect of aerial exposure on meat quality. In both figures, a loss of muscle and tubule structure indicated the possible damage done by the bacteria, however it should also be

noted that digestive enzymes released from the hepatopancreas after death will aid the breakdown of tissues.

2.4 DISCUSSION

2.4.1 Carbohydrate dynamics profile

The carbohydrate profile of *Nephrops norvegicus* is greatly altered by aerial exposure. The results have illustrated how the temperature of this exposure significantly affects the physiology of the animal.

There was a decrease in haemolymph pH levels with increasing aerial exposure; this decrease was more pronounced the higher the aerial temperature. Vermeer (1987) and Taylor & Whiteley (1989) both reported acidosis in decapods due to emersion. Sub-tidal crustaceans are not able to extract oxygen efficiently from air. DeFur & McMahon (1978) report that in *Cancer productus* gas exchange rate is reduced 5 fold in air, and Thomas (1954) reported that *Homarus vulgaris* only extracts one-seventh as much oxygen when in air. Vermeer (1987) reported that in the spiny lobster, *Panulirus argus*, gill bailers continued to beat in air, but the loss of fluid support caused the gill filaments to collapse. The rise in L-lactate, which accumulates in quantities sufficient to overwhelm the protein and bicarbonate-carbonic acid haemolymph buffering, as well as the rise in CO_2 which accumulates in the blood during emersion (Taylor & Whiteley, 1989), are believed to be the main causes of acidosis during emersion according to Taylor & Wheatly (1980). Tyler-Jones & Taylor (1988) calculated that after 3 h emersion approximately 75% of the internal acidosis was due to the accumulation of metabolic acid in the crayfish, *Austropotambius pallipes*. Observations by Bergmann *et al.* (2001) studying emersion and

subsequent re-immersion of *Liocarcinus depurator* led them also to conclude that the low pH was a result of metabolic acidosis.

A high or low pH can disrupt enzymatic reactions, ionic osmoregulatory control and cell membrane stability (Prosser, 1973). Jonas *et al.* (1962) found a close correlation between blood pH and mortality in trout. The pH was lowered with acids and death resulted when the pH of the blood dropped from 7.5 to 6.8. Fatalities did not occur if the same quantities of acids did not reduce the pH of the blood to 6.8. This indicated that it was the acidosis not the acids themselves causing the mortality.

It should also be noted that with some *N. norvegicus* experiencing very high haemolymph L-lactate levels, the pH levels remained about 7.8, suggesting that *N. norvegicus* may be able to compensate for this increase in L-lactate. Burnett (1988) suggests that compensation involves the calcium carbonate in the shell, which dissociates and the bicarbonate neutralises the acidosis, this in turn results in an increase in haemolymph calcium concentrations. Compensatory mechanisms may also include branchial water stores which accumulate a titratable base. Burnett suggested that alkalization of the branchial water maintains a steeper PCO_2 gradient across the gill and reduces the magnitude of the acidosis for a short period. DeFur & McMahon (1984) reported that the degree of compensation was greater in smaller *Cancer productus*. This may be due to the increased ability of the small crabs to aspirate water into their branchial chambers (DeFur & McMahon, 1984). In the limited observations made during this investigation, it was noted that it was the larger *N. norvegicus* that appeared to be compensating better. Further work should concentrate on the ability of *N. norvegicus* to regulate their haemolymph pH, possibly by measuring changes in bicarbonate levels, and the effect of size on this regulation.

When *N. norvegicus* are exposed to air they rapidly become progressively hypoxic, and in crustaceans it is believed the only major end product of anaerobic respiration is L-lactate (Teal & Carey, 1967; Phillips *et al.,* 1977; Gade, 1984; Albert & Ellington, 1985, Hill *et al.,* 1991a). Therefore an increase in haemolymph L-lactate concentration is indicative of anaerobic metabolism, suggesting that *N. norvegicus* cannot maintain an adequate supply of oxygen to the tissues when exposed to air. This requires the lobster to rely, at least partially, on anaerobic metabolism to meet its energy requirements during this period (Spicer *et al.* 1990).

There have been few studies that have examined the effects of long term aerial exposure on L-lactate concentrations. Spicer *et al.* (1990) exposed *N. norvegicus* to air for 18 h at 10°C and on ice. After 12 h exposure at 10°C they recorded similar increases to those observed in the present study, with haemolymph L-lactate rising to nearly 11 mmol.l^{-1}. Surprisingly, they observed no difference in the concentrations of L-lactate in the haemolymph of *N. norvegicus* kept on ice and at 10°C. They concluded that despite lower temperatures and thus lower metabolic rates, lobsters kept on ice may be more reliant on anaerobic metabolism to meet their energy demands, at least in the first few h.

In the present study L-lactate concentrations in the haemolymph of *N. norvegicus* exposed to air for 4 h at 25°C were more than double those observed in animals exposed for the same duration at 10°C and 15°C.

It is well established that the metabolic rate of the animal increases with the temperature of aerial exposure, increasing the energy demands and leading to greater accumulations of L-lactate. At the lower temperatures the increase in L-lactate accumulation is far slower, and also appears to plateau out after 8-12 h. This indicates that the animal is managing to perform partial aerobic metabolism as the gills dry out. Increases in oxygen uptake during

prolonged air exposure of *Janus edwardsii* (Taylor & Waldron, 1997) and other crustaceans (Taylor & Wheatly 1981, Innes *et al.*, 1986) suggest that as their gills dry out, their diffusion capacity, and thus their oxygen uptake increases markedly. If these increases in the oxygen uptake can offset the added accumulation of L-lactate from increased metabolic rates due to temperature, it may explain the apparent plateauing out of L-lactate concentrations. Tyler & Wheatly (1981) exposed *Austropotamobius pallipes* in air for 24 h (at 15°C) and observed both abdominal muscle and haemolymph L-lactate returning to concentrations observed in submerged animal after the early increases. They discussed the fate of this L-lactate and suggested that there was no evidence for the re-oxidation of L-lactate during aerial exposure, since oxygen consumption in air is maintained at a rate similar to that measured in settled, submerged crayfish. They concluded that the L-lactate may be sequestered into tissues other than abdominal muscles and that it is redistributed after emersion. It was also observed that L-lactate concentrations rose following immersion and that the oxygen consumption increased also. Recent research by Jackson *et al.* (2001) demonstrated L-lactate sequestration in the carapace of the crayfish, *Austropotamobius pallipes*, during exposure in air and concluded that the exoskeleton appears to provide a reserve of buffering capacity and a sink for L-lactate during anaerobic metabolism. A similar mechanism has been identified in pond turtles (Jackson, 1997).

Interestingly, the CHH data obtained during the 10°C aerial exposure trial show that CHH levels in the haemolymph actually fall again after prolonged exposure, while the corresponding L-lactate levels remain high, suggesting that the animal is resuming aerobic respiration after long periods of exposure, as the gills dry out. If CHH clearance rates occur faster than those for L-lactate the observed results can be explained. For this to be answered further work is required on the clearance rates of CHH from *N. norvegicus*

haemolymph following the return to aerobic metabolism. It is known that crustaceans do not have efficient systems for metabolising L-lactate, so its removal from the haemolymph is relatively protracted (Ellington, 1983; Bridges & Brand, 1980) possibly taking up to 24 h (chapter 4). It would be expected that as a hormone, it is not beneficial for the animal for CHH to remain active in the haemolymph for long periods, suggesting the need for rapid clearance.

At 15°C and 25°C CHH increased with the duration of exposure to air, and this increase was more pronounced at the higher temperature, closely mirroring the results from the L-lactate analysis. CHH is composed of 8- to 9-KDa neuropeptides produced in the X organ which can be selectively released from the sinus gland into the haemolymph (Santos & Keller, 1993a), after which it targets the hepatopancreatic plasma membranes (Kummer & Keller, 1993) the abdominal musculature (Santos & Keller, 1993b) and the haemocytes (Santos & Stefanello, 1991). At these locations it liberates glucose from glycogen stores through glycogenolysis. The hyperglycaemic hormone is released into the blood through exocytosis from the sinus gland axon terminals, and regulates blood sugar levels to meet the physiologically required metabolic energy needs during periods of hypoxia induced through aerial exposure. Since the metabolic rate increases with the temperature of aerial exposure this need is greater and so more hormone is released.

The increase in CHH in response to a thermal stress, as well as to aerial exposure was also observed by Chang *et al.* (1998), and Chung & Webster (1996). It was suggested by Chang *et al.* (1998) that this was due to the increased metabolic rate at higher temperatures. Few studies have assessed the response of CHH levels in *N. norvegicus* to stress. Stentiford *et al.* (2001b) reported mean haemolymph CHH concentrations as high as 106.6 $fmol.ml^{-1}$ in *N. norvegicus* heavily infected with the dinoflagellate parasite, *Hematodinium,* whereas the

peak recorded in the present study for *N. norvegicus* exposed for 4 h at 25 °C was far higher (421 fmol.ml^{-1}).

It has been widely reported that the glucose concentration in the haemolymph rises in response to stressors such as emersion, handling and disease (Dall, 1974; Spindler-Barth, 1976; Santos & Keller, 1993a; Hall & Van Ham, 1998). Hagerman *et al. (*1990), Spicer *et al.* (1990) and Stentiford *et al.* (2001b) all reported increases in haemolymph glucose concentrations in *N. norvegicus* following stress. The plasma CHH titre increases during emersion and subsequent hypoxia, and this leads to elevated plasma glucose levels (*Orconectes limosus,* Santos & Keller, 1993a; *Cancer pagurus,* Webster 1996; *Homarus americanus,* Chang *et al.* 1998). However in the present study with *N. norvegicus* a different response was observed. At 10°C, haemolymph glucose concentrations did not change significantly from those recorded in control animals, and at higher temperatures there was a general decrease in haemolymph glucose levels, which was more pronounced at 25°C. It is possible that an increase in haemolymph glucose levels during the initial exposure period could be masked due to the large intra-specific variation in the concentrations of circulating blood sugars in *Homarus americanus* found by Telford (1968) and in *N. norvegicus* by Hagerman *et al.* (1990), though these variations were not observed in other studies (Spicer *et al.,* 1990). After exposing *Carcinus maenas* to air, Johnson & Uglow (1985) observed a small hyperglycaemic response, which they attributed to the considerable intra-specific variation of glucose levels.

A reduction in haemolymph glucose concentrations following prolonged exposure to air was observed in *N. norvegicus* held on ice by Spicer *et al.* (1990), but only in lobsters exposed for 48 h. At higher temperatures, the metabolic rate will be elevated, leading to a more rapid depletion of glycogen concentrations, and an earlier cessation of glucose

release. Glucose is mainly re-supplied to the plasma via tissue-based glycogenolysis, and the main storage tissues for such polysaccharides in crustaceans are the hepatopancreas (Dall & Moriarty, 1983), the muscle (Schwoch, 1972), and the haemocytes (Johnson *et al.*, 1971). Once the tissue reserves have become exhausted the animal will be unable to re-supply glucose to the haemolymph and maintain carbohydrate homeostasis. CHH mobilises glucose from intracellular glycogen stores via glycogenolysis (Santos & Keller, 1993a), and under the anaerobic conditions associated with emersion, the liberated glucose could be converted intracellularly to L-lactate via glycolysis (Santos & Keller, 1993b), and not liberated to move out of the intracellular fraction into the haemolymph.

Abdominal muscle glycogen concentrations in *N. norvegicus* show a general decrease during aerial exposure, although there no clear effect of temperature was observed. The concentrations in control lobsters recorded during the present study are similar to those reported by Stentiford *et al.* (2001b), though large intraspecific variations exist. Baden *et al.* (1994) also reported a significant reduction in abdominal tail muscle glycogen in *N. norvegicus* exposed to environmental hypoxia, and it was noted that there was again high inter-individual variability.

To summarise, carbohydrate profiles are altered by the temperature and duration of aerial exposure. The high mortalities observed after exposure at high temperatures (25°C) may be a result of internal acidosis. The increase in L-lactate, and the associated proton, is too great and too rapid for the animal to compensate. In fish, it is the fall in blood pH that has been implicated as the cause of death after extensive stressing (Jonas *et al.*, 1962; Caillouet, 1968). Lowered blood pH adversely affects the activity of many of the tissues systems, causing loss of equilibrium and death (Caillouet, 1968). Severe depletion of the

carbohydrate profile of *N. norvegicus* during high temperature aerial exposure represents a form of physiological stress that the animal cannot recover from.

Since *N. norvegicus* is a commercially important species, further studies should investigate the effect of the altered carbohydrate profiles on taste. A large fall in sugars, increase in metabolic acids, and fall in pH may have an effect on taste and thus meat quality affecting the marketability of these valuable animals. This requires more detailed chemical analyses and sensory organoleptic measures. In the Pork Industry, stress prior to slaughter has been identified to be causing pale soft exudative (PSE) meat and dark firm and dry (DFD) meat. Both these conditions, caused by reduced glycogen content of the muscle; result in poor quality meat, with an inferior taste and shelf life (Chambers & Grandin, 2001; Pleva *et al.*, 1990). It is suggested that pigs and other animals should be rested prior to slaughter so ensure muscle glycogen is replaced as much as possible. The post mortem pH depends on the final glycogen content of the muscle, with a sweet to sour taste being caused by increased pH, illustrating the impact these variables can have on taste. Interestingly, in one of the few studies on crustacean meat quality, Boyd & Sumner (1973) noted that flesh from moribund lobsters was identified by a taste panel as being of inferior quality to the meat from livelier lobsters.

2.4.2 Immunological measures

The major findings of the immunological study are that air exposure at the higher temperatures causes significant reduction in total haemocyte counts (THC), a significant reduction in haemolymph prophenoloxidase levels and elevated haemolymph bacteraemia levels.

A number of studies have looked at the effect of hypoxia and emersion-induced hypoxia on the prophenoloxidase levels of Crustacea with different outcomes. Le Moullac *et al.* (1998) found that prophenoloxidase levels increased significantly and attributed this increase to the decreased amount of plasma inhibitors present in the haemolymph, even though THC also reduced. However, other research has found the opposite. Norton *et al.* (1999) and Cheng *et al.* (2002a) both reported decreases in prophenoloxidase levels following exposure to stresses, such as disease or emersion. Perazzolo & Barracco (1997) have determined from haemocyte lysate or serum using a variety of elicitors (non-self molecules such as ß-1,3-glucans which activate the conversion of prophenoloxidase), that most of the prophenoloxidase activity in crustaceans occurs within the granulocytes and semigranulocytes. The decrease in prophenoloxidase levels, in the present study, could possibly be attributed to the large fall in circulating haemocyte numbers which was also observed in the present study.

Prophenoloxidase is at least partially responsible for the recognition process of the defence mechanism in crustaceans (Söderhall, 1982; 1992; Ashida & Söderhall, 1984; Söderhall *et al.*, 1994). Phenoloxidase promotes hydroxylation of phenols and oxidation of o-phenols to quinines, necessary for melanisation. Melanisation is a response to pathogens and wound healing (Johansson & Söderhall, 1989). The reduction in circulating prophenoloxidase levels in *N. norvegicus* during aerial exposure concurs with previous work and suggests that it is an accurate measure of lobster health. Phenoloxidase is the terminal enzyme in the prophenoloxidase activating system, a major factor in the crustacean immune system (Cheng & Chen, 2002). Interestingly Cheng & Chen (2002) found that the reduction in prophenoloxidase activity was not a consequence of a reduction in the number of circulating haemocytes or altered differential haemocyte counts, which contradicts our own findings. However, as Evans (1999) reported the immune response to stress in crustaceans

changes with the duration of time after the initial stressor. Initially THC increase, with a corresponding increase in phenoloxidase activity, and later these parameters show reductions. With stressors of different severities or differing times elapsed until sampling after stress very different results will be obtained.

A number of studies have correlated decreased haemolymph pH levels with increased susceptibility to disease (Mikulski *et al.,* 2000; Holman *et al.,* 2004). This may be explained by findings that the optimum pH for prophenoloxidase activity in crustaceans lies between pH 8.0 and 9.0 (Gollas-Galvan *et al.,* 1999; Cardenas & Dankert, 2000).

The prophenoloxidase data show great variation, even in unstressed *N. norvegicus.* It is recommended that in future studies haemolymph protein levels are also measured so that enzyme activity can be expressed per mg of protein, as done by Gomez-Jimenez *et al.* (2000).

The number of circulating haemocytes in *N. norvegicus* decreases with increasing temperature of aerial exposure. This has generally been found in other studies, though Hawkins *et al.* (1993) reported elevated counts in clam, *Mercenaria mercenaria,* exposed during the tidal cycle, and Jussila *et al.* (1997) reported significantly higher THC in the rock lobster *Panulirus cygnus* during transport, which was attributed to stress. Hauton (1995) found similar increases when studying *C. maenas,* but attributed this small rise to a behavioural response following exposure, whereby the heart and ventilation rates drop, causing haemolymph to collect in the peripheral sinuses (the site of haemolymph sampling in most studies).

During hypoxia most researchers have observed the number of circulating haemocytes declining. Cheng *et al.* (2002b) found significant reductions in the freshwater prawn,

Macrobrachium rosenbergii, exposed to hypoxic conditions, and similar results were obtained by Le Moullac *et al.* (1998) with *Penaeus stylirostris.* In crustaceans, cellular defences rely on several haemocyte functions, such as coagulation, phagocytosis, encapsulation and wound healing (Johansson & Söderhall, 1989; Hose & Martin, 1989; Bachere *et al.,* 1995).

The monitoring of THC has been used for some time as a measure of stress in crustaceans (Mix & Sparks, 1980; Martin & Graves, 1985; Lorenzon *et al.,* 2001), and this study has shown that the duration and temperature of exposure has a significant effect on the THC of *N. norvegicus.* This is thought to reflect an immuno-suppression (Lorenzon *et al.,* 2001), which might provide a critical window for opportunistic pathogens such as *Vibrio* species to exploit the host and initiate spoilage.

There has been considerable discussion on the fate of the haemocytes following exposure to stress, of which there are several possibilities. A number of other studies have correlated the decline in circulating haemocytes with the injection of foreign substances such as bacteria (Smith & Söderhall, 1983; Persson *et al.,* 1987; Martin *et al.,* 1993; van de Braak *et al.,* 2002; Holman *et al.,* 2004); although this was not observed in all cases (Cheng & Chan, 2002). In the present study a rise in bacteraemia levels occurs concomitantly with a decline in THC. Following the rise in bacteraemia, haemocytes move to the site of invasion and kill the bacteria by mechanisms such as melanisation (Fontaine & Lightner, 1974; van de Braak *et al.,* 2002). Smith *et al.* (1984) and Martin *et al.* (1998) have shown that these aggregations grow in size to form larger aggregates or nodules, an observation that has also been made in the histological work of this chapter. The fate of these aggregates is unknown, but accumulations have been observed in the heart, the hepatopancreas and the connective tissue of crabs (Smith & Ratcliffe, 1980) and lobsters

(Factor & Beekman, 1980). In the present study, aggregations were only observed in the intertubule spaces of the hepatopancreas.

In vertebrates there is a substantial body of evidence linking a decrease in circulating leukocytes with an increase in the stress hormone cortisol, and that corticosteroids, secreted in response to stress, are responsible, at least in part, for the immuno-suppression observed in stressed fish (Barton & Iwama, 1991). The reasons for cortisol-induced immuno suppression of fish is yet to be clearly understood, but there is evidence to suggest that cortisol acts to inhibit interleukin-like factors necessary for the differentiation of lymphocytes from their precursor cells (Tripp *et al.*, 1987; Kaattari & Tripp, 1987). More recent papers have shown that cortisol induces apoptosis (pre-programmed cell death) (Weijts *et al.*, 1997; 1998; Saeij *et al.*, 2003). As well as reducing cell numbers, cortisol also reduces the activity of the antioxidants (GSH – reduced glutathione) within the cell. As GSH plays a major role in the protection against NO-mediated inhibition of lymphocyte proliferation, cortisol may render stressed animals more susceptible to the immunopathological effects of nitrous oxide (NO), an oxygen radical (Saeij *et al.*, 2003). Grimm (1985) also found that cortisol suppressed the mitogen-induced proliferation of cultured leukocytes from plaice, and suggested that this may be a mechanism by which stress increases susceptibility to disease.

It is interesting that a similar increase in the stress hormone in invertebrates (CHH) is correlated with a decrease in circulating haemocytes. Further research at a molecular level may answer some questions about what induces the decline in haemocytes. This decline is possibly due to the number of reasons outlined above.

Aerial exposure results in stress which manifests itself in changes in the physiology, immunology and microbiology of the animal. The combination of the range techniques

used in the present study makes it possible to begin the construction of a causal chain to explain why aerial exposure at high temperatures causes high fatalities in *N. norvegicus*. The high levels of bacteraemia were attained following immuno-suppression, which is consequent with the alterations in the carbohydrate profile caused by exposure at high temperatures.

The temperature of aerial exposure increases the stress suffered by *N. norvegicus* following capture, leading to the meat spoilage and increased mortality during the export process, resulting in the economic losses. In the summer period aerial exposure following landing should be minimised otherwise the animals will experience shifts in internal pH, affecting the taste profile, and immuno-suppression.

Table 2.1: Effect of temperature and duration of air exposure on the pH of the haemolymph of *Nephrops norvegicus*. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Tukey's Pair-wise comparisons. SD represents the standard deviation. Different letters between each time series of the same temperature exposure trial indicate significant differences between exposure times.

Figure 2.1: Effect of temperature and duration of air exposure on the percentage mortality rates of *Nephrops norvegicus*.

Exposure Temp.	10°C		15°C		25°C	
Exposure Time (h)	Mean	SD	Mean	SD	Mean	SD
0	7.88 a	0.13	7.90 a	0.05	7.95 a	0.05
4	7.60 b	0.24	7.26 b	0.16	6.57 b	0.22
8	7.62 b	0.28	7.26 b	0.24		
12	7.48 b	0.27	7.25 b	0.33		

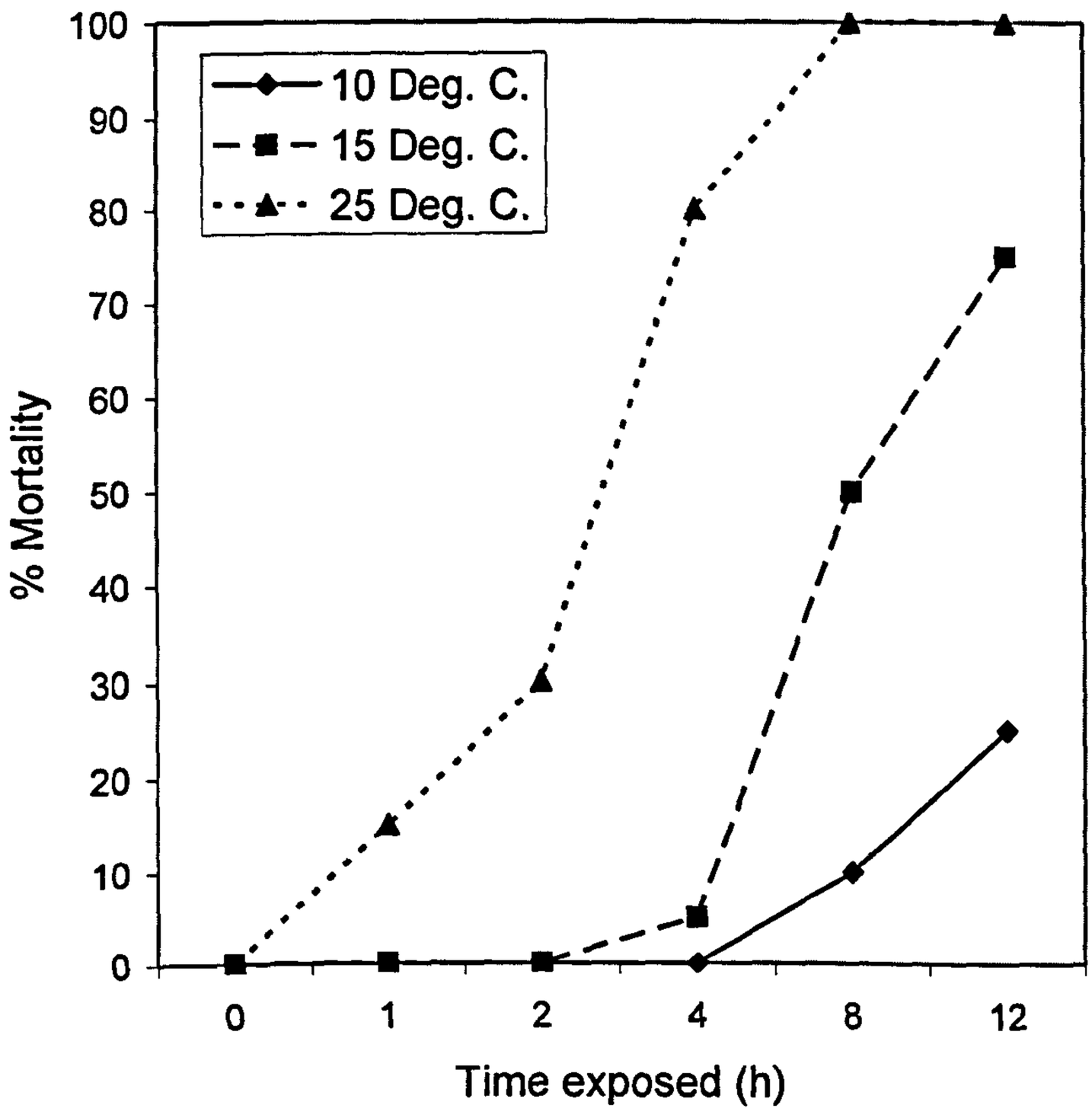
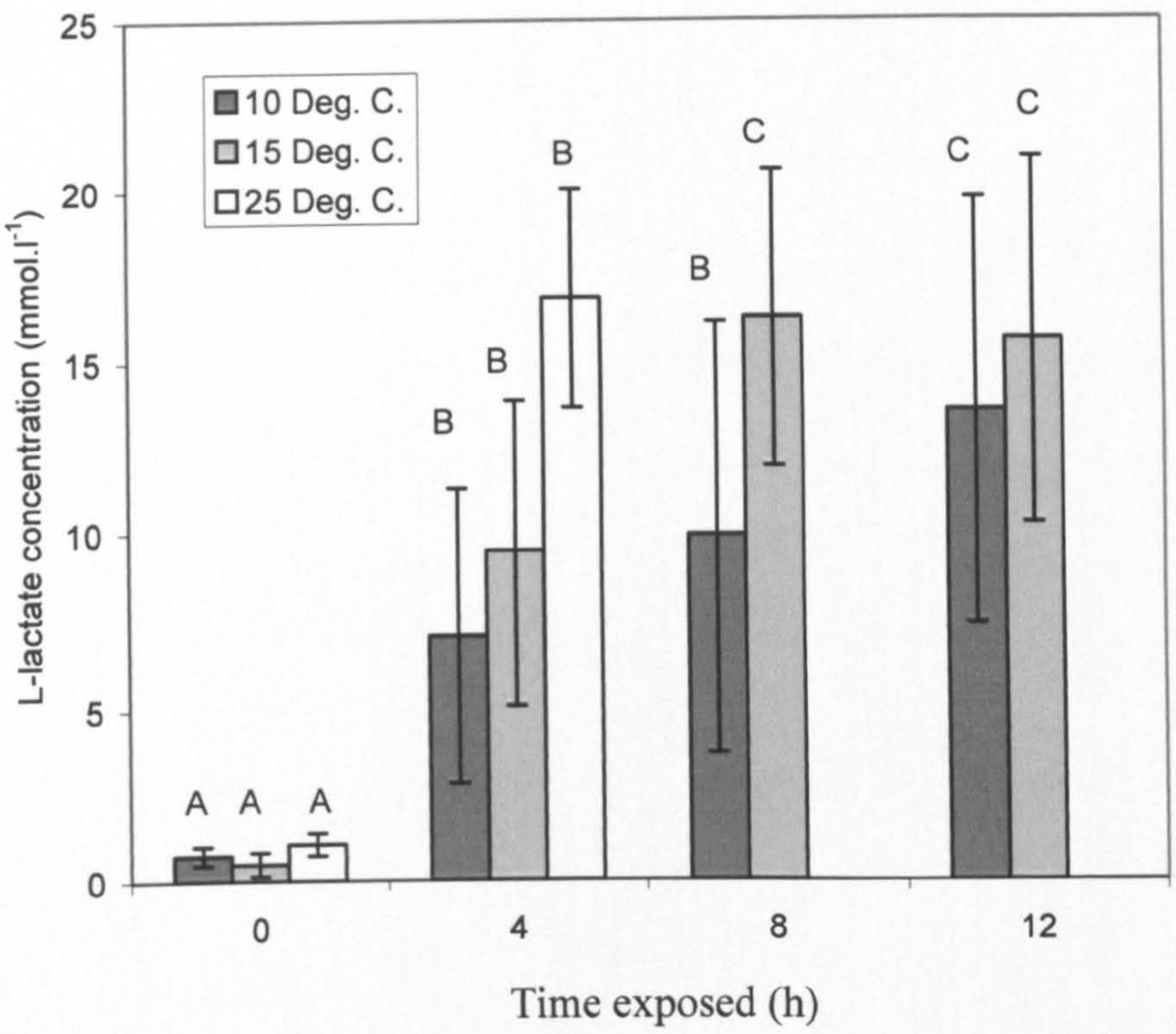


Figure 2.2: Effect of temperature and duration of air exposure on the mean L-lactate concentration in haemolymph of *Nephrops norvegicus*. Error bars represent standard deviations. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Tukey's Pair-wise comparisons.

Figure 2.3: Effect of temperature and duration of air exposure on median glucose concentration in the haemolymph of *Nephrops norvegicus*. Error bars represent inter-quartile variation. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Mann-Whitney Pair-wise comparisons.


10 Deg. C.
15 Deg. C.
25 Deg. C.
L-lactate concentration (mmol.l-1)
25
20
15
10
5
0
A A A
B B B
B B C
C C C
0
4
8
12
Time exposed (h)

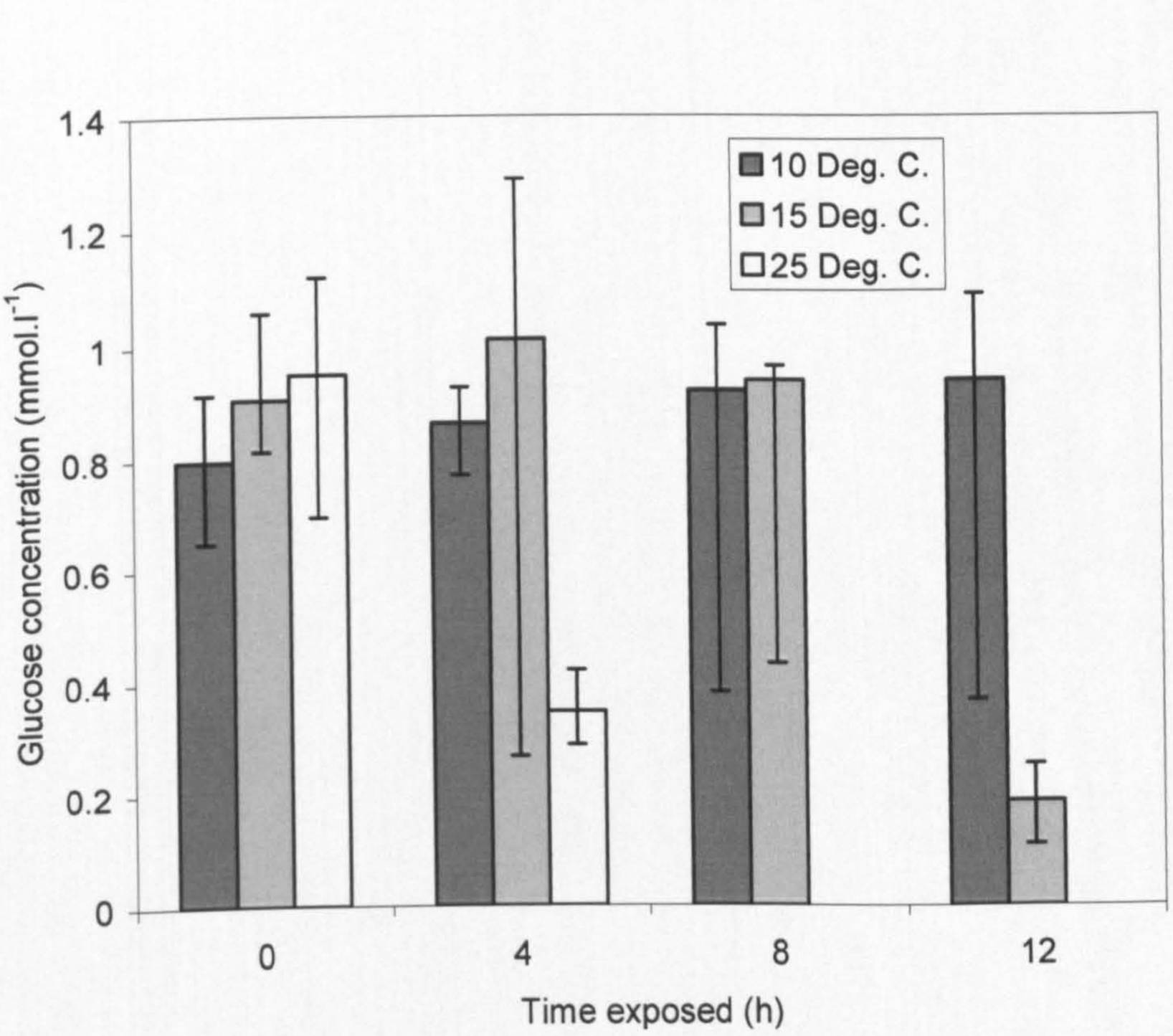
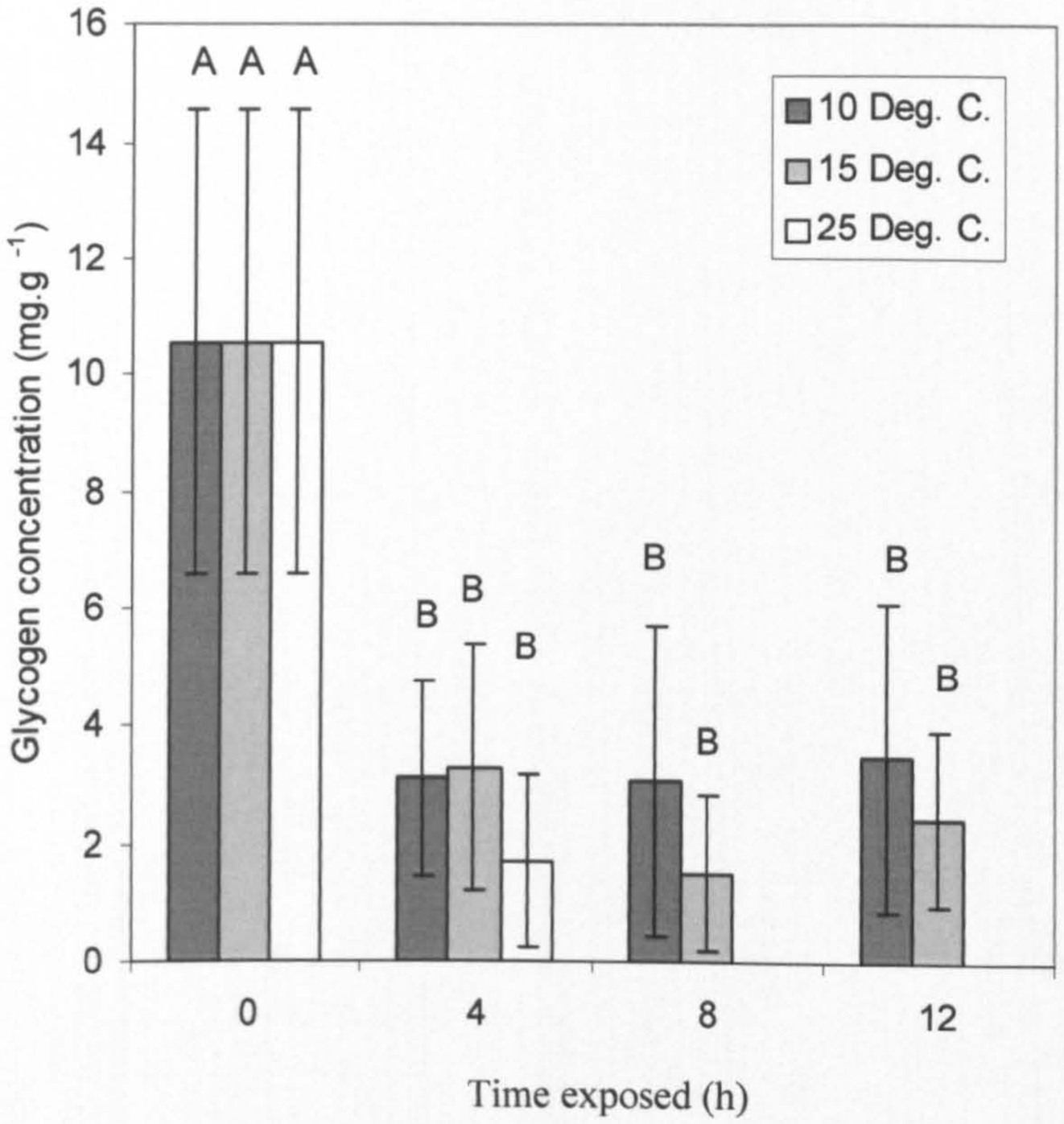

10 Deg. C.
15 Deg. C.
25 Deg. C.
Glucose concentration (mmol.l-1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
4
8
12
Time exposed (h)

Figure 2.4: Effect of temperature and duration of air exposure on the mean glycogen concentration of the tail muscle of *Nephrops norvegicus*. Error bars represent standard deviations. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Tukey's Pair-wise comparisons.

Figure 2.5: Effect of temperature and duration of air exposure on the mean CHH concentration of the haemolymph of *Nephrops norvegicus*. Error bars represent the standard deviations. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Tukey's Pair-wise comparisons.


16
14
12
10
8
6
4
2
0
Glycogen concentration (mg.g -1)
10 Deg. C.
15 Deg. C.
25 Deg. C.
A A A
B B B
B B B
0
4
8
12
Time exposed (h)

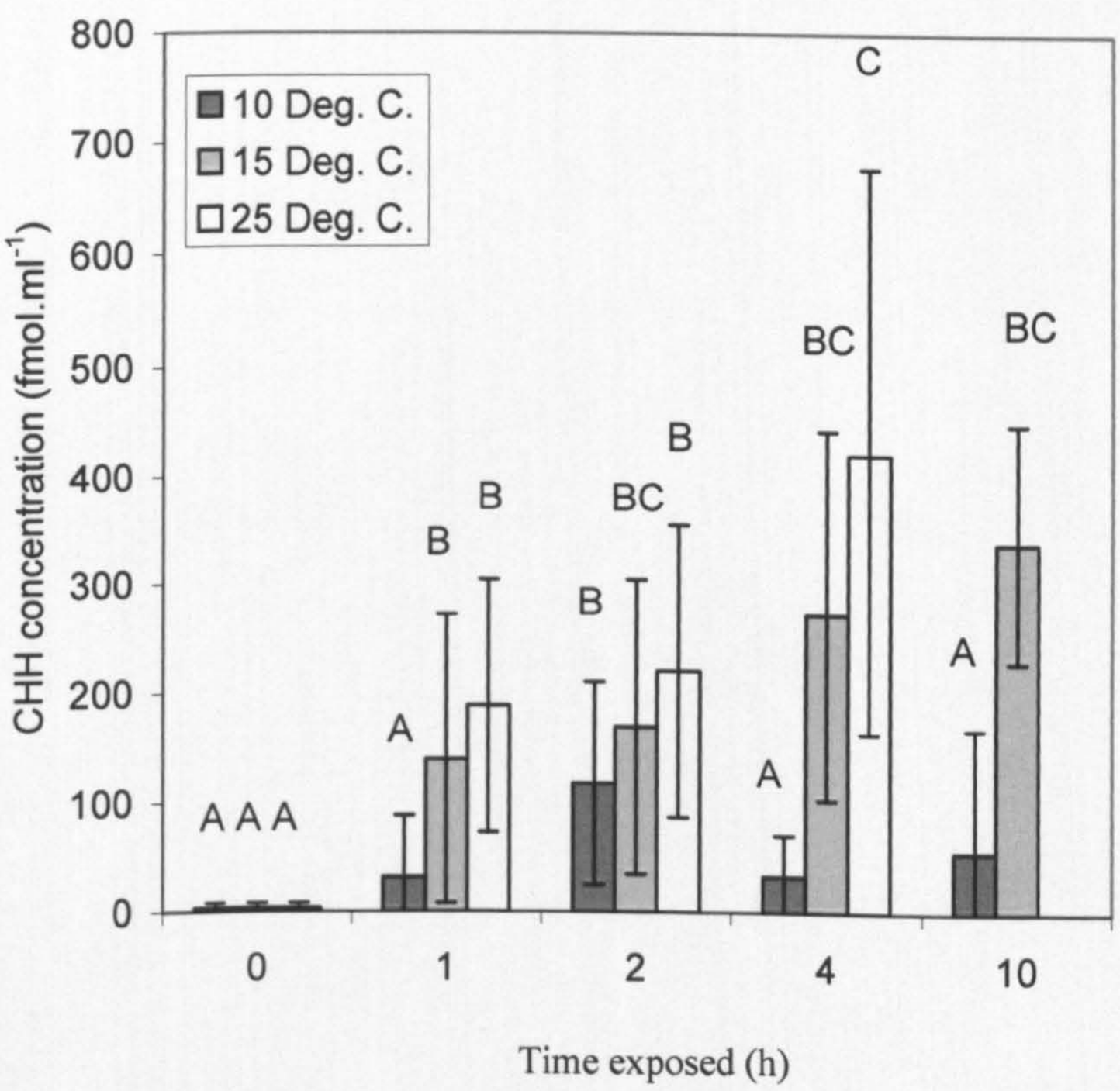
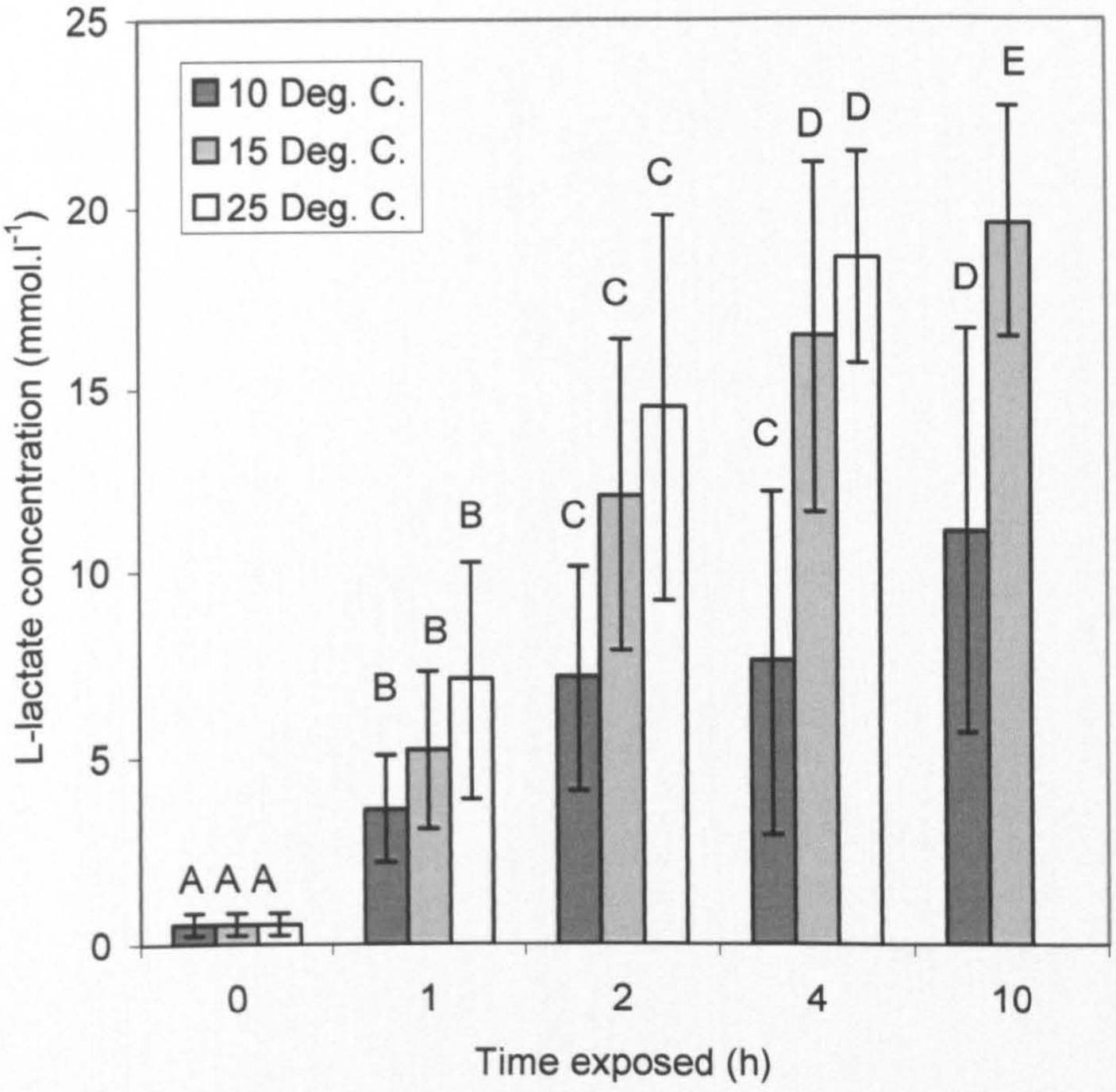

800
700
600
500
400
300
200
100
0
CHH concentration (fmol.ml-1)
10 Deg. C.
15 Deg. C.
25 Deg. C.
A A A
A
B B
B BC
B
A
BC
C
BC
0
1
2
4
10
Time exposed (h)

Figure 2.6: Effect of temperature and duration of air exposure on the mean L-lactate concentration of the haemolymph of *Nephrops norvegicus.* Taken concurrently with samples analysed for CHH levels. Error bars represent standard deviations. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Tukey's Pair-wise comparisons.

Figure 2.7: Effect of temperature and duration of air exposure on mean total haemocyte counts of *Nephrops norvegicus.* Error bars are standard deviations. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Tukey's Pair-wise comparisons.


25
20
15
10
5
0
L-lactate concentration (mmol.l-1)
10 Deg. C.
15 Deg. C.
25 Deg. C.
A A A
B
B
B
C
C
C
D
D
D
E
0
1
2
4
10
Time exposed (h)

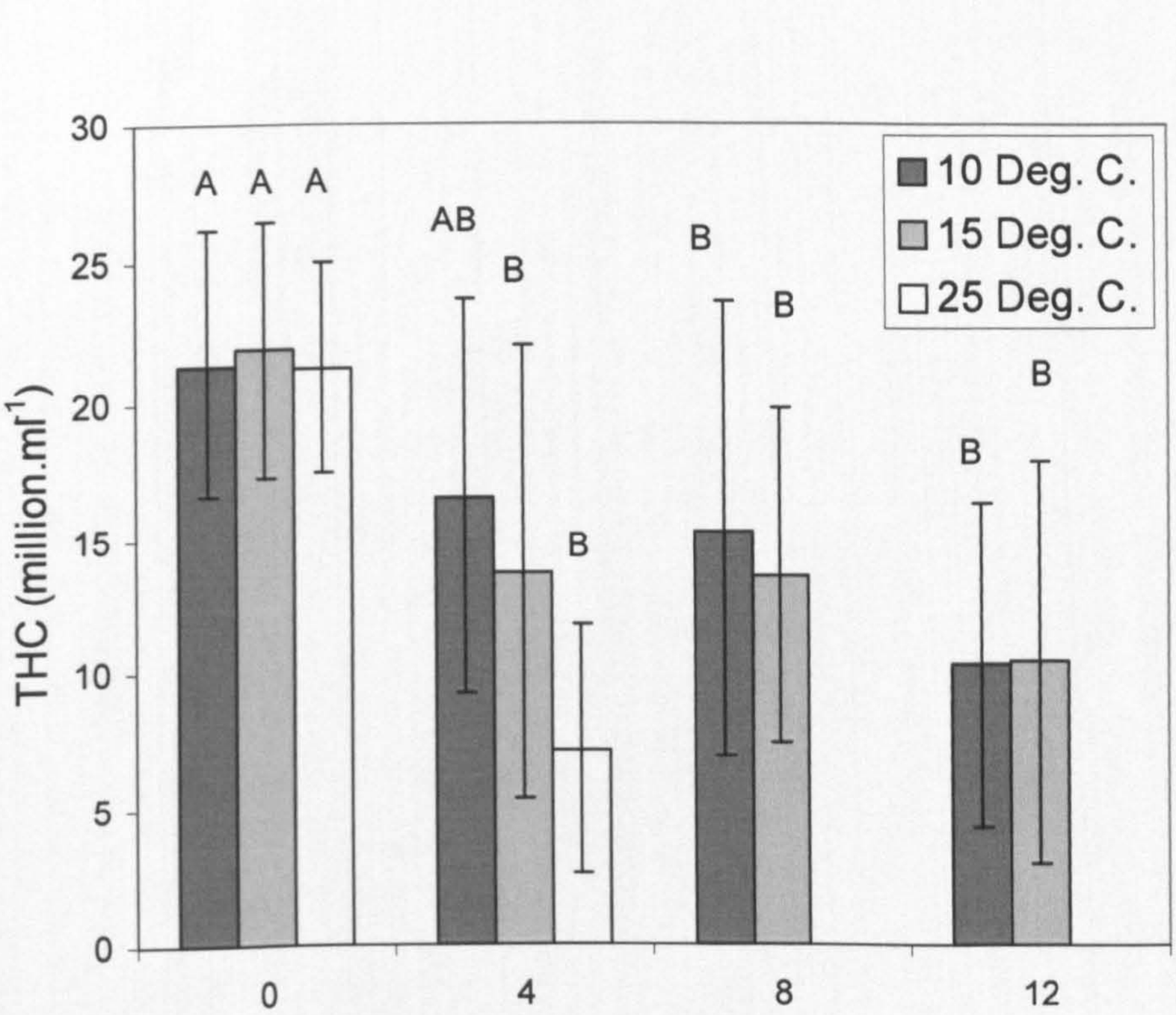
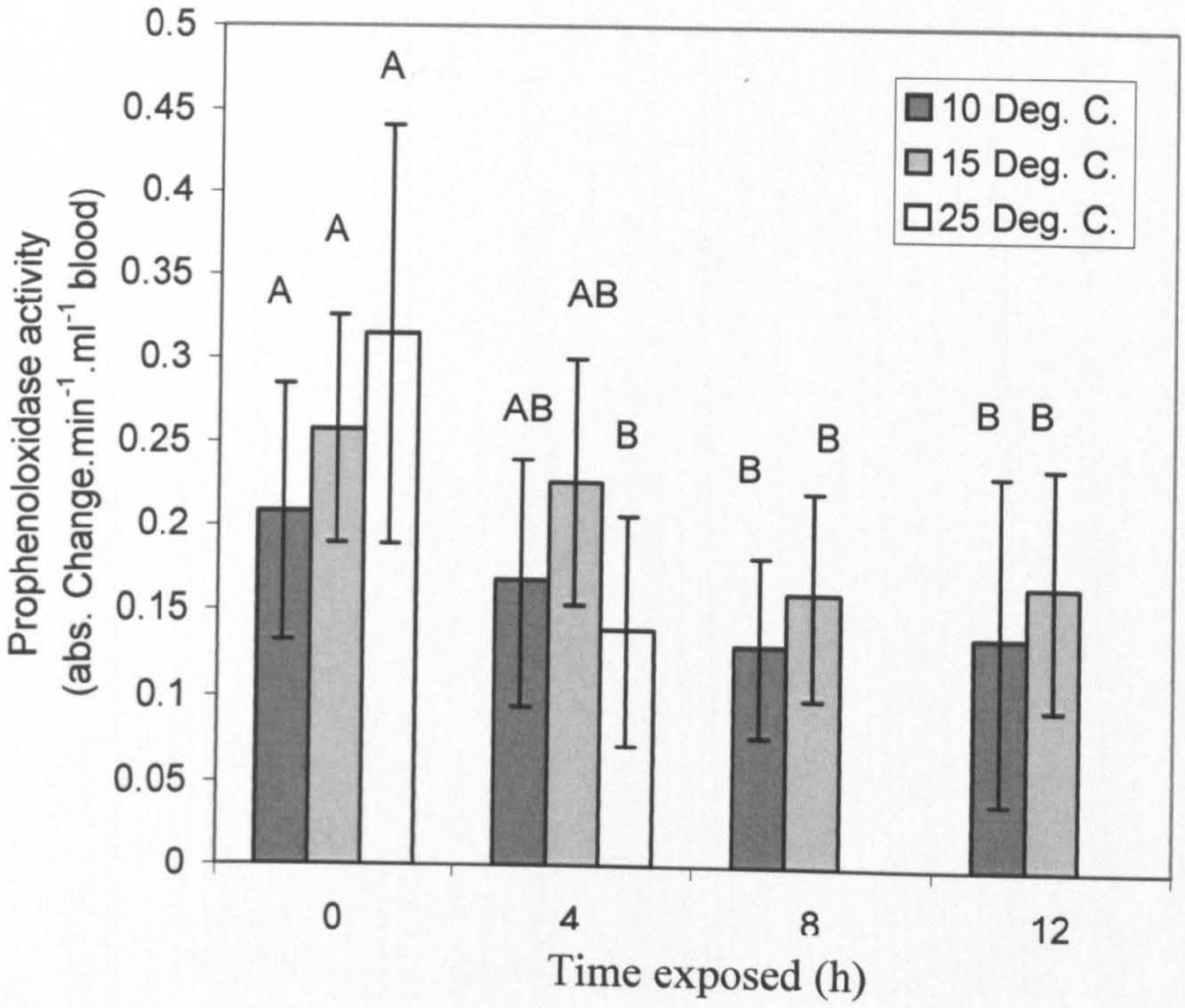

30
25
20
15
10
5
0
THC (million.ml-1)
10 Deg. C.
15 Deg. C.
25 Deg. C.
A A A
AB
B
B
B
B
B
B
B
0
4
8
12
Time exposed (h)

Figure 2.8: Effect of temperature and duration of air exposure on the mean prophenoloxidase activity in the haemolymph of *Nephrops norvegicus*. Error bars represent standard deviations. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Tukey's Pair-wise comparisons.

Figure 2.9: Effect of temperature and length of air exposure on the median bacterial concentrations of the haemolymph of *Nephrops norvegicus*. Error bars represent inter-quartile variation. Within each temperature exposure trial different letters indicate significant differences between the times, as indicated by Mann-Whitney Pair-wise comparisons


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Prophenoloxidase activity
(abs. Change.min-1.ml-1 blood)
10 Deg. C.
15 Deg. C.
25 Deg. C.
A
A
A
AB
AB
B
B
B
B
B
0
4
8
12
Time exposed (h)

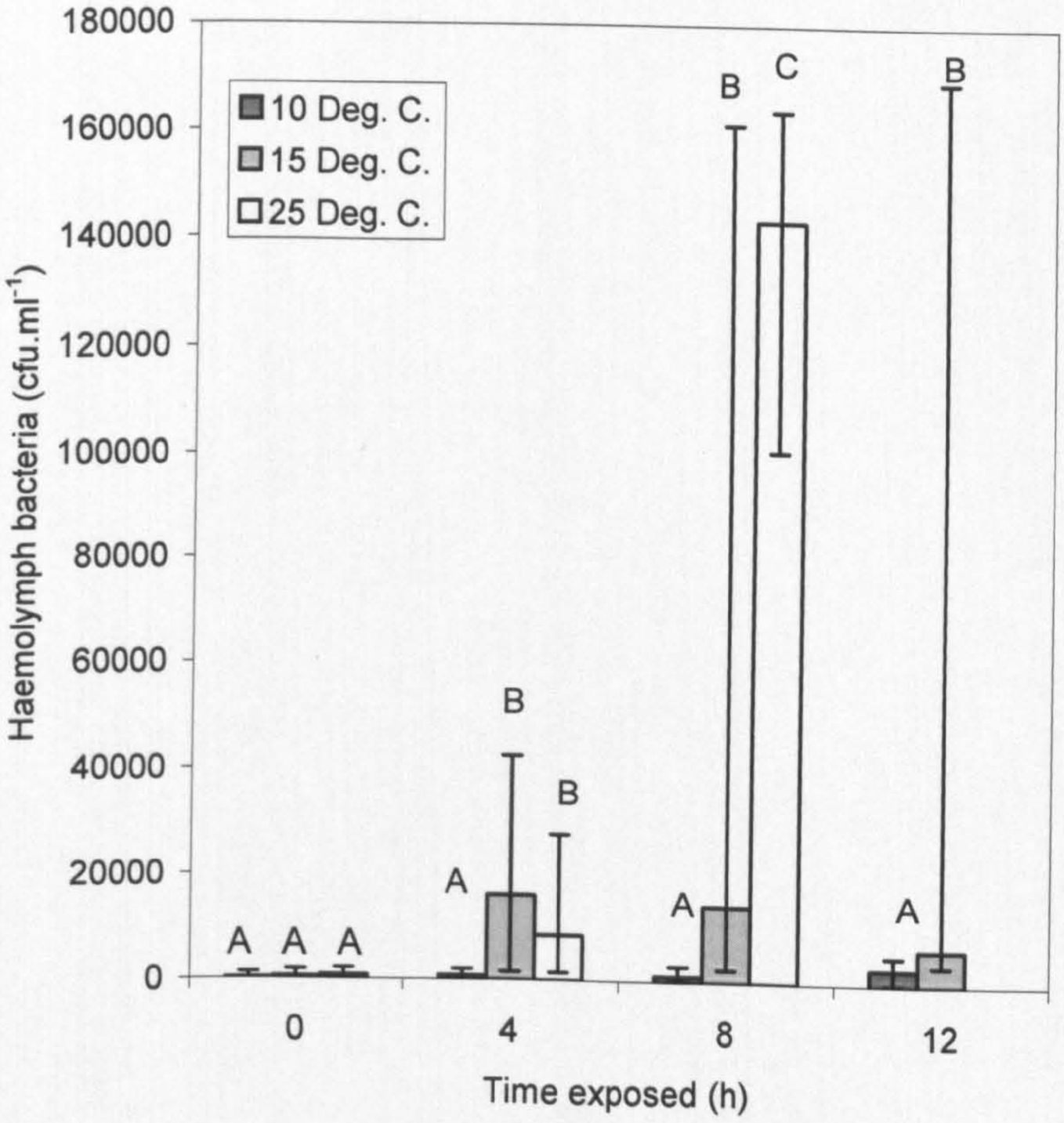
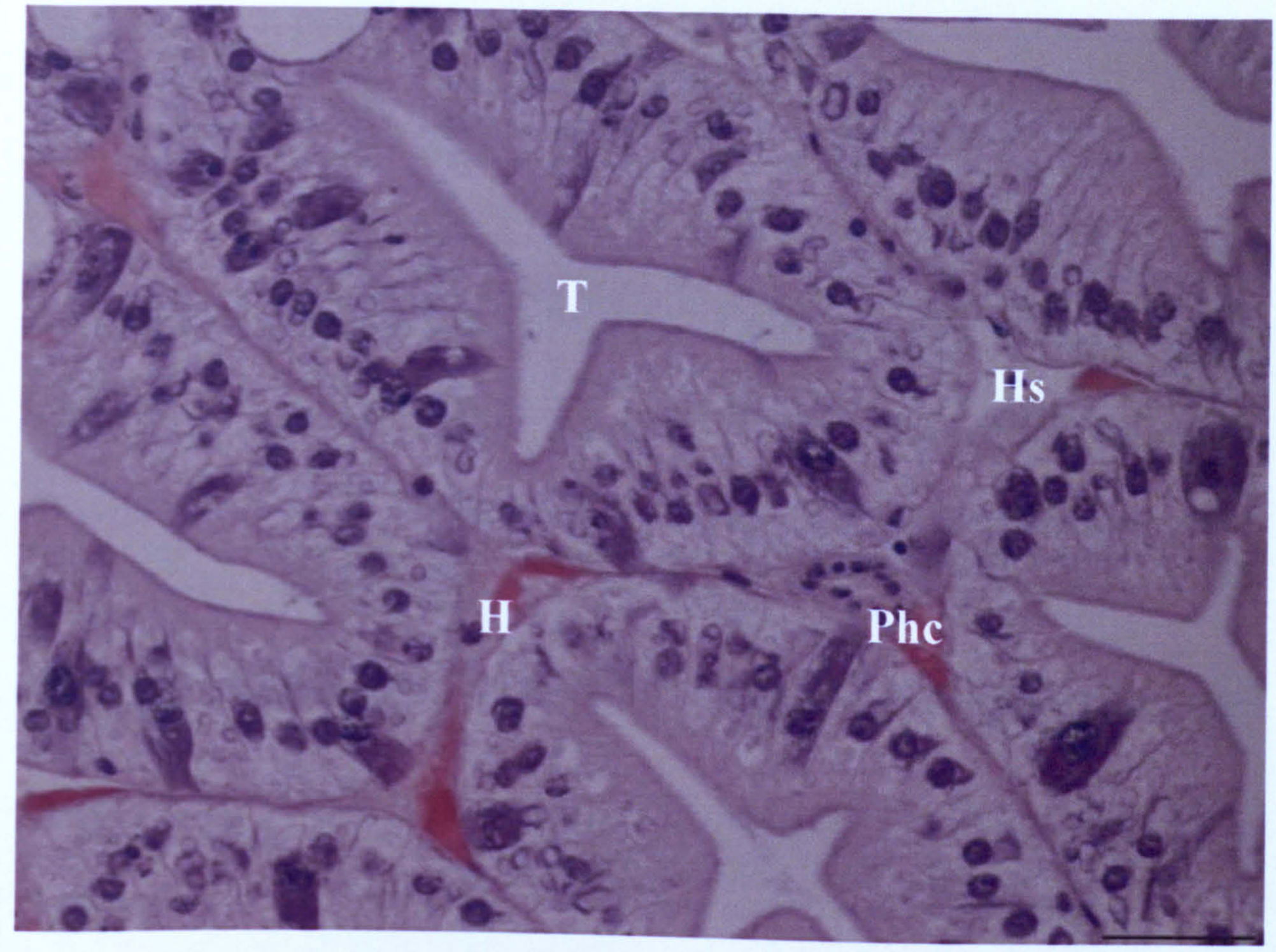

180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Haemolymph bacteria (cfu.ml-1)
10 Deg. C.
15 Deg. C.
25 Deg. C.
A
A
A
A
B
B
B
C
B
0
4
8
12
Time exposed (h)

Figure 2.10: Hepatopancreas from *N. norvegicus* exposed to air for 1 h at 25 °C. Note the relatively few haemocytes (H) in the haemal spaces (Hs) between the hepatopancreas tubules (T). Only phagocytic cells (Phc) lining the haemolymph vessels are evident. Scale Bar 25 μm. Tissue stained with haemotoxylin and eosin.

Figure 2.11: Hepatopancreas from *N. norvegicus* exposed to air for 3 h at 25 °C. Note the increased number of haemocytes (H) infiltrating the hepatopancreas tissues (HT) and filling the haemal spaces (Hs) between the hepatopancreas tubules (T). Scale Bar 50 μm. Tissue stained with haemotoxylin and eosin.


T
Hs
H
Phc

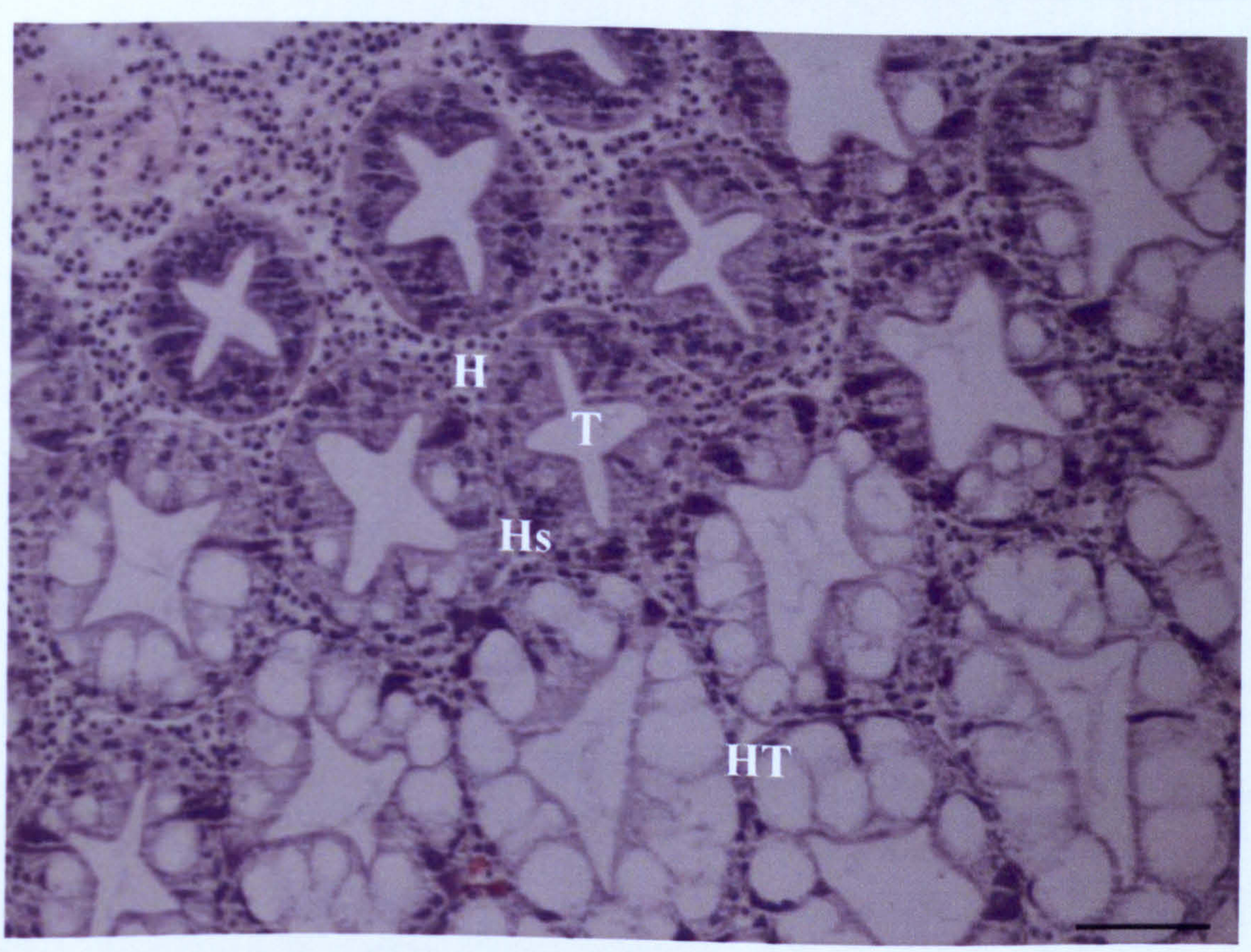
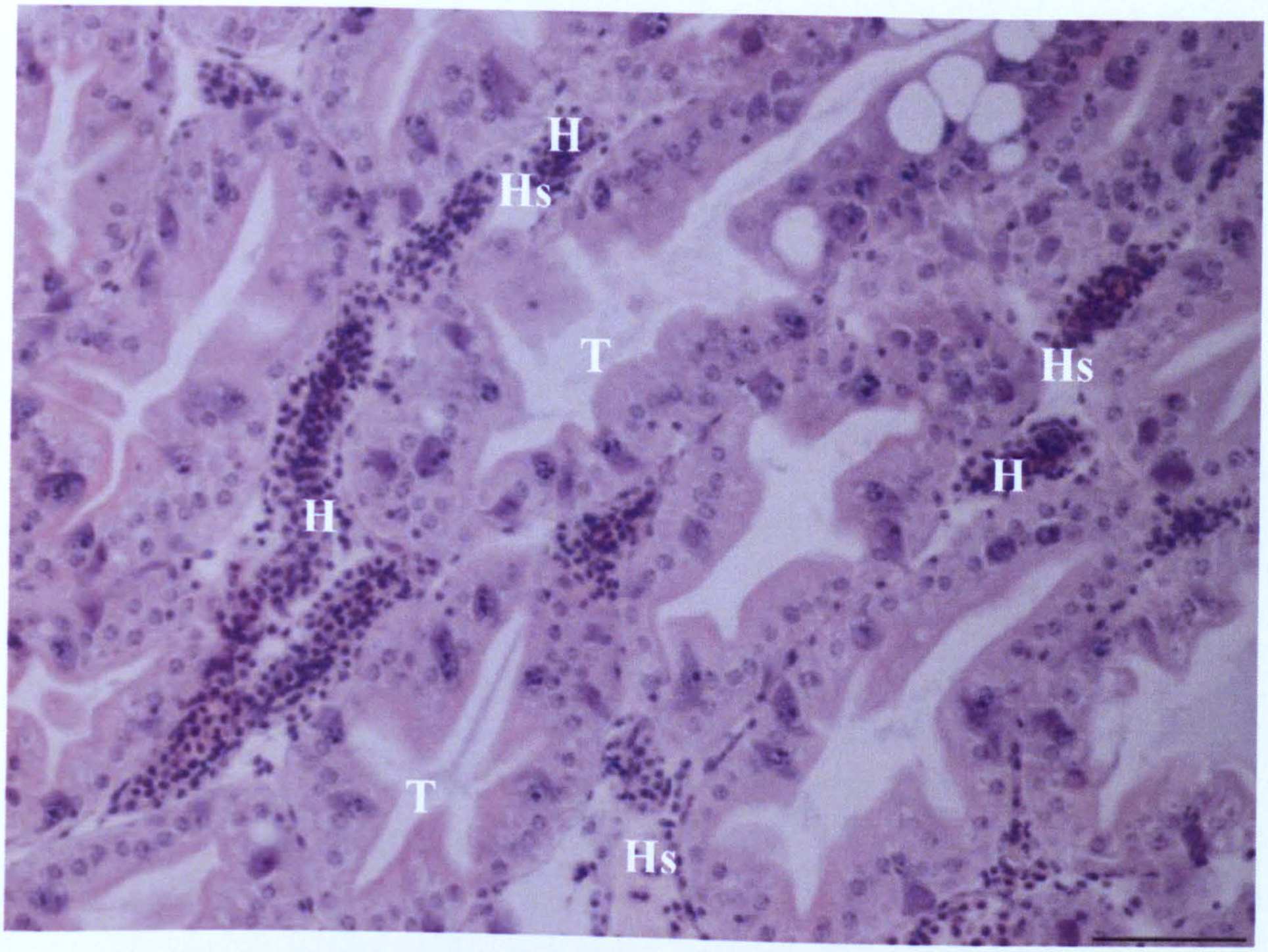

H
T
Hs
HT

Figure 2.12: Hepatopancreas from *N. norvegicus* exposed to air for 4 h at 25 °C. Compared to figure 2 the haemocytes (H) appear to be even more densely packed into the haemal spaces (Hs) between tubules (T). Scale Bar 50 μm. Tissue stained with haemotoxylin and eosin.

Figure 2.13: A section showing part of the hepatopancreas (Hp) from *N. norvegicus* exposed to air for 4 h at 25 °C. There appears to be haemocyte infiltration and early attempts at encapsulation (arrow). Scale Bar 25 μm. Tissue stained with haemotoxylin and eosin.


H
Hs
T
Hs
H
H
T
Hs

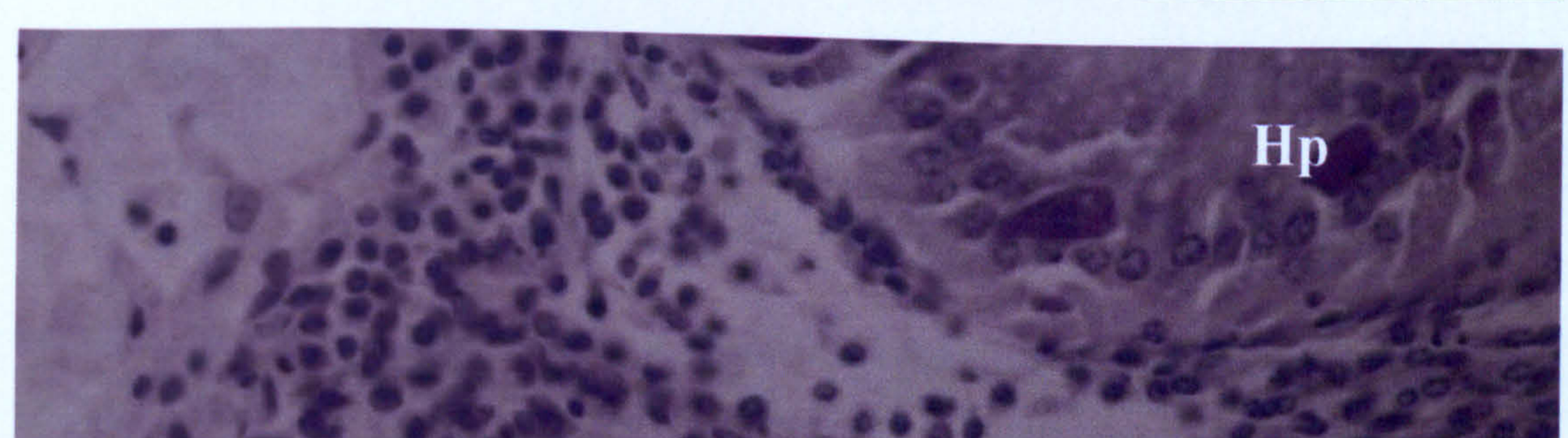
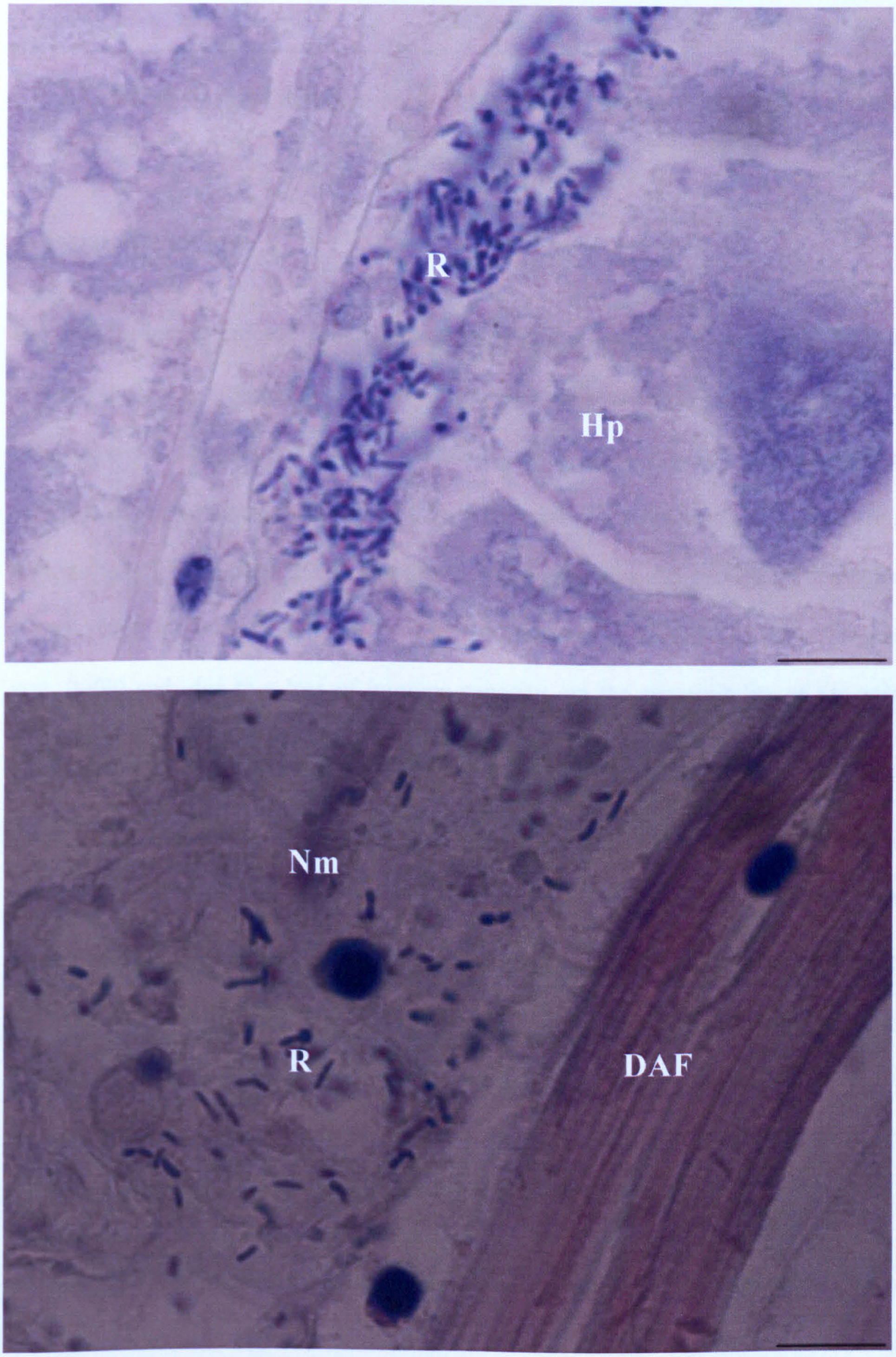

Hp

Figure 2.14: Hepatopancreas from a recently deceased *N. norvegicus* exposed for 8 h at 25 °C. Rod shaped bacteria (R) can be observed in the hepatopancreas (Hp). Scale Bar 10 μm (x100 oil emersion). Tissue stained with haemotoxylin and eosin.

Figure 2.15: Deep abdominal flexor (DAF) muscle from a recently deceased *N. norvegicus* exposed to air for 8 h at 25 °C. Rod shaped bacteria (R) can be observed in an area of necrotic abdominal musculature (Nm). Scale Bar 10 μm (x100 oil emersion). Tissue stained with haemotoxylin and eosin.


R
Hp
Nm
R
DAF

Chapter 3

The effects of trawl duration on the physiological status of the Norway lobster, *Nephrops norvegicus.*

3.1 INTRODUCTION

The trawl capture process for *Nephrops norvegicus* exposes the animals to numerous stresses, including struggling, mechanical damage and overcrowding in the net as well as aerial exposure, temperature fluctuations and handling after capture. These stresses can lead not only to physiological stress responses, which are often reversible (Uglow *et al.*, 1986; Taylor *et al.*, 1997; Speed, 2001), but also to more extensive and permanent damage to their tissues which is not reversible, and impacts strongly on both product quality (Stentiford & Neil, 2000) and, due to a loss of immune capacity, on the susceptibility of the animals to disease (Spotts & Lutz, 1981).

Over the past decade the fishery of *N. norvegicus* has developed into the most valuable on the west coast of Scotland (Bailey *et al.,* 1986; Scottish Fishery Statistics, 2001). However, after continuing growth of the fishery, quotas were reduced in 2000 because of concern about the cod by-catch (EU Fisheries Council, 2000) but have now been increased in both the North Sea and Scottish west coast waters (EU Fisheries Council, 2004). Where lobster catch limits (or quotas) are restrained, improving the quality of the catch is an important means of adding value to the product (Taylor *et al.*, 1997).

There have been few studies on the damage caused by trawling to *N. norvegicus,* and much of this research has centred on damage to small sized *N. norvegicus* which will be discarded after capture (Symonds & Simpson, 1971; Gueguen & Charau, 1975; Harris *et al.*, 1997). Past studies of escaped and discarded *N. norvegicus* indicated that more than

50% of the animals that had encountered the trawl had physical damage. A range of damage scales have been used in the past to estimate injuries sustained during trawl capture (Wassenberg & Hill, 1989; Mensink, 2000; Salini, 2000; Pranovi *et al.*, 2001; Troffe *et al.*, 2003). This approach allows for the relative fragility of the target animal to be assessed. In the present study a three-level scale was used for damage assessment.

When crustaceans are exposed to certain stresses (e.g. temperature change, osmotic shock), plasma levels of the Crustacean Hyperglycaemic Hormone (CHH) increase (Chang *et al.*, 1998). CHH stimulates glycogenolysis, which increases glucose availability in the haemolymph, and reduces glycogen levels within the tissues (Santos & Keller, 1993b). When the animal swims it utilises available glucose and thus lowers glucose concentrations in the haemolymph. This will induce release of CHH (by reducing the negative feedback from glucose) (Santos & Keller, 1993a), which in turn mobilises glucose from glycogen stores in the tissues, thereby becoming progressively depleted. This changing level of glycogen in the abdominal muscles can therefore act as an indicator of the amount of physical stress that the animal has suffered in the trawl.

During trawl capture and handling the increased energy demand can elevate the metabolic rate to a value that can exceed the ability of the respiratory system to deliver oxygen. As a result anaerobic metabolism will take place, causing the production of L-lactate and related metabolic end products. It has been hypothesised that the acidosis thus produced could cause a reduction in product quality, and an increased susceptibility to disease and death (Spotts & Lutz, 1981). The accumulation of L-lactate in the haemolymph of *N. norvegicus* following capture can therefore be a useful indicator of the physiological stress experienced by the animal. Creel capture of *N. norvegicus* is generally thought of as a low stress process that delivers the animal to the surface in better condition than trawling. However in one of the

few studies comparing creel and trawl caught *N. norvegicus*, Spicer *et al.* (1990) demonstrated elevated L-lactate concentrations and a hyperglycaemic response in the haemolymph of creeled individuals, suggesting it may be more stressful than originally thought.

Methods have been developed for monitoring the immune status of the animal, to provide an indication of whether it may be susceptible to bacterial infection. One of the more reliable methods is the Total Haemocyte Count (THC), since the circulating haemocytes play central roles in phagocytosis, capsule formation and killing of bacteria either intra- or extra-cellularly (Chisholm & Smith, 1992; Smith & Chisholm, 1992). Le Moullac *et al.* (1998), Gomez-Jimenez *et al.* (2000), Mikulski *et al.* (2000), Fotedar *et al.* (2001), and Cheng *et al.* (2002b) all concluded that air exposure or subsequent hypoxia had a significant adverse effect on the immune system and hence on the health status of lobsters. Several authors have suggested that haematological parameters such as total haemocyte counts might represent sensitive indicators of immuno suppression in marine decapods, such as *N. norvegicus* (Smith & Johnson, 1992; Hauton *et al.*, 1995; Jussila *et al.*, 1997).

In addition, data have been obtained on the extent of body damage caused to samples of *N. norvegicus* by trawls of different durations at different times of day, and the subsequent mortality rates of these animals held under controlled conditions.

The results included in this chapter have been included in a report detailing the two trials conducted by Seafish in September 2003 and May 2004 in conjunction with Scotprime Ltd.

The aim of this chapter is to compare the stress and physical damage experienced by *N. norvegicus* following different capture methods and trawl durations (1 and 5 h). This will

be performed using stress indicators of the health status of the animal, namely measures of circulating haemocytes, assays of the blood metabolite L-lactate and of muscle tissue glycogen, together with mortality and damage assessments. A damage index will allow *N. norvegicus* to be classified a damage category and allow the damage caused by trawls of different durations to be compared. Information on the mobilisation of carbohydrate reserves and the stress response in *N. norvegicus* will be provided by measures of haemolymph L-lactate and CHH concentrations in addition to abdominal muscle glycogen concentrations. An assessment of the immune competence of the animal will be provided by haemocyte counts.

3.2 METHODS

3.2.1 Trawling

The first trawling trial was performed on *"Aeolus"*, a commercial *Nephrops* trawler, on September 22nd and 23rd 2003 in the Hunterston Channel of the Clyde Sea Area. Trawls of either a short duration (1 hour) or a long duration (5 h) were performed either at dawn (am) or at dusk (pm) to coincide with periods of maximum burrow emergence. During the trials all trawl nets were towed at 2.5 knots. The trawling schedule was as follows:

Day 1 - Short Trawl am (**SAM**), Long Trawl pm (**LPM**).

Day 2 - Long Trawl am (**LAM**), Short Trawl pm (**SPM**).

On Day 1 haemolymph and muscle tissue samples for analysis were taken from 25 animals for both the SAM and the LPM trawls. The four trawls provided animals for the damage indices and the mortality rates, but no haemolymph or muscle tissue samples were taken on Day 2.

To provide reference measures from 'unstressed' animals, 25 *N. norvegicus* were creel-caught off Largs in October 2003 using RV "Aplysia" from the University Marine Biological Station Millport, and haemolymph and muscle tissue samples were taken in the same way.

The second trawling trial, again on *"Aeolus"* was performed at the end of May/beginning of June 2004 in the same area, using the same sampling regime. Creeled animals were also collected at the beginning of June from the same area as used in September 2003.

The second trial took place over four days with two trawls per day. The trawling schedule was as follows:

Day 1 - Long Trawl am **(LAM)**, Short Trawl pm **(SPM)**.

Day 2 - Short Trawl am **(SAM)**, Long Trawl pm **(LPM)**.

Day 3 - Long Trawl am **(LAM)**, Short Trawl pm **(SPM)**.

Day 4 - Short Trawl am **(SAM)**, Long Trawl pm **(LPM)**.

Haemolymph and muscle tissue samples for analysis were taken from 25 animals for the SAM, LPM, LAM and SPM trawls on Days 1 and 2. The four catches on Days 2 and Day 3 provided animals for the damage indices and the mortality rates, but no haemolymph or muscle tissue samples were taken on the last two days.

3.2.2 Sampling

Once the catch was landed a sample was submerged in a bucket of seawater and all sampling was completed within 45 minutes of the catch being landed. The catch was submerged in seawater as chapter 4 reveals that once submerged recovery is very slow and the desired parameters would vary little over a 45 minute period, while chapter 2 suggests that had the

catch been left exposed to air then the sample would have been exposed to a further stress. Haemolymph samples were taken from the base of the 5th pereiopod using a 25-gauge needle attached to a 1ml syringe, and subdivided for later analysis. All samples for metabolite assays were frozen in liquid nitrogen and transported in a dry shipping container to Glasgow for processing.

3.2.3 Measurement of haemolymph parameters

L-lactate

Refer to section 2.2.2 for the method for the determination of the L-lactate concentration of the haemolymph.

Muscle glycogen

Refer to section 2.2.2 for the method for the determination of the glycogen concentration in the abdominal muscle.

CHH

Refer to section 2.2.2 for the method for the determination of the CHH concentration in the haemolymph. Samples were only analysed for CHH levels following the May trials.

Total haemocyte counts (THC)

Refer to section 2.2.3 for the method for the estimation of THC of the haemolymph.

3.2.4 Data analysis

As long as the relevant criteria were met (normal distribution, homogeneity of variance) the data were analysed using ANOVA, and further comparisons between the treatment means

were made using Tukey's pairwise tests. Non-parametric data analysis was performed using Kruskal and Wallis One way ANOVA with further pairwise comparisons being made using the Mann-Whitney test.

3.2.5 Damage index

A Damage Index record sheet was drawn up to record physical damage suffered by *N. norvegicus* during trawling. The sex, carapace length and moult stage were recorded for each animal, along with an indication of damage to the rostrum, carapace, eyes, claws, abdomen, minor limbs. An assumption of this damage index was that *N. norvegicus* were classified as undamaged prior contact with the trawl net, and any damage observed on the deck is assumed to be a consequence of the trawling process. From these data animals were ascribed to a damage category according to the following criteria:

Undamaged Animals - those showing no visible damage to their external structure and no loss of limbs.

Lightly Damaged Animals - those exhibiting no more than two of these features:

- Loss of two or fewer walking legs
- Loss of only one claw
- Soft tissue punctures or very small punctures to the shell.
- The loss of the tip of the rostrum.

Heavily Damaged Animals - those exhibiting at least one of these criteria:

- Loss of more than two walking legs
- Loss of both claws
- Loss of an eye
- Compressed or cracked body parts/segments
- Major soft tissue damage (large wounds to the abdomen)
- Or those exhibiting three or more than three criteria of lightly damaged animals.

3.3.6 Mortality rate

Data on the survival of *N. norvegicus* following capture, sorting and landing were obtained to allow comparisons to be made between the mortality rates following the trawls of different durations. Following each trawl, 320 animals were placed into sets of 'prawn tubes' (segregated cartons which allow the storage of large number of animals separately, in a confined area), and were held submerged initially in seawater tanks on board, and later in filtered seawater (5°C, salinity 33) tanks at the premises of Scotprime Ltd, Ayr. On days 1,2,3,7 and 14 following capture, dead animals were removed and the amount and location of any damage recorded.

Damage analysis was also carried out on a random sample of *N. norvegicus* taken immediately on landing. The data pertaining to these animals were provided by Seafish (Seafish Industry Authority, a UK Fishery Organisation).

3.3 RESULTS

3.3.1 Haemolymph parameters

Total haemocyte counts (THCs)

Figure 3.1 illustrates the mean THCs obtained from animals immediately after landing from the SAM and LPM trawls in the September trial. The mean THC obtained in a separate study from a group of creeled animals immediately after landing is also shown, as it provides a measure of the 'unstressed' condition.

The ANOVA demonstrates that at least one of the THC counts is significantly different from the others (F value 8.54, $p<0.001$, d.f. 74). Tukey's pairwise comparisons demonstrate show that the THCs from the creeled controls and the SAM trawled *N. norvegicus* did not differ significantly, but that there was a significant reduction in THCs from animals in the LPM trawl compared with those in the SAM trawl.

Figure 3.2 illustrates the mean THCs obtained from animals immediately after landing from the 1 hour trawls (SAM and SPM) and the 5 hour trawls (LAM and LPM) in the May trial. The mean THC count obtained from animals caught in a separate creeling study around the same date is also shown. Although the animals caught by the two 5 hour trawls (LAM and LPM) had lower THCs than those caught by the two 1 hour trawls (SAM and SPM) and the creeled *N. norvegicus* they were not significantly reduced. In fact none of the groups were significantly different from each other (ANOVA F value 2.27, $p = 0.068$, d.f. 124).

To summarise the results of the two trials, in all cases animals caught by the longer trawls had lower THCs than did animals from the shorter trawls, but in only one case, during the September trial, did this difference prove to be statistically significant.

L-lactate

In the September trial, following a trawl of 1 hour (SPM) the L-lactate concentrations in the haemolymph of animals was elevated around 8-fold compared with the values for the haemolymph of creeled animals (mean 1.28mM, SD ±0.19) (Fig. 3.3). After a trawl of 5 h duration (LAM), L-lactate concentrations were also elevated (mean 11.92mM, SD ±3.77). ANOVA analysis shows that mean haemolymph L-lactate concentrations differed significantly between the three treatments (F value 171.24, $p < 0.001$, d.f. 74). Tukey's pairwise tests confirmed that the mean values for the two trawled groups were both significantly higher than that of creeled animals, but were not significantly different from each other.

In the May trial the L-lactate concentrations in the haemolymph of the trawled animals were again elevated, compared with the values for the creeled animals (Fig. 3.4). ANOVA analysis showed that the mean haemolymph L-lactate concentrations did differ significantly between the treatments (F value 171.24, $p < 0.001$, d.f. 124). Tukey's pairwise comparisons confirmed that the mean value for creeled animals was significantly lower than that of the trawled animals, regardless of trawl duration. In addition, animals from one of the dusk trawls (SPM) had significantly lower haemolymph L-lactate concentrations than did those from the two dawn trawls (SAM and LAM). The animals from the other dusk trawl (LPM) also had a lower haemolymph L-lactate concentration than those from the dawn trawls, although in this case the differences were not statistically significant.

Glycogen

In the September trial there was a large intra-specific variability in the concentration of glycogen within the muscles of *N. norvegicus*, as reflected in the large standard deviation for

the creeled group (mean 11.18 $mg.g^{-1}$; SD ±8.78). After trawling for either 1 hour or 5 h, glycogen concentrations in the muscle were also somewhat variable, but they were reduced in comparison to the creeled controls (LPM: mean 7.09 $mg.g^{-1}$; 2.72; SAM: mean 6.20 $mg.g^{-1}$; SD ±3.048) (Fig. 3.5). ANOVA analysis demonstrated that mean glycogen levels for each of the treatments differed significantly from each other (F value 5.44, p=0.006, d.f. 74). Tukey's pairwise tests confirmed that creeled animals had a significantly higher tissue glycogen content than trawled animals, but that no difference existed between animals from the 1h and 5h trawls.

In the May trial, muscle glycogen concentrations (Fig. 3.6) were much lower than the values observed in the September trial. A Kruskal and Wallis ANOVA of the medians indicated a significant difference between treatment medians (H = 24.35, associated p value = <0.001). Further analysis using a Mann Whitney test showed that all other treatments differed significantly from the high value (relatively) obtained for the LAM trawl (median 2.991). However, none of the other trawl durations or capture methods produced a significant difference.

CHH

The CHH analysis was performed only on animals captured during the May trial. Since these data were not normally distributed (Fig. 3.7), they were analysed with the Kruskal and Wallis non-parametric test. This showed that there was a significant difference in the data (H=42.22 with an associated p value of <0.001). Further pairwise comparisons indicated that animals obtained by trawling had significantly higher CHH concentrations than did creeled animals, but that no significant differences existed between trawls of differing durations.

3.3.2 catch data

Tables 3.1 and 3.2 show the composition of the catches, expressed in terms of sex, size and moult state, from the different trawls of the September and May trials respectively, that provided animals for the measurements of damage and mortality. The results show that each trawl catch from the September trial had a very low percentage of soft animals and was composed largely of males, while in the May trial the percentage of soft animals was generally in excess of 90%, with one catch (SAM) containing predominantly males while the other catches contained mainly females.

The mean weight of the total catch, including the retained and discarded *N. norvegicus* together with other species, for the autumn trials was 346 kg and 113 kg for the long and short trawls, respectively, while in the spring it was 577 kg and 262 kg, respectively.

3.3.3 Damage indices

During the trials, damage analysis was performed on over 4000 *N. norvegicus* (1030 and 1815 taken during random sampling in the autumn and spring, respectively, and 1200 assessed during mortality studies).

The damage indices from the September and May trials are presented in Table 3.3 as percentages, categorised according to sex, hardness, season of capture and trawl duration, trawl duration, and size class. The amount of damage incurred by animals during the trawls has been compared using the Chi-squared test, applied to the actual observations.

Of the sexes, females demonstrated an increased susceptibility to damage compared with the males (χ^2 probability <0.001), and of the size classes, the larger animals, greater than 45 mm CL, appeared less susceptible to damage (χ^2 probability <0.001). This was based

on fewer observations in the higher size categories, but was still enough to satisfy the assumptions of the Chi squared test.

Unsurprisingly, recently-moulted soft animals were more susceptible to damage (with only 43% being undamaged) compared with hard animals (53% undamaged) (χ^2 Probability <0.001). In the third hardness category (termed "jelly") only 5% were undamaged, and nearly 60% were highly damaged. However, this category of just- moulted animals comprised less than 2% of the total retained *Nephrops* catch.

In both September and May there was a significantly greater proportion of *N. norvegicus* classified as highly damaged following the long trawls than following the short trawls (χ^2 probability 0.02 and <.0001, for September and May, respectively).

The amount of trawling damage was also affected by the season. Thus a significantly greater proportion of animals were highly or lightly damaged in May than in September (χ^2 probability 0.02).

3.3.4 Mortality

Data are presented as cumulative mortality rates. In the September trial, mortalities increased with time over the first 7 days for all catches (Fig. 3.8). After the first 24 h the catches from the two short trawls (SAM and SPM) had lower levels of mortality than did the catches from the two long trawls (LAM and LPM). However, over the following days the mortality rate of the SPM catch rose more rapidly than for the other catches, so that after 7 days it corresponded more closely to the level for the catches from the long trawls (at >70%). The catch from the SAM trawl had a much lower mortality (<40%).

A similar pattern of results was obtained in the May trial (Fig. 3.9). Thus, after 14 days the mean mortality for the four trawls was 57.0%, which is not significantly different from the value of 72.7% for the four trawls in September (t value 1.278, p value 0.278, d.f 4). Also, after 24 h the catches from the two short trawls (SAM and SPM) had lower levels of mortality than those from the long trawls (LAM and LPM) (Fig 3.9). Moreover, after 7 days the level of mortality in the SPM catch corresponded more closely to a level of the catches from the long trawls (around 60%), while the value for the SAM catch remained below 40% (Fig. 3.9).

Figure 3.10 shows the mortalities over the 14-day period of measurement for animals assigned to each damage category. The degree of mortality increased with the amount of damage incurred (χ^2 probability <0.001), illustrating the negative impact of physical damage on the post capture survival of *N. norvegicus*.

It should also be noted that from all trawls around 50% of *N. norvegicus* were classified as undamaged, whereas the number that survived after 14 days was in most cases smaller. This, as well as the mortalities observed in undamaged animals, suggests that factors other than physical damage were responsible for their deaths.

To summarise, the mortality data demonstrate that reducing the duration of the trawl does reduce the subsequent mortality rates, but only if capture is followed by a long recovery period submerged in seawater. During both trials when a short trawl was followed by such a period of recovery, then substantially lower mortalities occurred.

3.4 DISCUSSION

3.4.1 Haemolymph parameters

Total haemocyte counts (THCs)

The monitoring of total haemocyte counts (THCs) has been used for some time as a measure of stress in crustaceans (Mix & Sparks, 1980; Martin & Graves, 1985; Lorenzon *et al.*, 2001), and the September trial demonstrated that the duration of the trawl has a significant effect on the THCs of *N. norvegicus*. This is thought to reflect an immuno-suppression (Lorenzon *et al.*, 2001), which might provide a critical window for opportunistic pathogens such as *Vibrio* species to exploit the host and initiate spoilage (even if THCs return to normal following the chronic stress of a trawl).

However, in the May trials there was no significant difference between the THCs of creeled animals or any of the trawl combinations. These findings suggest that the stresses from trawling are not sufficient in themselves to cause a systematic reduction in circulating haemocytes beyond that due to such factors as life stage, moult cycle, level of nutrition, disease and physiological or environmental conditions (Persson *et al.*, 1987; Söderhall *et al.*, 1988; Truscott & White, 1990; Smith & Johnson, 1992). In this measure, emersion of the animals following capture is a more powerful stressor, as it causes significant reduction in THCs (Chapter 2). In future trials it is strongly recommended that a control group of animals is obtained by creeling on or adjacent to the trawled ground, so that their THCs accurately represent the values of "unstressed" members of the same population. In the two trials that make up this study, the creeling locations were dictated by economic and logistical considerations. Specifically, creeling was restricted to locations within Clyde 'protected waters', i.e. north of a line from Portencross on the Ayrshire mainland to

Skipness on the Kintyre peninsula, passing 1 mile south of Little Cumbrae Island, as this is the operating zone stipulated by the Maritime and Coastguard Agency (MCA) for the vessel, *R.V. Aplysia*. In practice, creeling could only take place in areas within this zone where trawling did not regularly occur, or at times when trawlers were absent.

L-lactate

The haemolymph L-lactate concentrations reported in the present study demonstrate that there is a greater than 8-fold increase following trawling, compared with the creeled controls. Spotts & Lutz (1981) reported a similar 6-fold increase when they exhausted the fresh water prawn *Macrobrachium rosenbergii.* Some of this L-lactate is produced as a result of the extreme metabolic demand placed on the animal both by its attempts to swim away from the net, and by its struggling movements within the net. Both these reactions result from tail-flipping, which involves activation of the whole abdominal musculature, and requires the production of energy by anaerobic processes since oxygen delivery to the tissues cannot meet the required demand. L-lactate is the major end-product of anaerobic metabolism in crustaceans (Ellington, 1983), and accumulates in the haemolymph (Stentiford *et al.*, 2001b).

The results indicate that increasing the duration of the trawl from 1 hour to 5 h does not cause a greater accumulation of L-lactate in the haemolymph. This may reflect the fact that the animals eventually cease to struggle, and are not therefore subjected to further physiological stresses. However, it also suggests that while tightly packed within the cod-end of the net they do not resume aerobic respiration to an extent that is sufficient to clear L-lactate from their haemolymph. These results are consistent with the finding that crustaceans can take as long as 24-48 h to recover to unstressed levels (McMahon *et al.*, 1978; Crear & Forteath, 2001; Chapter 4).

The significance of the measured elevation of L-lactate concentrations is that it affects the survivability of crustaceans. Taylor and Spicer (1987) described a L-lactate-induced mortality of *Palaemon serratus,* and concluded that it was most probably caused by a breakdown of the acid-base regulatory system. L-lactate is also known to induce release of the hormone Crustacean Hyperglycaemic Hormone (CHH), which mobilises glucose from tissue glycogen stores (Sedlmeier, 1985). Therefore elevated L-lactate levels for prolonged periods may disrupt the dynamics of carbohydrate storage and release. Some evidence for this was provided by the measurements of glycogen levels made in this study.

In both trials the time of landing had a significant effect on L-lactate concentrations, with *N. norvegicus* landed in the morning having higher concentrations. There are a number of possibilities for this observation, but further studies are needed to substantiate these. The morning trawls were lifted after sunrise, always in bright sunlight, and it is possible that the higher temperatures and/or eye damage induced by this bright day light could induce further stress in the animals. Generally, *N. norvegicus* were exposed to air for less than 5 minutes and sampled within 30 minutes; however, these short periods of air exposure at high temperatures may cause elevated L-lactate levels. However, it has been established that even such brief exposures of the eye of *N. norvegicus* to light can irreversibly damage a large proportion of the retina and dioptric apparatus (Shelton *et al.,* 1985; Gaten, 1988; Gaten *et al.,* 1990). Chapman *et al.* (2000) concluded that light induced eye damage in *N. norvegicus* is permanent, but does not seem to influence their long term survival, growth or reproduction. Moreover, it is unknown if there is a stress response of the animal associated with eye damage.

In Crustacea there is a circadian rhythm in the concentrations of CHH and glucose in the haemolymph, with peak glucose concentrations appearing several h after the onset of

darkness (Hamann, 1974; Strolenberg, 1979; Reddy *et al.*, 1981; Kallen *et al.*, 1990). It is believed that this peak coincides with the peak foraging periods of the animal, allowing it to meet physiological energy requirements (for a review see Kleinholz, 1985). This circadian rhythm could be the cause of the increased concentrations of L-lactate observed in the morning trawls, as the *N. norvegicus* caught in the evening trawls are better adjusted to cope with the physiological stress. The height and duration of this peak are species-specific and are affected by seasonal influences as well as by physiological events (e.g. moulting) (Kallen *et al.*, 1990). Further research is required on the phenomenon in *N. norvegicus* in order to discover if this circadian rhythm is responsible for the elevated L-lactate concentrations observed following morning trawls.

Glycogen

The values obtained for glycogen reserves of "unstressed" animals show a large variation between individuals taken from the same location at the same time. This large variability in body condition may be due to a number of factors, but its existence makes it difficult to compare the effects of different treatments, such as the method of capture, on glycogen levels.

Nevertheless, during the September trials a statistically significant decrease in glycogen concentrations in abdominal muscles was found in both the trawled groups, compared with the creel-caught controls. This suggests the following scenario. In response to the stresses of trawling, and in part due to the accumulation of L-lactate in the haemolymph, the hormone CHH is released into the circulation. It acts on various carbohydrate stores around the body, probably in both the hepatopancreas and the muscle, to cause the conversion of glycogen to glucose and the release of the latter into the haemolymph. Similar interpretations have been made during crustacean aerial exposure studies by Santos

& Keller (1993b). The association of increasing haemolymph L-lactate levels and decreasing tissue glycogen levels makes it possible to suggest that measurements of L-lactate alone, which rise from baseline values and hence are not as prone to inter-individual variability, may be sufficient to indicate the degree to which animals have been subjected to stress during trawling.

Comparison of the May and September trials highlights the large seasonal variation that occurs in muscle glycogen levels of *N. norvegicus*, a fact that has previously been reported by Baden *et al.* (1994), who concluded that the variation was probably due to the seasonal abundance of food on the sea floor. However, these very low levels in May, combined with the large inter-individual variation, have obscured any differences associated with trawl duration, even when compared with creeled animals. It should also be noted that although the animals were in a poorer condition in May, as judged by their glycogen reserves, this did not cause a measurable increase in their mortality after 14 days, averaged out across the four trawls, compared with September data.

The possibility exists that these variations in glycogen levels may affect the taste of the tail meat of *N. norvegicus*. This requires more directed studies looking at a combination of more detailed chemical analyses and sensory organoleptic measures.

CHH

The data have shown a clear effect of trawling on CHH concentrations in the haemolymph of *N. norvegicus*. There have been few other studies on the effect of stress on *N. norvegicus* CHH concentrations. Stentiford *et al.* (2001b) studied the effect of the parasite *Hematodinium* in *N. norvegicus* and discovered that as the parasite utilised the haemolymph glucose reserves, more CHH was released for glycolysis. They found very

similar levels to that found in this study. In Chapter 2 of the thesis it was demonstrated that following emersion at high temperatures, CHH levels rose higher than those observed here. Interestingly, both L-lactate and CHH rose far more in simulated post capture procedures than during the capture process, suggesting that the post capture aerial exposure may possibly be stressing *N. norvegicus* more than the capture process itself. The CHH data suggest that decreasing the trawl duration may have little effect on the stress experienced by *N. norvegicus* during the capture process, and that the post capture process may indeed be the more stressful process.

The control of carbohydrate dynamics in the haemolymph and tissues of crustaceans is known to be exerted mainly by circulating levels of CHH (Sedlmeier, 1985). CHH stimulates glycogenolysis, which increases glucose availability in the haemolymph and reduces glycogen levels within the tissues (Santos & Keller, 1993b). When the animal swims it utilises available glucose and thus lowers glucose concentrations in the haemolymph. This will induce release of CHH (by reducing the negative feedback from glucose) (Santos & Keller, 1993a) that in turn mobilises glucose from glycogen stores in the tissues, which thereby become progressively depleted. To summarise, CHH-induced hyperglycaemia may be involved in the 'flight or fight' response in these animals.

All the data from the creeling studies indicate that *N. norvegicus* are exposed to little physiological or immunological stress during the creeling process. The L-lactate and CHH levels measured in *N. norvegicus* shortly after creeling are comparable to those found in animals recovered in the aquaria for two weeks following trawl capture (Chapter 2). This has illustrated the suitability of creeling as a low stress comparison in trawling stress trials. These results however contrast with those of Spicer *et al.* (1990) who reported greater L-lactate concentrations and a greater hyperglycaemic response in the haemolymph of *N.*

norvegicus immediately after creeling than after trawling. The reasons for this are unclear, the creels were situated at the same location in both investigations, however Spicer *et al.* (1990) only trawled for 30 minutes, and the density of the individuals in the creels was not reported. These factors may account for the contrasting results obtained.

3.4.2 Damage indices

The damage assessment data confirm that a greater proportion of animals are categorised as heavily damaged following longer trawls and following trawling in the spring.

There have been few studies on the damage caused to *N. norvegicus* by trawling, and most of these have concentrated on damage to small sized *N. norvegicus* that are discarded after capture (Symonds & Simpson, 1971; Gueguen & Charau, 1975; Harrris *et al.*, 1997). These previous studies of escaped and discarded *N. norvegicus* have indicated that more than 50% of the animals that encountered the trawl had suffered physical damage. Moreover, in the study by Harris *et al.* (1997), around 15% of the animals had a crushed or punctured cephalothorax and/or abdomen. The present study has obtained very similar values for both the general and specific measures of damage.

With trawl landings, damage can occur in the trawl net itself, due to crushing or to the abrasive action of other species or objects in the cod-end (Lancaster & Fridd, 2002), and during hauling, due to the pressure differences experienced (ICES, 1994).

During the May trials, trawling of *N. norvegicus* resulted in a significantly greater degree of damage. It is known that *N. norvegicus* females moult with a seasonal pattern depending on their size, but that the males do not follow a seasonal pattern. Bailey (1984) found in the Clyde Sea Area that small females showed a peak of moulting in the early summer whereas larger females moulted later in the year, during the autumn. It was also

reported that females in the size class 21-33 mm CL showed peaks of moulting in both the spring and winter as females approached maturity. Sarda (1991) has also shown that mature females are found, albeit in small numbers, all year round in the Catalan Sea. Mouat (2002) reported that during the September following moulting and copulation, female *N. norvegicus* remain mainly in their burrows incubating the eggs and so are unavailable to the fishery.

A similar seasonal pattern of damage was observed by Smith & Howell (1987) who studied the effects of trawl capture on American lobsters, *Homarus americanus*. They observed that damage varied seasonally and was greater during months when moulting was occurring.

During the present study 97% of *N. norvegicus* caught in the September trial were classified as "hard" compared with only 10% in May, when 87% of the catch were classified as "soft". It has been demonstrated that the larger number of soft animals during the spring period resulted in more physical damage during the May trials, suggesting that soft animals are more susceptible to damage. Interestingly during the May trials only 3% of *N. norvegicus* caught were classified as "jelly" or very soft, suggesting that these very soft animals may change the behaviour and stay in burrows during this vulnerable stage.

It has been hypothesised that physical damage will provide a route of entry for bacterial pathogens. Thus, the type and extent of damage to individual *N. norvegicus* will likely affect survivorship if they are to be kept alive for marketing purposes. This has been found in other Crustacea. Stevens (1990) investigated the survival of trawl-caught king crab (*Paralithodes camtschaticus*) and tanner crab (*Chinoecetes bairdi* and *C. opilio*) in the Bering Sea. After injuries to limbs and the cephlothorax, survival rates in experimental tanks were 50% and 75%, respectively, for the two species. Lancaster & Fridd (2002)

looked at the damage to undersized *C. crangon* following trawling, and reported low mortalities and only the occasional loss of telson or antennae after the catch was sorted by a mechanical riddle. In the Belgian *C. crangon* fishery, Mistakidis (1958) found that a maximum of 25% of discarded shrimps did not survive the trawling and sorting process, mainly as a consequence of desiccation. Troffe *et al.* (2003) reported that the survival of the humpback shrimp (*Pandalus hypsinotus*) released after trawling or trapping depends on the extent of the body region-specific anatomical damage that has occurred and its functional importance.

The data obtained in the present study suggest that physical damage, although differing significantly between trawls of different durations, does not account for the large difference in mortality rates observed in Figures 4 and 5. Physical damage indices may be a good tool for assessing the suitability of the catch for acceptance to the market, but do not provide an accurate measure of future mortality rates. A more reliable indication may be given by measures of internal state of the animal, such as the L-lactate concentration and Total Haemocyte Counts.

A number of different damage scales have been used in the past to estimate injuries sustained during trawl capture (Wassenberg & Hill, 1989; Mensink, 2000; Salini, 2000; Pranovi *et al.*, 2001; & Troffe *et al.*, 2003). This approach allows for the relative fragility of the target animal to be assessed. In the present study a three-level scale was used for damage assessment, which was capable of representing differences in the physical damage caused by the various treatments. Within the three-level scale there was a clear relationship between the extent of damage and the corresponding survival time, suggesting that the assignment to a category accurately represented the health status of *N. norvegicus*.

3.4.3 Mortality

During both trials the animals that were landed at dawn fared better in terms of survival than did those landed at dusk. An explanation for this may relate to the fact that, after initial sorting on the deck, the catch from each trawl was placed in seawater tanks, and remained submerged until arrival at the processing plant in Ayr, when the animals were further handled and exposed. This period of submergence was around 12 h for the dawn catch, but was no more than 3 h for the dusk catch. It can be hypothesised that it is this greater period of submergence between landing and re-handling at the processing plant that explains the lower mortality rates in the dawn catch. If so, this would highlight the importance of recovery periods in the post capture protocol. The oxygen debt and respiratory acidosis (following trawling and subsequent aerial exposure) are reduced during the periods that *N. norvegicus* are submerged in the seawater tanks, but if lobsters are further stressed, by re-handling or aerial exposure before this recovery is complete, the acidiotic stress will be compounded, which Taylor *et al.* (1997) predicted may potentially reduce survival time.

It is noticeable that during both trials it was also the catch from the shorter trial (SAM) that had the better survival, indicating that both trawl duration and the post capture protocol are important in determining the extent of stress experienced by trawl-caught *N. norvegicus.*

Due to limitations of the sampling process, the causes of death were not determined in this study. Future studies should therefore attempt to detail gross clinical signs of the animal at the time of death. The economic impact of such conditions as idiopathic muscle necrosis (IMN) and bacterial necrosis can then be further understood.

3.4.4 Conclusions

The physiological stresses of trawling can be monitored by measuring haemolymph L-lactate concentrations and tissue glycogen concentrations, although the former measure is more easily interpreted. L-lactate concentrations increase significantly as a result of trawl capture, and remain elevated, with no further increase, when animals are held in the net for up to 5 h.

The immune status of the animal is conveniently assessed by total haemocyte counts. This indicates a trend towards progressive loss of immune capacity with increasing durations of trawling, although the values were in many cases not statistically significant.

While the chain of events linking elevated L-lactate concentrations, release of CHH and immuno-suppression remain to be determined, it can nevertheless be concluded that the process of trawling will increase the susceptibility of animals to invasion by spoilage bacteria, leading to tail necrosis. This process will be further exacerbated by exposure to the air or to elevated temperature during post capture procedures.

The mortality rates indicate that trawl duration does have a significant negative effect on the survivability of the catch. However, the importance of the post capture protocol is evident, reflected in the large difference in mortality rates between landings at dawn and at dusk.

These two trials allow a best practice for securing maximum survival and minimum stress to be proposed. From the data it is suggested that trawls should be of short duration, the net should be lifted in the dark, then before they are re-handled the animals should be allowed a long recovery period (*ca* 8 h) while submerged in water on board the vessel or at the port.

Table 3.1:

The percentage of males (Male) and soft animals (Soft), and the mean carapace length (CL) (mm) of the four trawls during the September trial. SAM, short morning (am) trawl; LPM, long afternoon (pm) trawl; LAM, long morning (am) trawl; SPM, short afternoon (pm) trawl.

	SAM	LPM	LAM	SPM
% Male	79.22	78.37	66.67	88.56
% Soft	1.1	1.9	0.8	0.9
CL (mm)	32.696	33.627	35.597	35.672
± S.D	± 3.498	± 3.212	± 3.239	± 3.454

Table 3.2:

The percentage of males (Male) and soft animals (Soft), and the mean carapace length (CL) (mm) of the four trawls during the May trial. SAM, short morning (am) trawl; LPM, long afternoon (pm) trawl; LAM, long morning (am) trawl; SPM, short afternoon (pm) trawl.

	SAM	LPM	LAM	SPM
% Male	75.70	28.65	40.84	31.55
% Soft	92.3	96.8	86.1	92.4
CL (mm)	35.790	35.645	33.563	35.273
± S.D	± 3.417	± 4.089	± 3.867	± 4.099

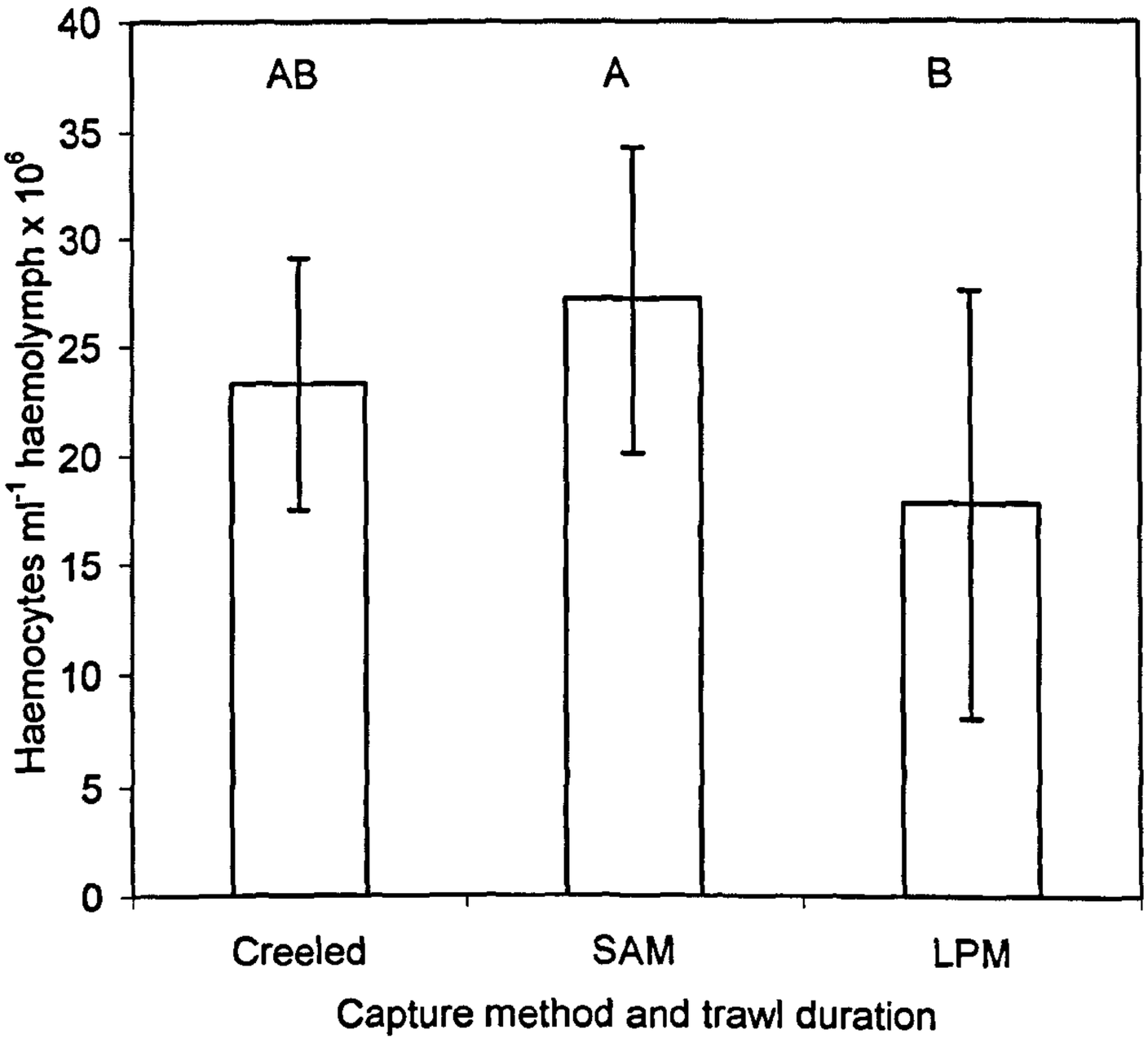
Table 3.3

The susceptibility to damage, expressed as percentage, of *N. norvegicus* categorised into different groups according to sex, size class (CL mm), hardness, season of capture and trawl duration and trawl duration.

	% of catch	% Highly damaged	% Lightly damaged	% Undamaged
Sex				
Male	32.9	14.2	34.5	51.3
Female	30.9	18.6	43.7	37.7
Unclassified	36.2	15.7	34.3	50
Size Class				
25-29	2.6	19.4	45.8	34.7
30-34	45.8	15.2	36.1	48.7
35-39	41.6	18.2	37.1	44.7
40-44	7.9	12.2	39.2	48.6
44-49	1.7	6.2	46.9	46.9
Hardness				
Hard	41.9	13.6	33.1	53.3
Soft	56.0	16.5	40.3	43.2
Jelly	2.1	58.3	36.7	5.0
Season and Trawl duration				
Autumn long	23.0	18.1	33.4	48.5
Autumn short	13.2	11.7	35.9	52.4
Spring long	32.2	20.1	39.6	40.3
Spring short	31.6	12.7	38.1	49.2
Trawl duration				
Long	55.2	19.2	37.0	43.8
Short	44.8	12.4	37.5	50.1

Figure 3.1: The effect of different capture methods and trawl duration on the total haemocyte counts (THCs) of *N. norvegicus* during the September 2003 trial. SAM, short (1h) trawl at dawn; LPM, long (5h) trawl at dusk. N = 25 for each group. Within each trawling trial different letters indicate significant differences between the times, as indicated by Tukey's pairwise comparisons. Error bars represent standard deviations.

Figure 3.2: The effect of different capture methods and trawl duration on the total haemocyte counts (THCs) of *N. norvegicus* during the May 2004 trial. SAM, short (1h) trawl at dawn; SPM, short trawl at dusk; LAM, long (5h) trawl at dawn; LPM, long trawl at dusk. N = 25 for each group. Within each trawling trial different letters indicate significant differences between the times, as indicated by Tukey's pairwise comparisons. Error bars represent standard deviations.


40
35
30
25
20
15
10
5
0
AB
A
B
Haemocytes ml-1 haemolymph x 10^6
Creeled
SAM
LPM
Capture method and trawl duration

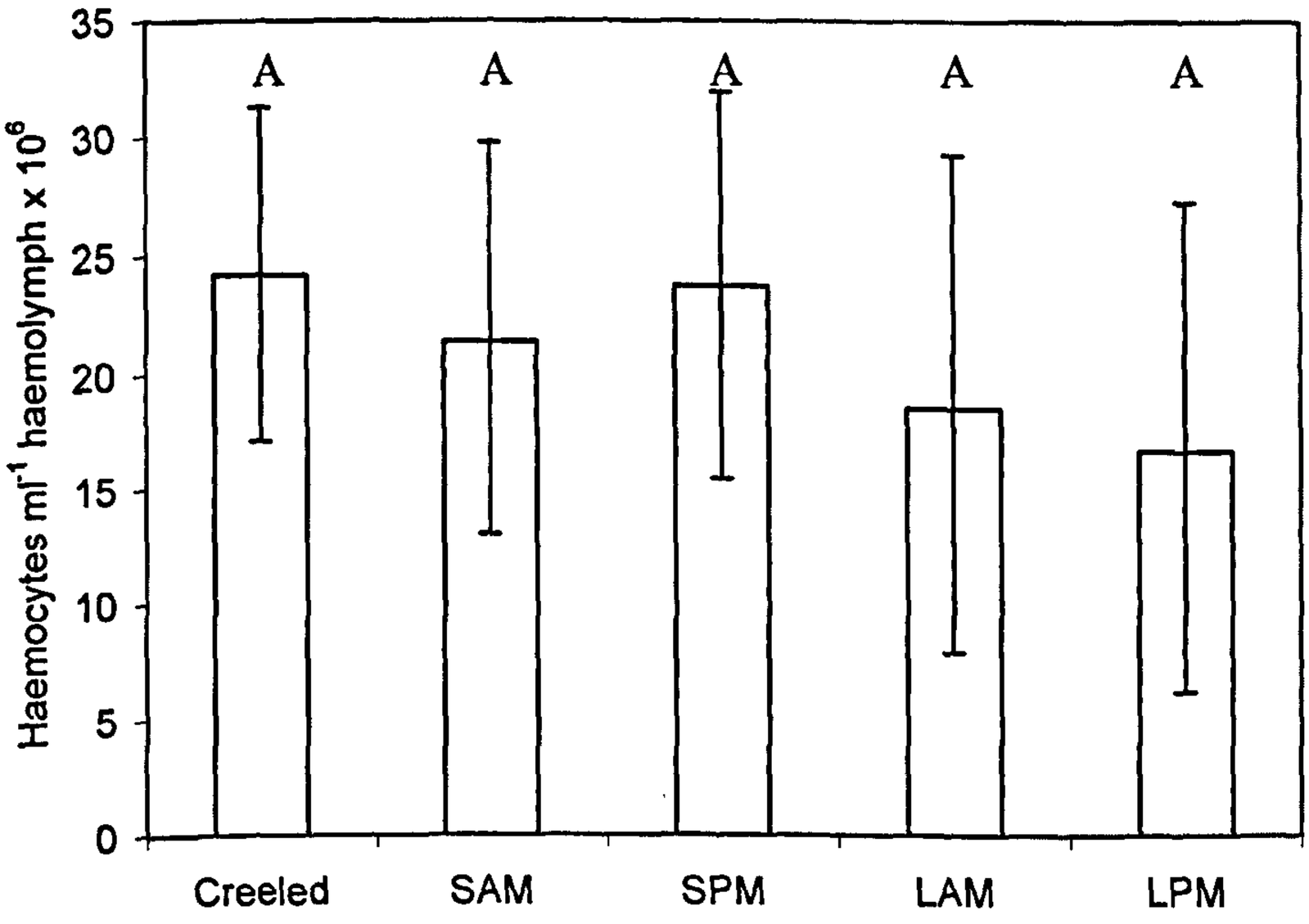
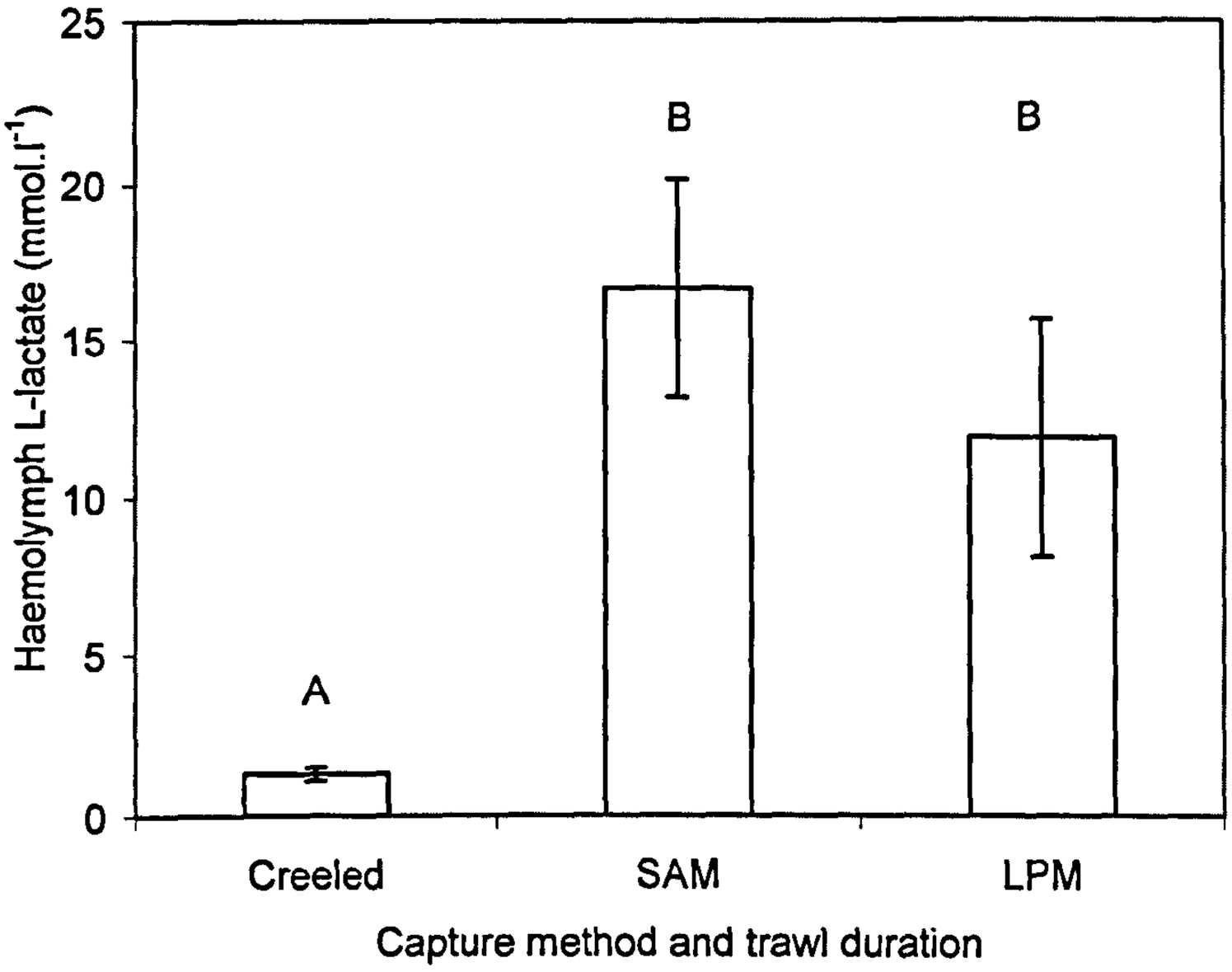

35
30
25
20
15
10
5
0
A
A
A
A
A
Haemocytes ml-1 haemolymph x 10^6
Creeled
SAM
SPM
LAM
LPM
Capture method and trawl duration

Figure 3.3: The effect of different capture methods and trawl durations on the haemolymph L-lactate concentrations (mM) of *N. norvegicus* during the September 2003 trial. SAM, short (1h) trawl at dawn; LPM, long (5h) trawl at dusk. N = 25 for each group. Within each trawling trial different letters indicate significant differences between the times, as indicated by Tukey's pairwise comparisons. Error bars represent standard deviations.

Figure 3.4: The effect of different capture methods and trawl durations on the haemolymph lactic acid concentrations (mM) of *N. norvegicus* during the May 2004 trial. SAM, short (1h) trawl at dawn; SPM, short trawl at dusk; LAM, long (5h) trawl at dawn; LPM, long trawl at dusk. N = 25 for each group. Within each trawling trial different letters indicate significant differences between the times, as indicated by Tukey's pairwise comparisons. Error bars represent standard deviations.


25
20
15
10
5
0
Haemolymph L-lactate (mmol.l-1)
A
B
B
Creeled
SAM
LPM
Capture method and trawl duration

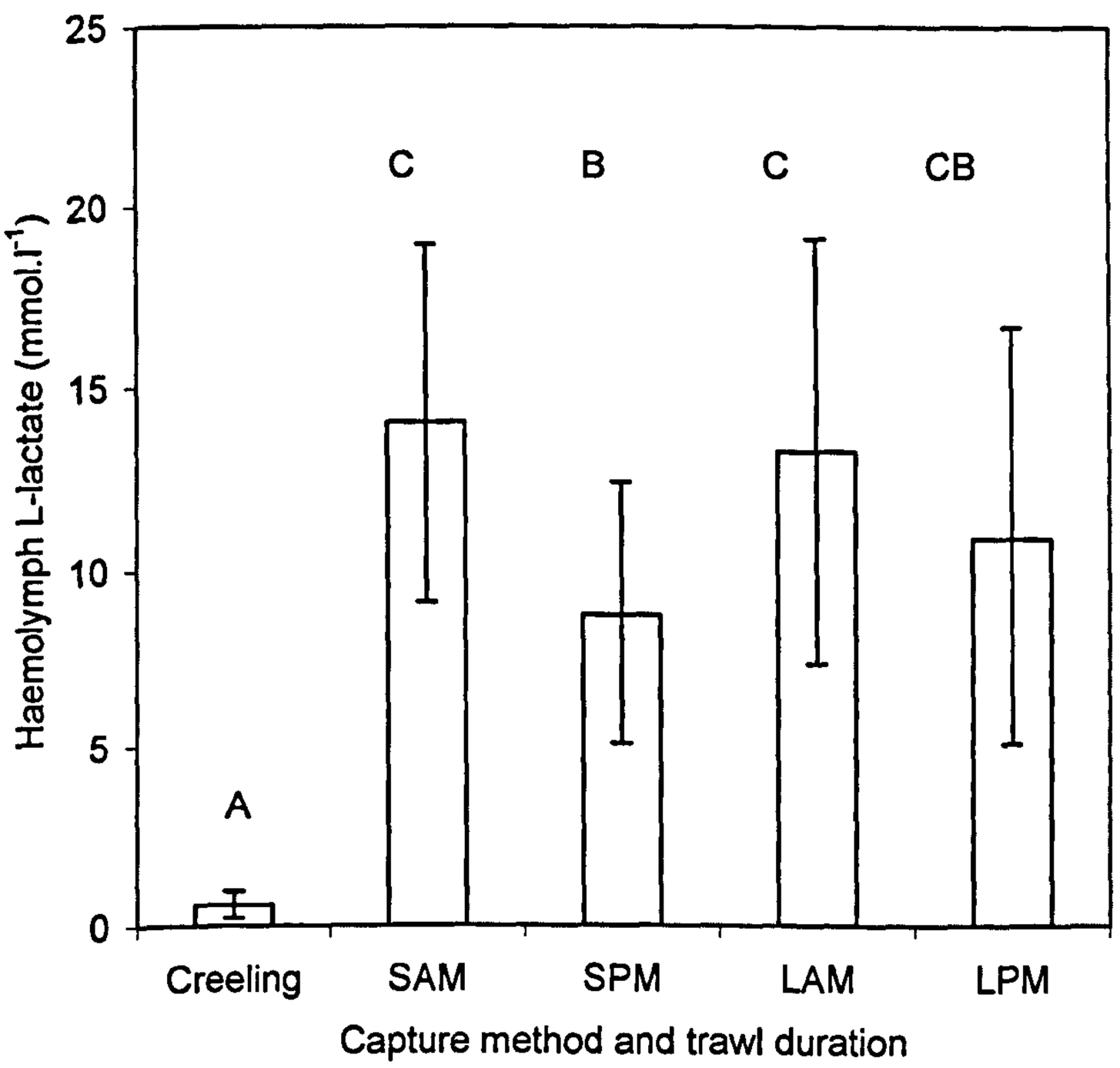
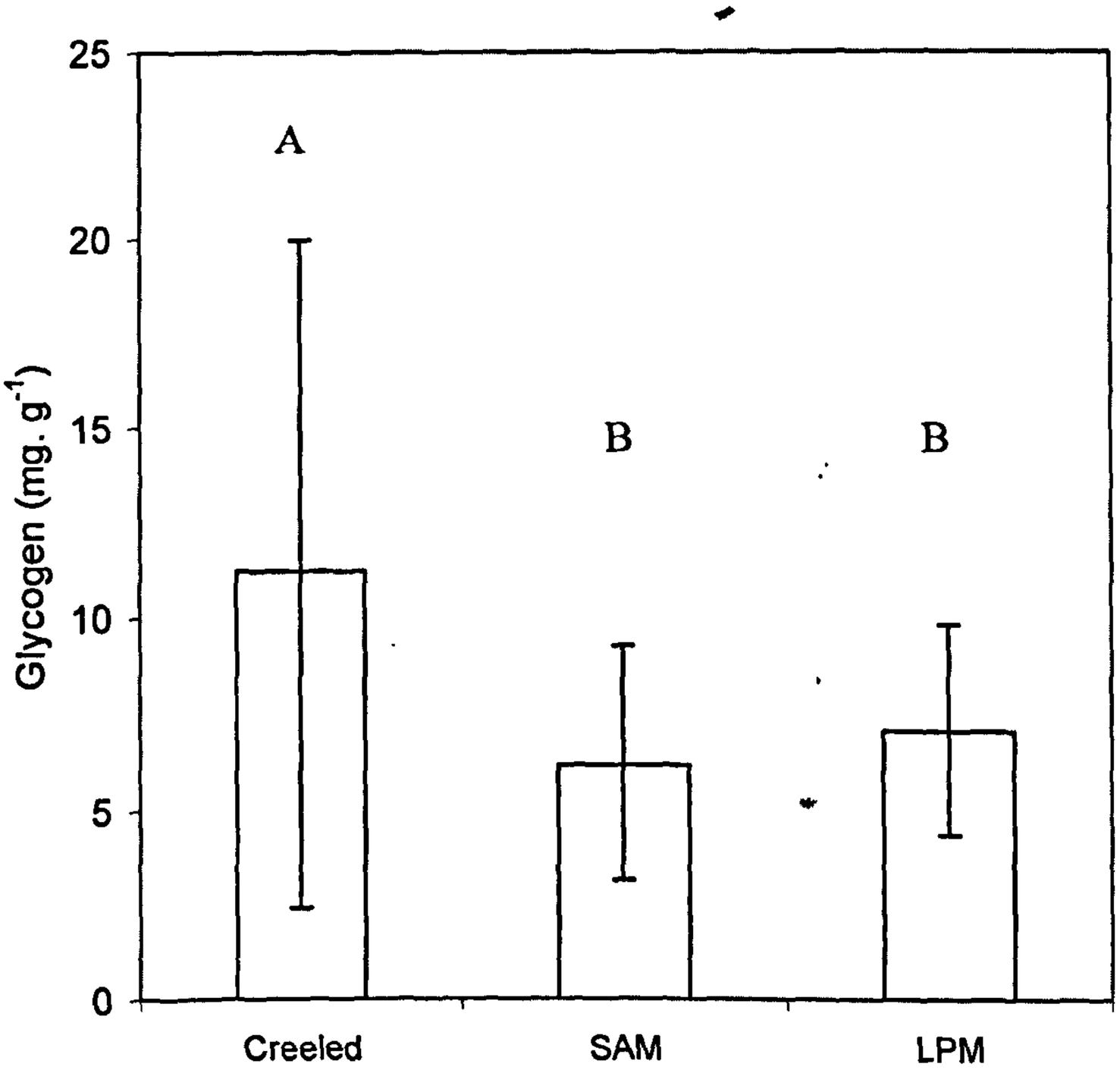

25
20
15
10
5
0
Haemolymph L-lactate (mmol.l-1)
A
C
B
C
CB
Creeling
SAM
SPM
LAM
LPM
Capture method and trawl duration

Figure 3.5: The effect of different capture methods and trawl durations on the levels glycogen ($mg.g^{-1}$ tissue) in the abdominal muscles of *N. norvegicus* during the September 2003 trial. SAM, short (1h) trawl at dawn; LPM, long (5h) trawl at dusk. N = 25 for each group. Within each trawling trial different letters indicate significant differences between the times, as indicated by Tukey's pairwise comparisons. Error bars represent standard deviations.

Figure 3.6: The effect of different capture methods and trawl durations on the levels glycogen ($mg.g^{-1}$ tissue) in the abdominal muscles of *N. norvegicus* during the May 2004 trial. SAM, short (1h) trawl at dawn; SPM, short trawl at dusk; LAM, long (5h) trawl at dawn; LPM, long trawl at dusk. N = 25 for each group. Within each trawling trial different letters indicate significant differences between the times, as indicated by Mann-Whitney pairwise comparisons. Error bars represent the interquartile range.


25
20
15
10
5
0
Glycogen (mg. g-1)
A
B
B
Creeled
SAM
LPM
Capture method and trawl duration

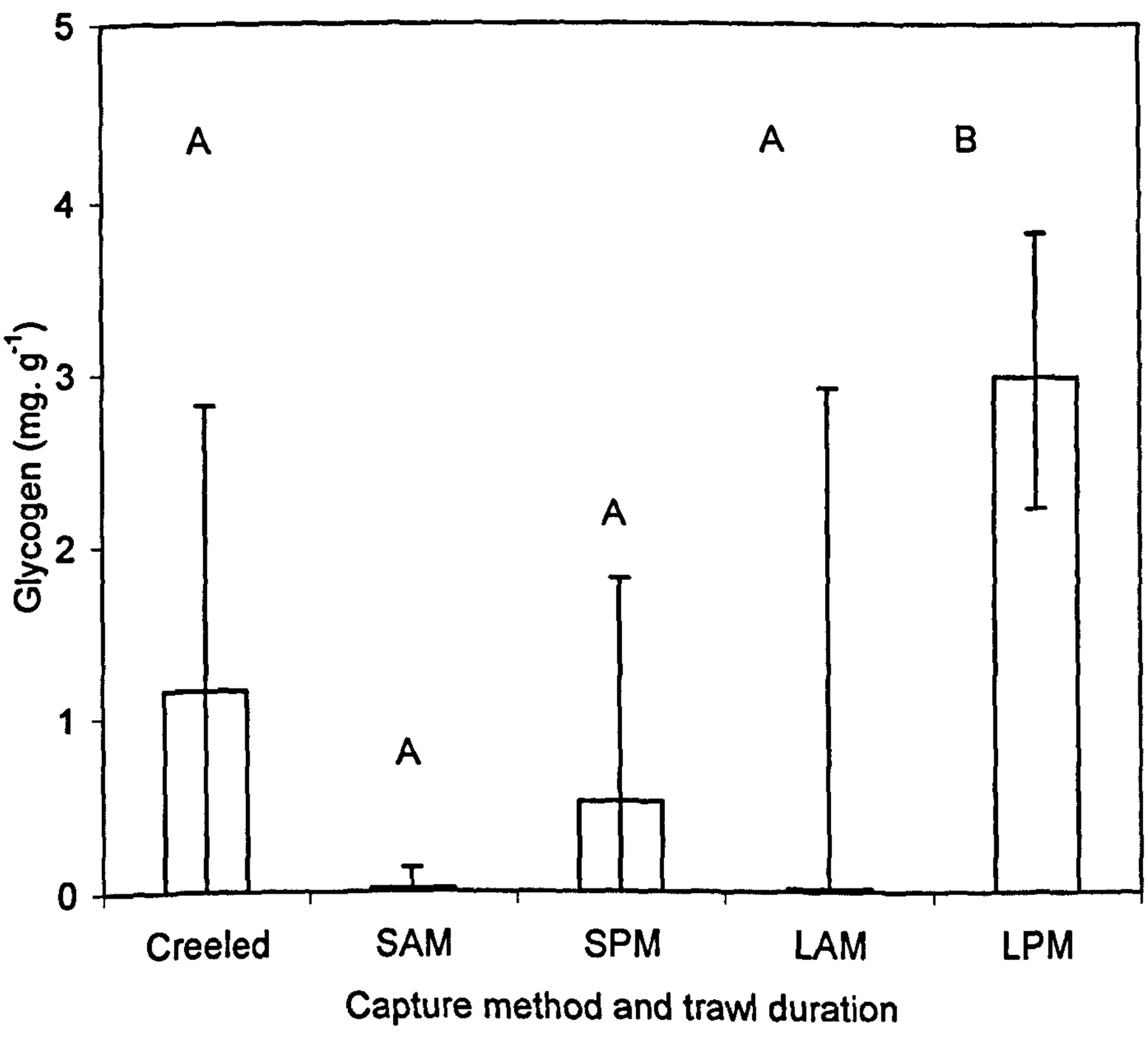
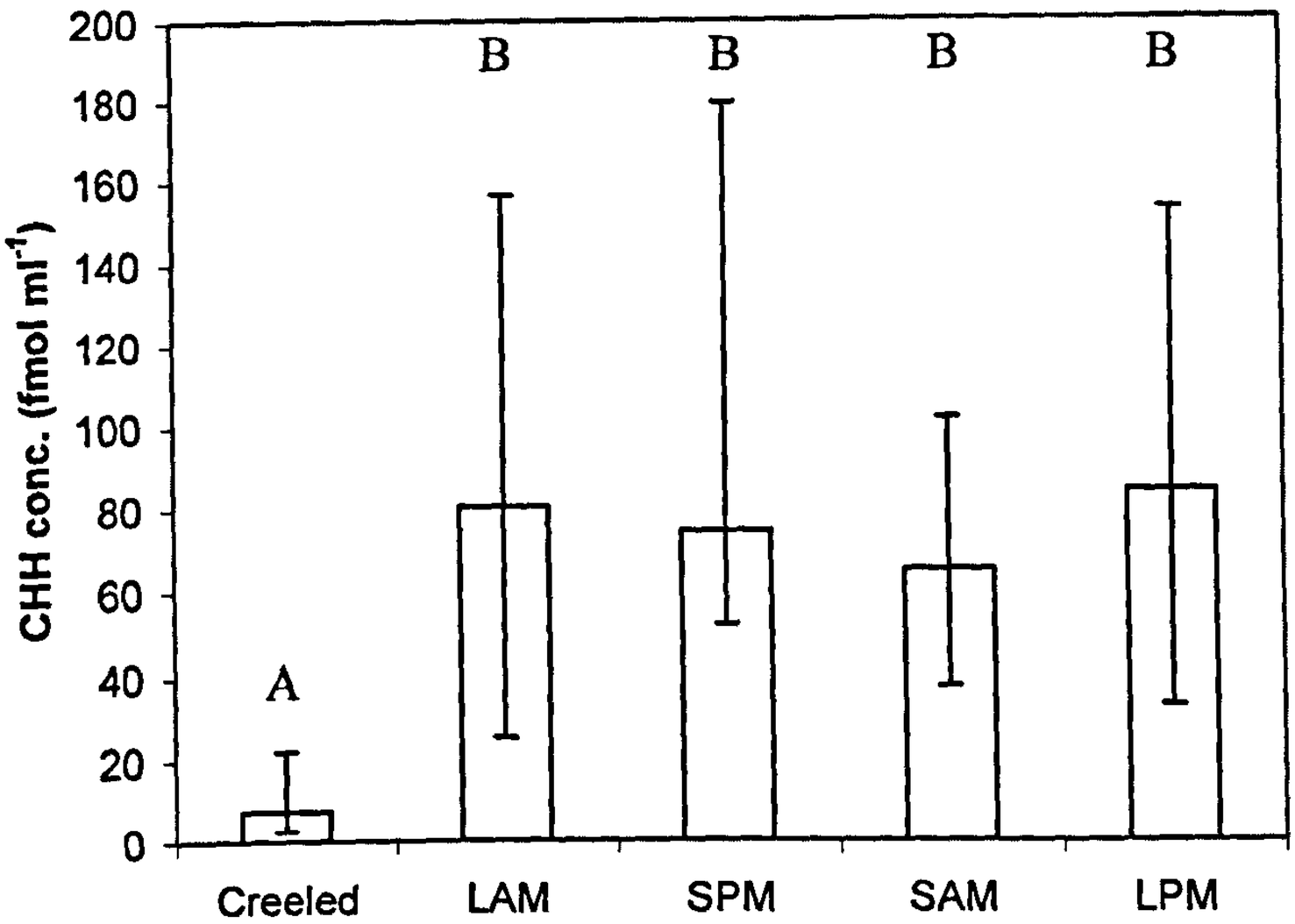

5
4
3
2
1
0
Glycogen (mg. g-1)
A
A
A
A
B
Creeled
SAM
SPM
LAM
LPM
Capture method and trawl duration

Figure 3.7: The effect of different capture methods and trawl durations on the median concentration of the crustacean hyperglycaemic hormone (CHH) (fmol.ml^{-1}) in the haemolymph of *N. norvegicus* during the May 2004 trial. SAM, short (1h) trawl at dawn; SPM, short trawl at dusk; LAM, long (5h) trawl at dawn; LPM, long trawl at dusk. N = 25 for each group. Within each trawling trial different letters indicate significant differences between the times, as indicated by Mann-Whitney pairwise comparisons. Error bars represent the interquartile range.

Figure 3.8: Cumulative mortality rates (%) of *N. norvegicus* following the four trawls up to 14 days after landing for the September Trial. SAM, short (1h) trawl at dawn; SPM, short trawl at dusk; LAM, long (5h) trawl at dawn; LPM, long trawl at dusk.


200
180
160
140
120
100
80
60
40
20
0
CHH conc. (fmol ml-1)
B
B
B
B
A
Creeled
LAM
SPM
SAM
LPM
Capture method and trawl duration

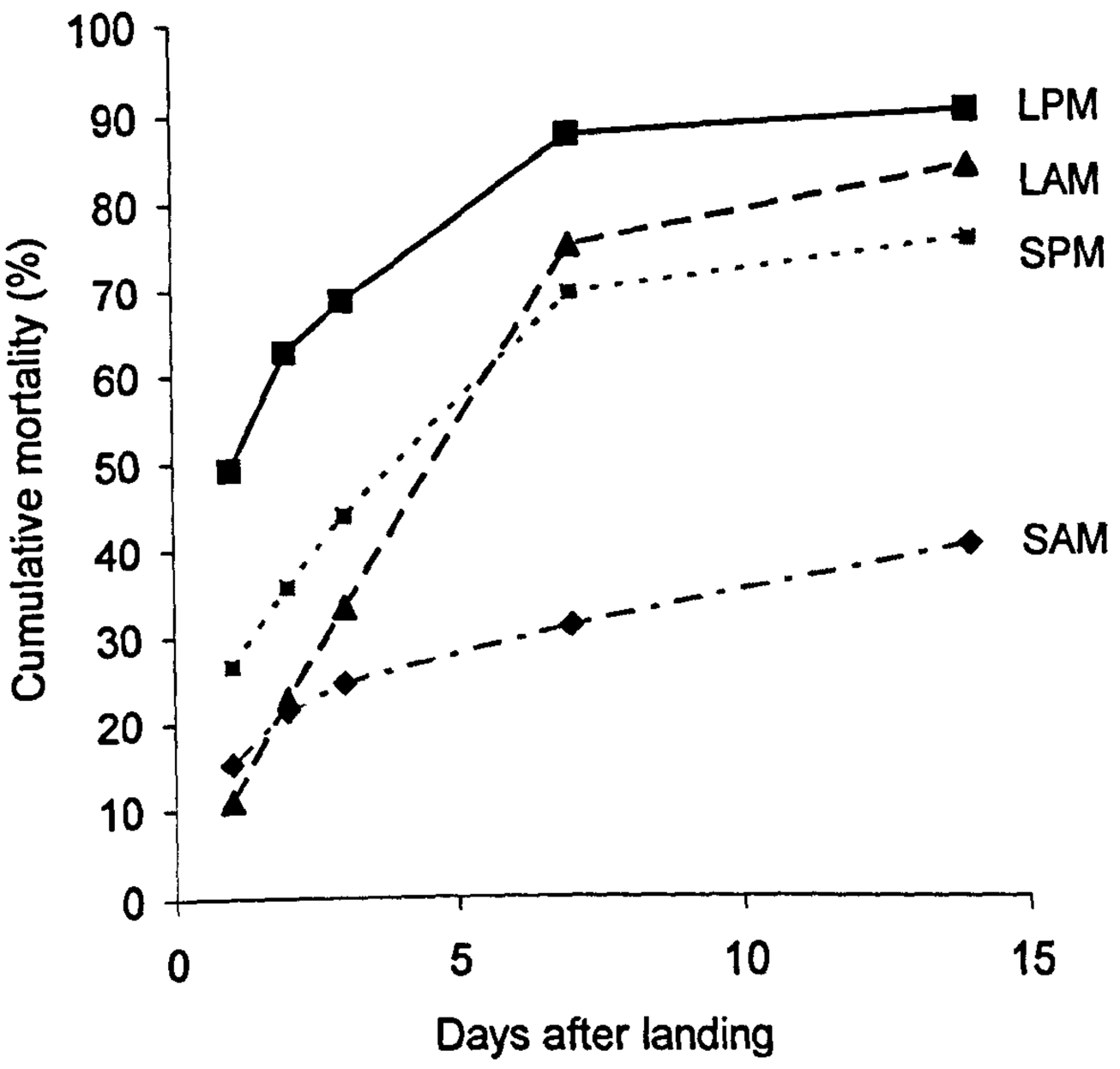
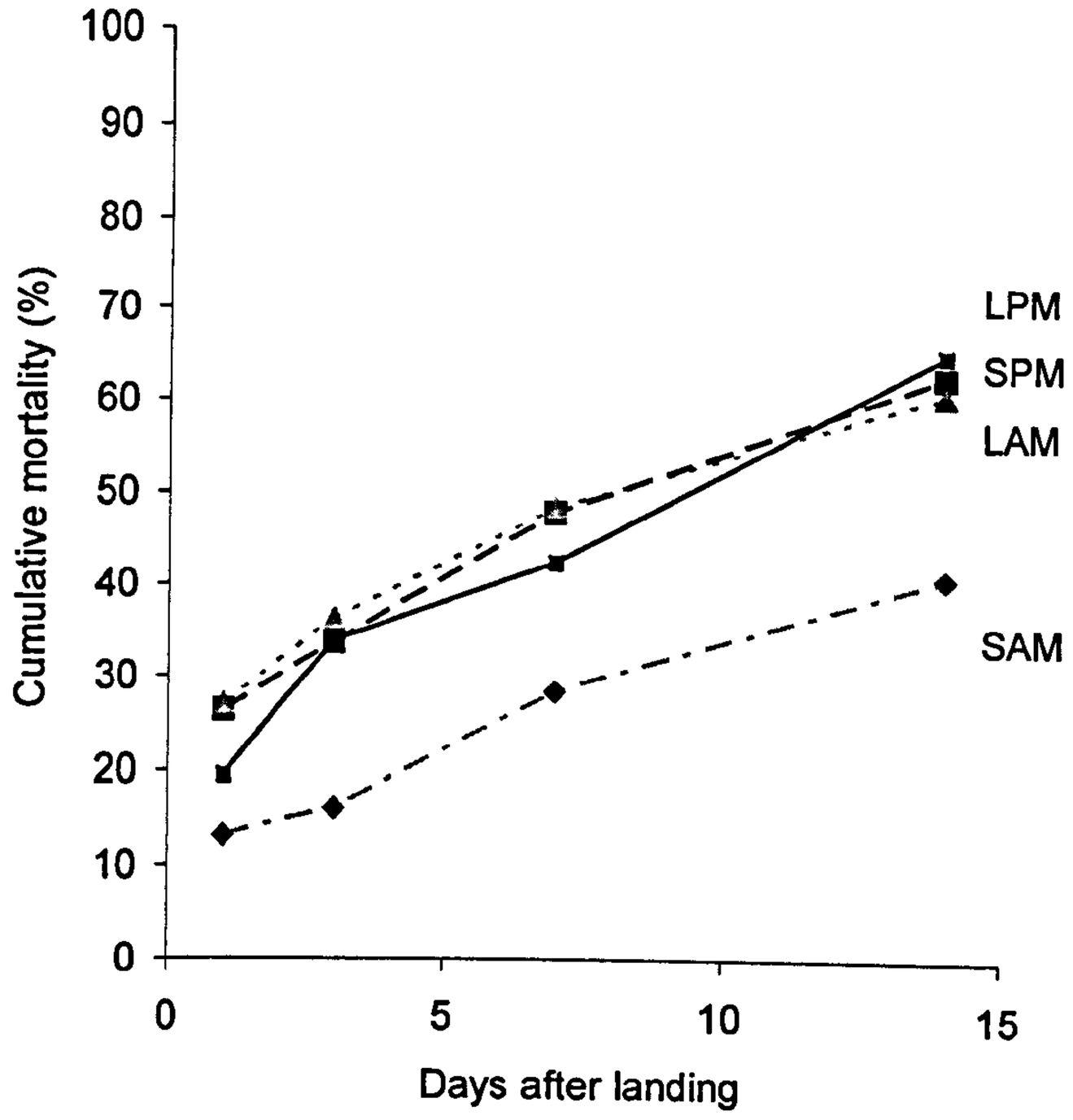
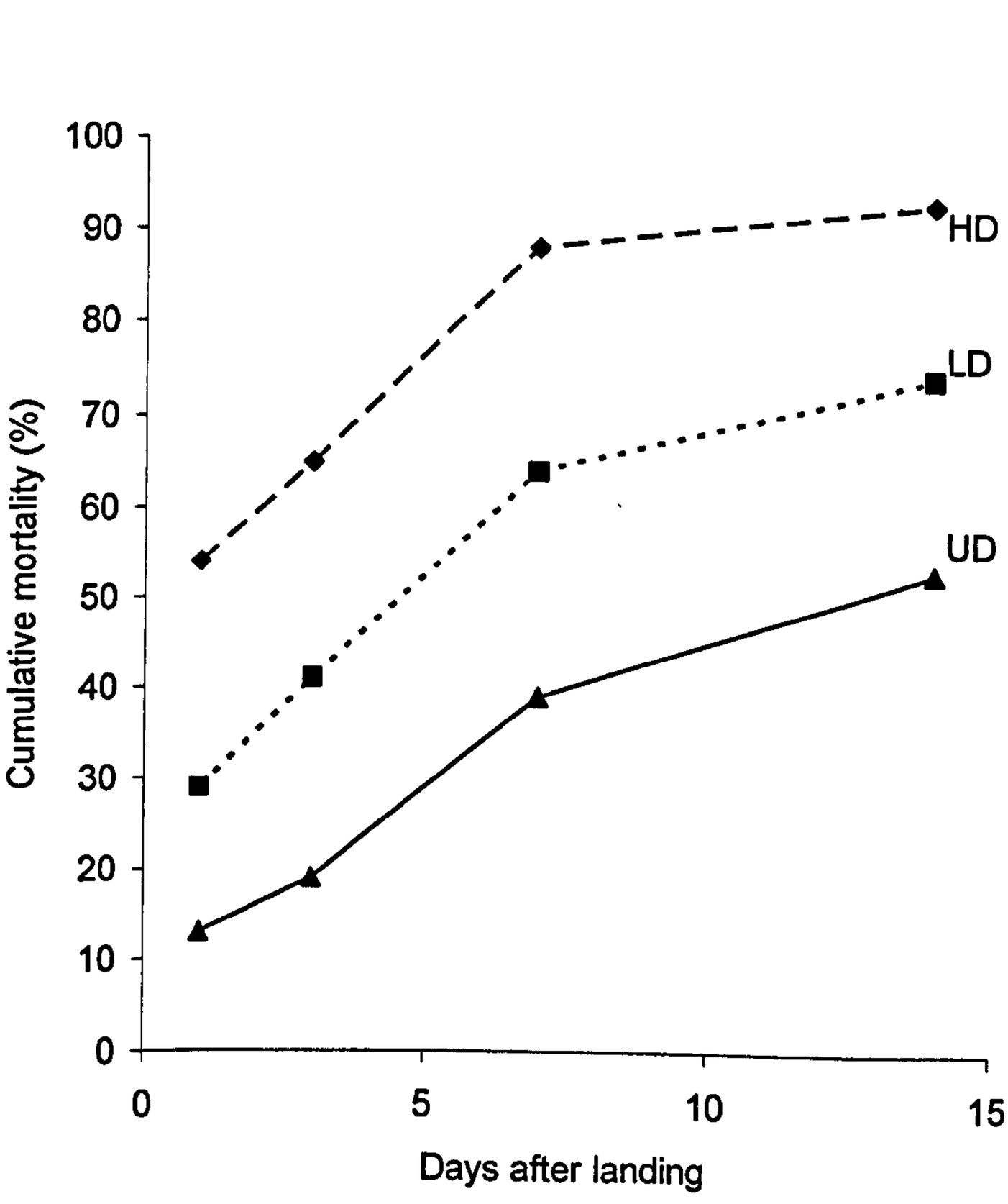

100
90
80
70
60
50
40
30
20
10
0
Cumulative mortality (%)
LPM
LAM
SPM
SAM
0
5
10
15
Days after landing

Figure 3.9: Cumulative mortality rates (%) of *N. norvegicus* following the four trawls up to 14 days after landing for the May trial. SAM, short (1h) trawl at dawn; SPM, short trawl at dusk; LAM, long (5h) trawl at dawn; LPM, long trawl at dusk.

Figure 3.10: Cumulative mortality rates (%) of *N. norvegicus* from the three different damage categories following trawling during the September 2003 and May 2004 trials, for up to 14 days after landing. HD, heavily damaged; LD, lightly damaged; UD, undamaged.


100
90
80
70
60
50
40
30
20
10
0
Cumulative mortality (%)
0
5
10
15
Days after landing
LPM
SPM
LAM
SAM


100
90
80
70
60
50
40
30
20
10
0
Cumulative mortality (%)
0
5
10
15
Days after landing
HD
LD
UD

Chapter 4

The recovery of the Norway lobster, *Nephrops norvegicus*, following trawl capture and aerial exposure.

4.1 INTRODUCTION

During the capture and post capture period, *N. norvegicus* are exposed to air at several stages, for up to 280 minutes (Bergmann *et al.*, 2001), and such stresses can make the animal too weak to enter the live export process. Following exposure to these stressors *N. norvegicus* will exhibit elevated haemolymph L-lactate and glucose concentrations (Spicer *et al.*, 1990; chapter 2), reduced concentrations of abdominal muscle glycogen (Baden *et al.*, 1994; chapter 2) and a decrease in the number of circulating haemocytes (Lorenzon *et al.*, 2001; chapter 2). Severe depletion of the carbohydrate profile of *N. norvegicus* during high temperature aerial exposure represents a form of physiological stress that the animal cannot recover from (Stentiford *et al.*, 2001b). Paterson & Spanoghe (1997) concluded "that almost anything that can be measured in Crustacea may be a stress indicator of some kind."

Several authors have suggested that haematological parameters such as total haemocyte counts might represent sensitive indicators of immuno suppression in marine decapods, such as *N. norvegicus* (Smith & Johnson, 1992; Hauton *et al.*, 1995; Jussila *et al.*, 1997). The reduction in the number of circulating haemocytes may leave the lobster immune system temporarily suppressed providing a window of opportunity to bacterial pathogens resulting in meat spoilage or death (Lorenzon *et al.*, 2001).

An adequate recovery period involving immersion in seawater before each subsequent handling and transport process is essential to avoid compounding the stress syndrome

(Taylor *et al.,* 1997). Generally, physiological disturbances are fully reversed within 24 h of re-immersion (Crear & Forteath, 2001).

There have been a number of studies documenting the return of the carbohydrate profile of decapod crustaceans to unstressed levels following a physiological stress (See Mauro & Malecha, 1984; Albert & Ellington, 1985; Taylor & Spicer, 1987; Taylor & Whiteley, 1989; Hill *et al.,* 1991a; Paterson *et al.,* 1997; Hall & van Ham, 1998; Bergmann *et al.,* 2001; Crear & Forteath, 2001). However, few of these have studied *N. norvegicus* or looked at the dual stressors of the capture process and the post capture periods. It is hoped that the results detailed here, combined with the comprehensive data collected on the capture (chapter 3) and the post capture (chapter 2) periods, will enable recovery rates to be predicted. This knowledge will aid the industry in maximising the returns from the fishery, and reduce the mortalities experienced during transport, as suggested by Taylor *et al.* (1997) in a workshop report on the physiology and live transport of lobsters.

To date, few studies have looked at the recovery of *N. norvegicus* carbohydrate profiles or immune health status following trawl capture and subsequent handling. The aim of this study was to discover the duration required for selected haemolymph parameters to return to pre-stress levels. It is hoped this will provide guidance to the Scottish *Nephrops* fishery and facilitate in the maintenance of lobsters fit for live export.

4.2 MATERIALS AND METHODS

Collection of lobsters

Norway lobsters, *N. norvegicus* were obtained by an otter trawl (70 mm mesh size) of 2 h duration from the Hunterston Channel south of the Isle of Cumbrae in the Clyde Sea area, Scotland, UK (55.41° N, 04.56° W).

4.2.1 Experimental design

On landing, 20 *N. norvegicus,* showing no visible damage, were sampled. The remainder of the catch was left for a further 3 h in air on the deck of *R.V Aora* (air temperature 17°C). After this period, undamaged *N. norvegicus* were again sampled before being moved to circulating seawater tanks (10°C, 33ppt salinity, there was no additional oxygenation/aeration of the tanks) at the University Marine Biological Station, Millport. After re-immersion in seawater a further 20 lobsters were sampled after 8, 20, 32 and 44 h post landing. Only male *N. norvegicus* (size range of 30 – 40 mm carapace length) showing no signs of visible damage were used for the experiments. All the animals were in the intermoult stage as defined by Aiken (1980). Haemolymph was removed from the sinus at the base of the 5th pereiopod using a 25 gauge needle and syringe and then subdivided for later analysis. After removal of haemolymph all animals were sacrificed. There was no sequential sampling in this study.

One hundred and sixty *N. norvegicus* were taken from the deck of *R.V Aora* in medium sized "Prawn-tubes" (commercial export cartons allowing the transportation and storage of a large number of *N. norvegicus* separately in a confined area) provided by Scotprime Seafoods. The animals were not fed for the duration of the experiment.

Haemolymph samples were collected and analysed for glucose, L-lactate, total haemocyte counts (THC), and abdominal muscle glycogen concentrations as described previously in chapter 2. Abdominal muscle tissue was removed by cutting out the second abdominal segment and stored for later analysis by freezing in liquid nitrogen.

4.2.2 Metabolites and cellular indicators of stress

4.2.3 Glucose

Refer to section 2.2.2 for the method for the determination of the glucose concentration of the haemolymph.

4.2.4 L-lactate

Refer to section 2.2.2 for the method for the determination of the L-lactate concentration of the haemolymph.

4.2.5 Muscle glycogen

Refer to section 2.2.2 for the method for the determination of the glycogen concentration in the abdominal muscle.

4.2.6 Total haemocyte counts (THC)

Refer to section 2.2.3 for the method for the estimation of THC of the haemolymph.

4.2.7 Data analysis

As long as the relevant criteria were met, the data were analysed using ANOVA or T tests, and further comparisons between the treatment means were made using Tukey's or Fisher's pairwise tests. The Anderson Darling Normality test was used to test if the data were normally distributed. Non-parametric data were analysed using Kruskal and Wallis One way ANOVA with further pairwise comparisons being made using the Mann-Whitney test.

4.3 RESULTS

Throughout the experimental period, dead *N. norvegicus* were removed from the "prawn-tubes". Of 160 animals held for the recovery period, 24 died.

4.3.1 Muscle glycogen

Abdominal muscle glycogen concentrations (Fig. 4.1) showed significant variations over the experimental period (F value 7.68, p <0.001, d.f. 96). Following the physiological stress of trawling, muscle glycogen was at a reduced level of 4.86 $mg.g^{-1}$ (significantly lower values than recovered *N. norvegicus*). After being exposed to air there was no apparent change, however after 6 h immersed in seawater a significant reduction in muscle glycogen concentrations was then observed (1.658 $mg.g^{-1}$). As the duration of the recovery increases, muscle glycogen concentrations showed a significant increase to reach a maximum of 12.06 $mg.g^{-1}$after 42 h immersed, although the increase between after 30 h immersed (8.65 $mg.g^{-1}$) to 42 h was not significant. The results of the pair wise comparisons are indicated on Figure 4.1. There was a considerable intra-specific variation in *N. norvegicus* muscle glycogen concentration, indicated by large standard deviation.

4.3.2 Haemolymph glucose

Haemolymph glucose concentrations in *N. norvegicus* showed significant variations over the experimental period (F value 11.52, p <0.001, d.f. 110) (Fig 4.2). From an initial level of 101.13 $ug.ml^{-1}$ in animals immediately after trawling, a significant increase occurred after the *N. norvegicus* have been exposed for 2 h (175.03 $ug.ml^{-1}$), this remained significantly elevated following 6 h in seawater (8 h after trawl capture). After 18 h immersed, haemolymph glucose concentrations reduced significantly from the value observed following the period of aerial exposure, to 125.58 $ug.ml^{-1}$, over the following 24

h concentrations gradually decreased to a low point of 101.03 ug.ml^{-1}, although no further significant reductions were observed. Interestingly, glucose concentrations after trawling were not significantly different from those of haemolymph samples collected after 42 h of recovery. The results of the pair wise comparisons are indicated in Figure 4.2. There was considerable intraspecific variation in the haemolymph glucose concentrations of *N. norvegicus*, as indicated by the large standard deviation.

4.3.3 Haemolymph L-lactate

Haemolymph L-lactate concentrations varied significantly over the experimental periods (F value 41.43, p <0.001, d.f. 108) (Fig. 4.3). Initial values following trawling were already elevated (8.10 mM), and following 2 h aerial exposure increased significantly (15.18 mM). After 6 h in seawater the L-lactate concentrations did not change significantly, but then decreased to a significantly lower level than was observed both post trawling and post aerial exposure, after 18 h of immersion in seawater (3.198 mM). L-lactate concentrations continued to decrease to less than 1 mM after 32 h recovery in sea water, although no further significant reductions were observed. The results of the pair wise comparisons are indicated in Figure 4.3. There was considerable intraspecific variation in *N. norvegicus* exhibiting high haemolymph L-lactate concentrations, indicated by large standard deviation.

4.3.4 Total haemocyte counts (THC)

The total haemocyte counts varied significantly over the experimental period (F value 3.92, p 0.003, d.f. 108) (Fig. 4.4). After 2 h exposure in air the THC values were lower than those recorded immediately following trawling but this reduction was not significant. Following emersion for 6 h in seawater (8 h after trawling) the number of circulating

haemocytes increased to a value that was significantly higher than that observed in *N. norvegicus* after the period of aerial exposure. The THCs remained elevated for the duration of the experimental period. Haemocyte counts in samples collected immediately after trawling were lower than those recorded following recovery, but these differences were not significant. Large standard deviation bars indicate large variability in the THCs of the recovered animals. The results of the Tukey's pair wise comparison are indicated in Figure 4.4.

4.4 DISCUSSION

The carbohydrate profile of *Nephrops norvegicus* is significantly altered following the trawl capture and subsequent handling and aerial exposure of animals during the post capture period, where they exhibit elevated haemolymph glucose and L-lactate concentrations as well as reduced tissue glycogen concentrations. Furthermore, their total haemocyte counts are significantly reduced, indicating that they are immuno-suppressed.

Previous studies have demonstrated that the removal rate of haemolymph L-lactate following re-immersion in water varies greatly amongst species. Mauro & Malecha (1984) recorded haemolymph L-lactate levels in *Macrobrachium rosenbergii* exposed to hypoxic conditions, returning to levels comparable to those of normoxic controls within 3 h, while Hill *et al.* (1991a) reported that the haemolymph L-lactate concentrations in *Carcinus maenas* exposed to environmental anoxia were still significantly higher than in control animals after 28 h of recovery in fully aerated sea water. Crear & Fortheath (2001) demonstrated that recovery of *Panulirus cygnus* after aerial exposure was dependant on the oxygen concentration of the water during the recovery period. The present study illustrates that it can take over 30 h following landing after they have been returned to seawater until the carbohydrate profile (glycogen, glucose and L-lactate) of *N. norvegicus* returns to

unstressed levels. This agrees with the study of *Homarus vulgaris* by McMahon *et al.* (1978) which demonstrated that it may take between 24-48 h for acid-base balance to return to pre-stress levels.

During the capture and post capture period *N. norvegicus* are exposed to functional (exercise during trawl capture) and environmental (aerial exposure) hypoxia (see Spicer *et al.*, 1990). In the present study, L-lactate was elevated following trawling, and continued to increase during aerial exposure, then decreased throughout the recovery period. It was previously thought by McDonald *et al.* (1979) that because of the low body temperature and open circulatory system of crustaceans, the peak concentrations of L-lactate do not occur until 1-2 h after the stress. A number of other studies (Onnen & Zebe, 1983; Head & Baldwin, 1986; Hill *et al.*, 1991a; Whiteley & Taylor, 1992; Crear & Forteath, 2001) observed significant increases in L-lactate concentrations in the tissues or haemolymph 1-2 h after re-immersion.

It has been suggested that this increase in haemolymph L-lactate is due to release of L-lactate previously held in the tissues during the stress (Taylor & Wheatly, 1981; Waldron, 1991). However, observations by Hill *et al.* (1991b) and Head & Baldwin (1986) suggest that L-lactate release from the tissues does not fully explain the increased haemolymph concentrations. An alternative possibility is that increased L-lactate production on re-immersion is a result of the high energy demand, requiring a contribution from both anaerobic and aerobic metabolism (Head & Baldwin, 1986; Gruschczyk & Kamp, 1990; Whiteley & Taylor, 1992).

Crear & Forteath (2001) studied the effect of different oxygen concentrations during re-immersion on *P. cygnus* and discovered that the relative increase in L-lactate concentration during the first hour was dependant on the dissolved oxygen concentration of the water. In

situations where insufficient oxygen resulted in an inability of the animal to fuel the aerobic portion of the energy requirements of recovery, the excess was made up via anaerobic metabolism. This led them to conclude that the increase in L-lactate following re-immersion is a result of continued use of anaerobic energy sources, rather than the release of L-lactate from tissues. It was also suggested by Onnen & Zebe (1983) that the use of anaerobic metabolism during recovery ensures that muscle function is restored rapidly. In the present study no significant increase in haemolymph L-lactate concentrations was observed following re-immersion, but this was probably a result of the sampling times. Haemolymph parameters were not sampled until 6 h after re-immersion, interestingly, variations in muscle glycogen were observed during this period.

In invertebrates, L-lactate is slowly converted back to tissue glycogen under aerobic conditions by the process of gluconeogenesis (Ellington, 1983). This process is both slower and less efficient than the Cori cycle in mammals (Schluman & Landau, 1992). Radioisotope studies undertaken by Hill *et al.* (1991b) indicated that most of the labelled L-lactate remained in the animal and appeared as glycogen, while a small portion was oxidised. This led them to conclude that there was no evidence for the excretion of L-lactate. Elevated L-lactate levels caused by internal hypoxia stimulate the release of CHH, which results in increased glycogenolysis (Santos & Keller, 1993b), resulting in the release of glucose.

Following trawling, haemolymph glucose concentrations were similar to those observed after 48 h recovery, even though the corresponding L-lactate levels were high. Spicer *et al.* (1990) also observed low glucose concentrations, and concluded that the stress during trawling was not sufficient to provoke activity, or that lobsters met the increased metabolic demands via aerobic metabolism. In the present study it is interesting that L-lactate

concentrations of the haemolymph are elevated following the internal hypoxia induced through trawling, yet glucose remained relatively low. However, Hill *et al.* (1991a) also observed elevated L-lactate concentrations in the haemolymph during a period of anoxia with no change in the glucose concentrations, possibly indicating that the animal, though experiencing internal hypoxia, was not sufficiently stressed. A repeat study analysing the concentrations of the crustacean hyperglycaemic hormone (CHH) in the haemolymph may be more informative.

Haemolymph glucose concentrations decreased throughout the recovery period and after 20 h of recovery, and were significantly lower than those of lobsters that had been exposed to air following trawling. The period of recovery from hyperglycaemia has not been well studied, but the results obtained in the present study were similar to those recorded for *P. cygnus* by Crear & Forteath (2001) and by Spanoghe & Bourne (1997), and for *Palaemon elegans* and *P. serratus* following acute hypoxia (Taylor & Spicer, 1987). Crear & Forteath (2001) reported that the rate of recovery to unstressed glucose concentrations was greatest at water oxygen concentrations of 70-80% and above, illustrating the importance of the holding conditions during the recovery period.

Following aerial exposure, an increased concentration of glucose and L-lactate in the haemolymph of *N. norvegicus* was observed, as well as a decrease in abdominal muscle glycogen. In crustaceans, the carbohydrate profile is known to be mainly under the control of CHH levels (Sedlmeier, 1985). Interestingly, the lowest observed glycogen concentrations were observed 6 h after re-immersion. This may be a consequence of the continued anaerobic metabolism after re-immersion as previously discussed (Onnen & Zebe, 1983; Head & Baldwin, 1986; Hill *et al.*, 1991b; Whiteley & Taylor, 1992; Crear & Forteath, 2001). This would result in elevated L-lactate concentrations, with corresponding

reductions in tissue glycogen concentrations. After this initial reduction in tissue glycogen concentrations there was a progressive increase through the recovery period. Previous studies have observed the seasonal variations in muscle glycogen reserves (Baden *et al.*, 1994). In the spring when glycogen reserves are at their lowest following the food scarcity of the winter, repletion may occur over a shorter period. The present study was carried out in August. The impact of the seasonality of glycogen reserves on the time required for the repletion of muscle glycogen levels should be studied in future.

There has been much discussion about the source of the utilised glycogen. There are three possibilities: the haemolymph, the muscle tissue, and the hepatopancreas. Keller & Andrew (1973) observed a reduction in abdominal muscle glycogen concentrations of the crayfish, *Orconectes limosus*, after injection of CHH, but no change in the concentrations in the hepatopancreas. Similar results were reported by Telford (1975) for *Cambarus robustus*. However these findings are not consistent with the findings of Ramamurthi *et al.* (1968), researching endocrine control of glycogen synthesis in crabs, and Parvathy (1972), studying *Ocypode platytarsis*, observed significant reductions of glycogen only in the hepatopancreas, while Seidlmeier (1985) concluded that both the hepatopancreas and abdominal muscle tissue are sources of energy during anaerobiosis. In this investigation the glycogen concentration of the abdominal muscle was measured because it is understood to be a particularly important store of glycogen as it is more readily accessible when anaerobic metabolism is initiated (Baden *et al.*, 1994; Honke & Scheer, 1970; Stetten, 1982). It is also the tissue that is most desired commercially, and significant alterations of abdominal muscle glycogen will affect taste (Spotts & Lutz, 1981).

It should also be noted that haemocytes contain large amounts of polysaccharides in the form of glycogen (Johnson *et al.*, 1971; Johnson & Davies, 1972). Therefore, future

studies on depletion and repletion of glycogen concentrations should include all three of these possible sources of glycogen (haemolymph, hepatopancreas and abdominal muscle).

Following aerial exposure there was marked hyperglycaemia in the haemolymph of *N. norvegicus*, as has been previously observed in *Libinia emarginata* (Kleinholz, 1949), *Homarus americanus* (Telford, 1968), *Liocarcinus puber* (Johnson & Uglow, 1985), and *Panulirus cygnus* (Crear & Forteath, 2001). This increase may be due to internal hypoxia and handling stress as the lobsters were moved ashore during this period, as has been observed by Telford (1973). Marsden (1994) reported that although the capture and post capture treatment of *Penaeus monodon* resulted in elevated haemolymph glucose concentrations, the absolute change was very variable. The degree of change in circulating sugar concentrations as a result of a stress will be partially dependant on the animal's nutritional state and may explain why glucose levels in Crustacea can be highly variable (Hall & van Ham, 1998). Large variations in the concentrations of glucose and glycogen were observed in the present study. Hall & van Ham (1998) suggest that the nutritional status of prawns used in stress studies needs to be as uniform as possible to obtain meaningful results. However in studies, such as this, where recently captured crustaceans are the subject species, this is not possible.

The reason why knowledge of this recovery period is so important is that following re-immersion the oxygen debt and respiratory acidosis are reduced, but if lobsters are further stressed, by re-handling or further periods of aerial exposure, the acidiotic stress is compounded and pH regulation is compromised. Following this treatment, acid-base stress is consequently more severe and survival times in air potentially reduced (Taylor *et al.*, 1997). A reduction of haemolymph pH badly affects many tissue processes, causing loss of equilibrium and death (Caillouet, 1968).

At the end of the recovery period the number of circulating haemocytes was not significantly different from that measured in *N. norvegicus* immediately following trawling. However following the period of aerial exposure after trawling, a significant decrease was observed and haemocyte numbers had increased significantly after 20 h of recovery.

In crustaceans, cellular defences rely on haemocytes, which perform several functions such as coagulation, phagocytosis, encapsulation and wound healing (Johansson & Söderhall, 1989; Hose & Martin, 1989; Bachere *et al.*, 1995). The decline in the number of circulating haemocytes following an exposure to stress has been observed by a number of researchers (Mix & Sparks, 1980; Martin & Graves, 1985; Lorenzon *et al.*, 2001), and is thought to reflect an immuno-suppression (Lorenzon *et al.*, 2001).

The fate of the haemocytes has been extensively discussed, and it appears there are a several possibilities. Cornick & Stewart (1968) concluded that there is an increased phagocytosis and sequestration in the tissues during exposure to stress, though Lorenzon *et al.* (2001) suggested that the observed reduction in the number of circulating haemocytes may be due to cell lysis, or degranulation, or to a generalised stress response as proposed by Smith & Johnson (1992) and Smith *et al.* (1995). This might provide a critical window for opportunistic pathogens such as *Vibrio* species to exploit the host and initiate spoilage. However, a number of other studies have observed a decline in the number of circulating haemocytes following the injection of foreign substances such as bacteria (Smith & Söderhall, 1983; Persson *et al.*, 1987; Martin *et al.*, 1993; van de Braak *et al.*, 2002; Holman *et al.*, 2004). Martin *et al.* (1998) reported that in the presence of bacteria the haemocytes of *Homarus americanus* rapidly formed nodules that would be occluded in the gills, such aggregations in the tissues were also observed by Smith *et al.* (1984), Smith &

Ratcliffe (1980), and Factor & Beekman (1980). These results suggest that reduced THCs reflect a response to bacterial invasion, rather than an immuno-suppression that allows bacteria to invade. It is obviously important that the causality of the relationship between decreased circulating haemocyte numbers and increased bacterial numbers in the tail meat of necrotic animals is established.

There have been few studies documenting the recovery from stress-induced alterations to the haemocyte profile in crustaceans. Evans *et al.* (1999) reported that haemocytes returned to pre-stressed levels 24 h after being re-submerged. Although they observed significant increases in THCs following exposure to acute stressors. Lorenzon *et al.* (2001) observed significant decreases in the number of circulating haemocytes following the injection of lipopolysaccharide (LPS – component of gram negative bacteria cell walls) into *N. norvegicus* and observed a decrease in haemocyte numbers that lasted for 24 h and an incomplete recovery of haemocyte numbers.

Following removal from the stress, the numbers of haemocytes in *N. norvegicus* returned to unstressed levels. A number of possible ways by which this may have occurred have been suggested. Firstly, the haemocytes may be released back from the tissues and also from aggregations. Other suggestions are that in the more stress-resistant individuals a reserve pool of sessile haemocytes could be mobilised (Lorenzon *et al.*, 2001), or that haemocytes are recruited from haemopoietic proliferation (Johanssson *et al.*, 2000) or by haemocyte division in the haemolymph (Sequeira *et al.*, 1996). Because haemocyte numbers have been shown to vary significantly in crustaceans (Persson *et al.*, 1987; Lorenzon *et al.*, 2001), new haemocytes need to be proportionally produced to compensate for haemocyte losses. Haemocytes are continually released from specialised haemopoietic tissue that has been identified in a number of crustaceans (Ghiretti-Magaldi *et al.*, 1997;

Hose *et al.*, 1992; Martin *et al.*, 1993; Chaga *et al.*, 1995). Sequeira *et al.* (1996) observed that in *Penaeus monodon* LPS induced an increase of naturally proliferating haemocytes, suggesting that they may compensate for the observed decrease in circulating haemocytes associated with stress. There has been much research on the defensive role of haemocytes in crustaceans, but the knowledge about the haemopoietic tissue itself is limited.

This chapter has indicated the possible duration required for the recovery of the immune status of *N. norvegicus* following capture and post capture stressors. However, a more detailed study on the recovery of the immune status of *N. norvegicus* following exposure to stressors associated with the capture and post capture processes should be undertaken, encompassing more variables such as total protein concentration of the haemolymph, prophenoloxidase activity, differential haemocyte counts, and haemolymph bacteraemia levels.

As well as implications for the post capture transport of *N. norvegicus* in providing estimates of when recovered lobsters are recovered sufficiently to enable them to tolerate further exposure or stress to ensure maximum survival and resistance from disease, there are also implications for the taste and quality of the meat, as the altered carbohydrate profile returns to pre-stressed levels following recovery. Chambers & Grandin (2001) suggest that pigs and other animals should be rested prior to slaughter to ensure muscle glycogen is replaced as much as possible. The data obtained here suggest that *N. norvegicus* should be left submerged and unstressed for at least 24 h following their capture and subsequent handling. Transporting or handling the lobsters before this period will further alter the carbohydrate profile and increase the window of opportunity for invading bacteria to cause spoilage of the meat.

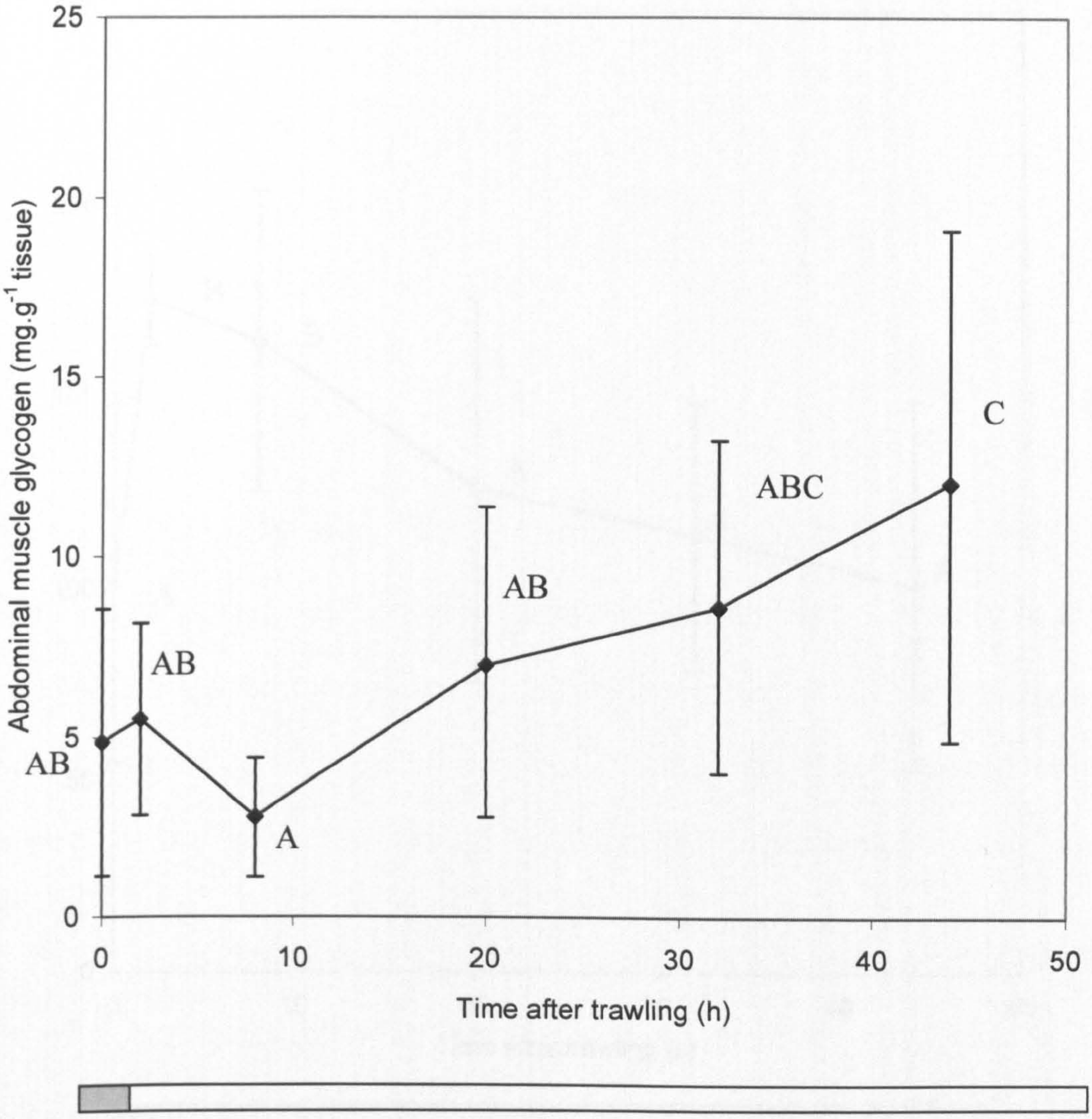


Figure 4.1: The variation in muscle glycogen concentrations of *N. norvegicus* following trawling, aerial exposure and recovery in seawater (values are means ± sd). Data points with the same letter are not significantly different as determined from Tukey's Pair wise analysis. The bar below the graph illustrates the treatment conditions at each sampling period, the shaded section indicates that the animal was exposed to air (17°C) and the open section indicates immersion in seawater.

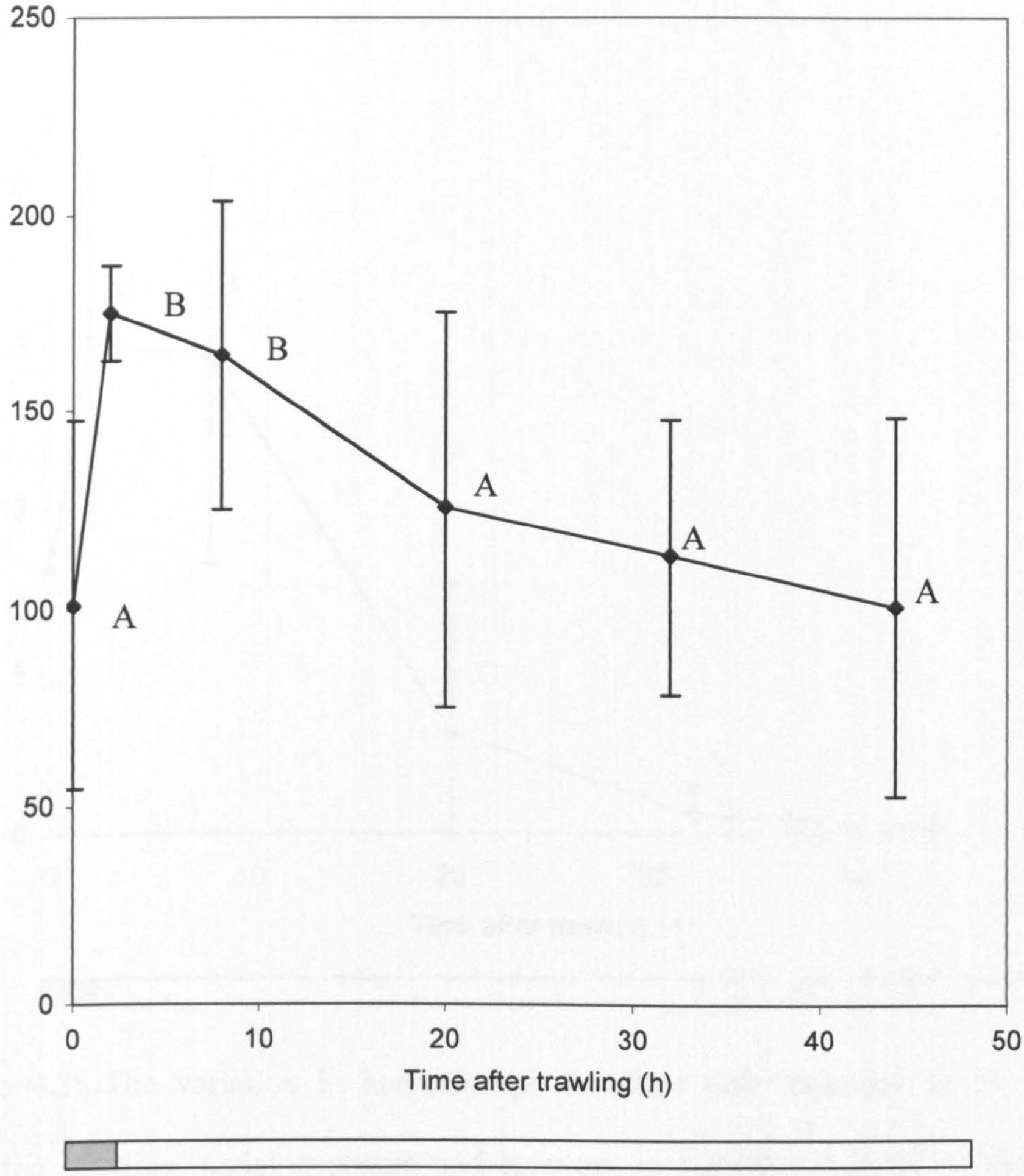


Figure 4.2: The change in haemolymph glucose concentrations of *N. norvegicus* following trawling, aerial exposure and recovery in seawater (values are means ± sd). Data points with the same letter are not significantly different as determined from Tukey's Pair wise analysis. The bar below the graph illustrates the treatment conditions at each sampling period, the shaded section indicates the animal was exposed to air (17°C) and the open section indicates immersion in seawater.

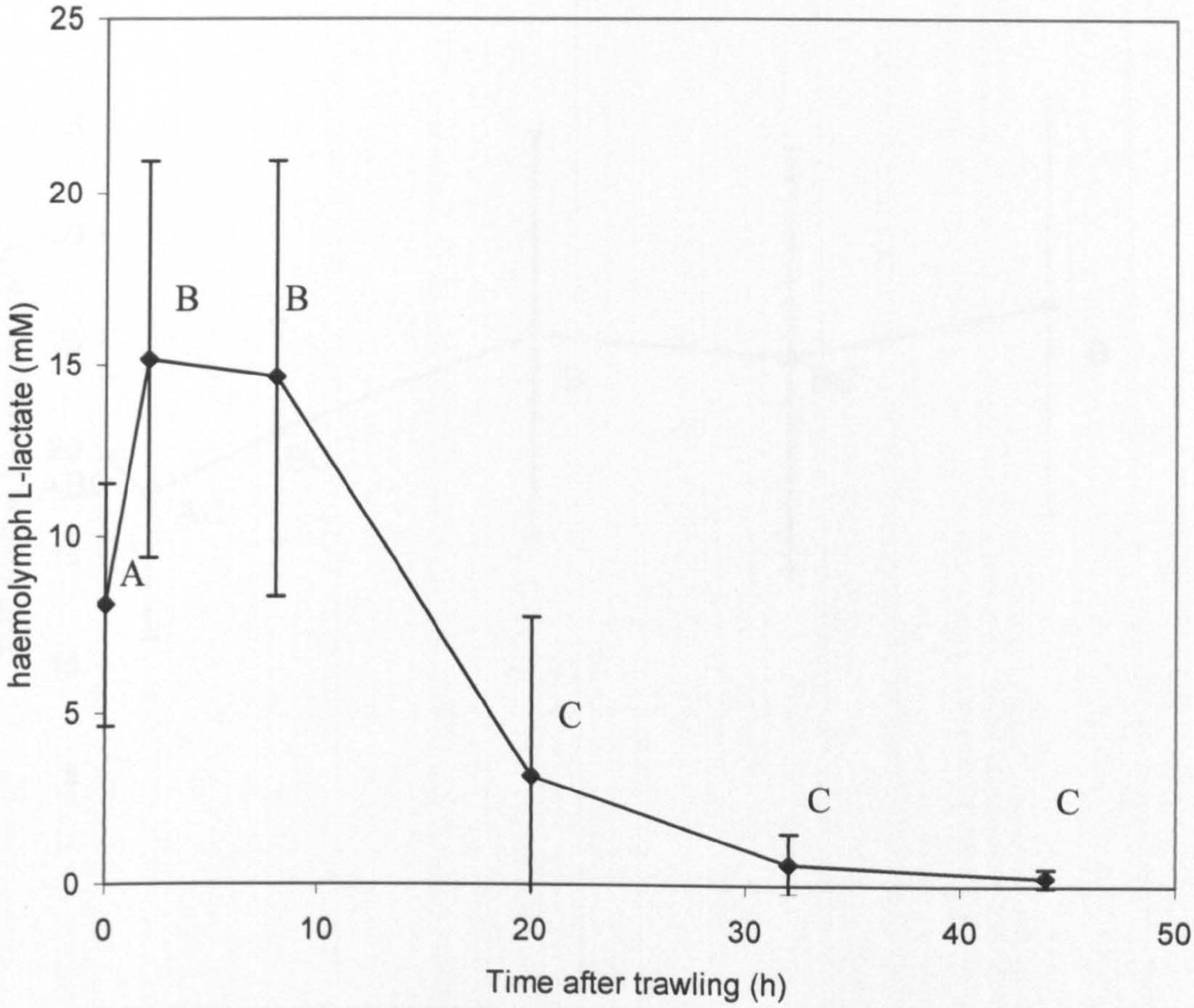


Figure 4.3: The variation in haemolymph L-lactate concentrations of *N. norvegicus* following trawling, aerial exposure and recovery in seawater (values are means ± sd). Data points with the same letter are not significantly different as determined from Tukey's Pair wise analysis. The bar below the graph illustrates the treatment conditions at each sampling period, the shaded section indicates the animal was exposed to air (17°C) and the open section indicates immersion in seawater.

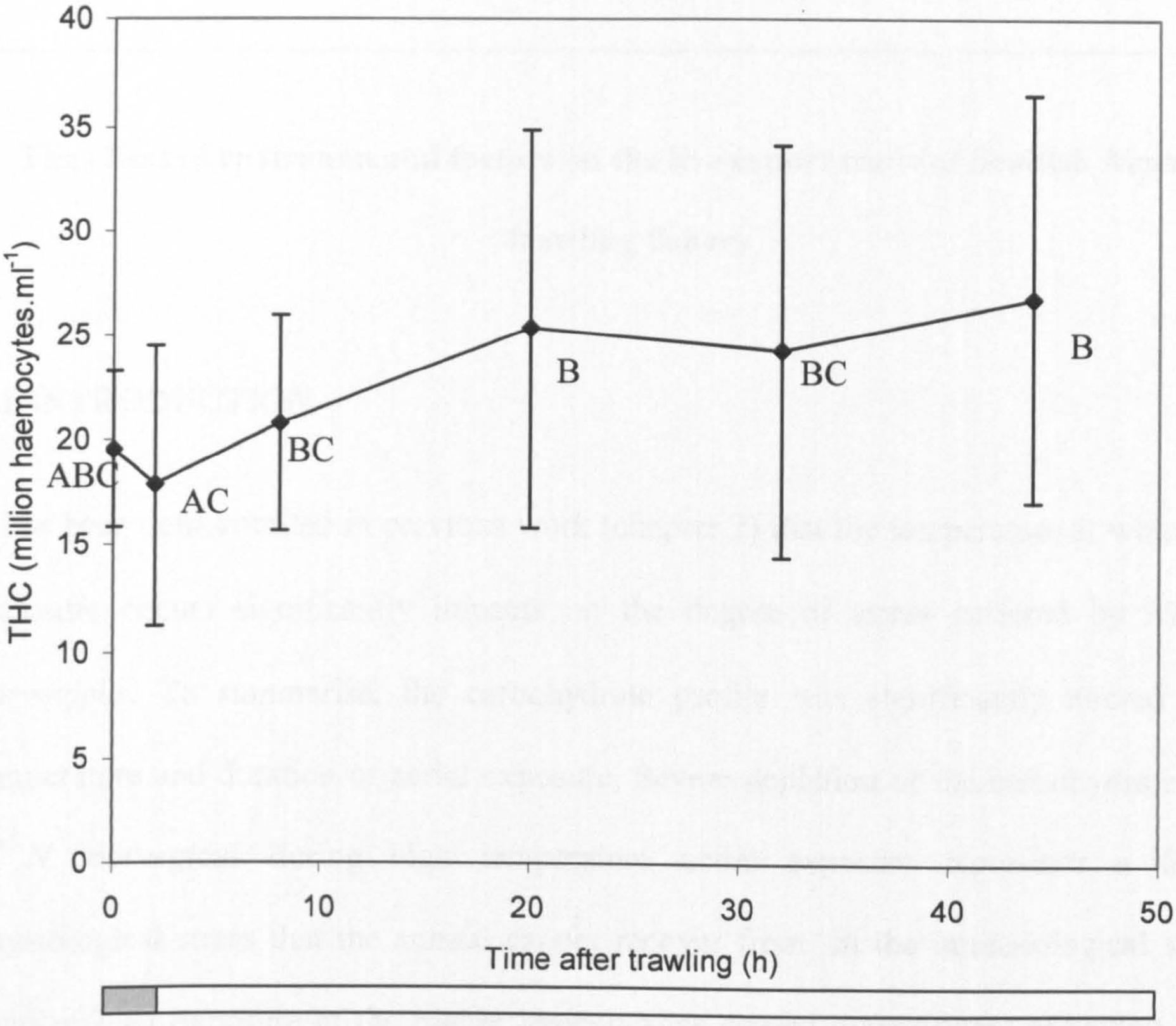


Figure 4.4: The variation in total haemocyte counts (THC) following trawling, aerial exposure and recovery in seawater (values are means ± sd). Data points with the same letter are not significantly different as determined from Tukey's Pair wise analysis. The bar below the graph illustrates the treatment conditions at each sampling period, the shaded section indicates the animal was exposed to air (17°C) and the open section indicates immersion in seawater.

Chapter 5

The effect of environmental factors on the live export trade of Scottish *Nephrops* trawling fishery

5.1 INTRODUCTION

It has been demonstrated in previous work (chapter 2) that the temperature at which aerial exposure occurs significantly impacts on the degree of stress suffered by *Nephrops norvegicus*. To summarise, the carbohydrate profile was significantly altered by the temperature and duration of aerial exposure. Severe depletion of the carbohydrate profile of *N. norvegicus* during high temperature aerial exposure represents a form of physiological stress that the animal cannot recover from. In the immunological study of chapter 2 air exposure at the higher temperatures caused a significant reduction in total haemocyte counts (THC), a significant reduction in haemolymph phenoloxidase activity and elevated haemolymph bacteraemia levels. The consequence of these alterations were the high mortalities observed after aerial exposure at high temperatures (25°C). This chapter aims to demonstrate if the susceptibility of *N. norvegicus* to elevated aerial temperatures is observed in the *Nephrops* fishery, specifically on the live export trade.

Following trawl capture *N. norvegicus* are exposed to air during sorting and then submerged in seawater tanks on the deck of the boat, before being further exposed to the elements during transportation to the processing plants on flat bed trucks. At the processing plant, *N. norvegicus* are categorised into sizes and also as "live" (sent for live export) or "dead" (rejected for the live trade and sold as whole animals or tailed for "scampi"). The objective of this study was to compare the effect of environmental factors

(wind speed, aerial temperature) on the day of capture with the amount of rejected animals from that catch once at the processing plant. This study was conducted over a 6 month period from November 2002 until April 2003 using the landings from two boats in the Clyde Sea area. Specific objectives were to correlate through statistical analysis meteorological variables with the quantity of animals rejected for the live export trade over the study period. It is hoped that information obtained during this study will provide guidance to the industry so optimum returns from the fishery can be achieved.

There have been no previous work correlating climatic variables with the mortality and morbidity of *N. norvegicus* destined for the live transport trade. A similar study was conducted by Spanoghe & Bourne (1997) to ascertain the affect of a range of environmental factors on the morbidity and mortality of the western rock lobster, *Panulirus cygnus*. It was found that holding time in the export cartons, ambient temperature within the export cartons, and chilling period before packing the lobsters had the greatest impact on the morbidity and mortality observed.

5.2 MATERIALS AND METHODS

Between November 2002 and April 2003 catch data were collated from the landings of two *Nephrops* trawlers, *Avalon* and *Dunan Star*, at Carradale Harbour, north of Campbeltown in the Firth of Clyde, West of Scotland. The catch data contained information on the weight of *N. norvegicus* classified as "live" or "dead" from each size class (small, medium or large) following arrival at the processing plant in Ayr. The *Avalon* is a twin-rigged trawler with a crew of 4, while the *Dunan Star* has only one rig and 3 crew members. A similar post capture treatment is used on both boats: *N. norvegicus* are exposed to air during sorting and then placed into "prawn tubes" (commercial shipping cartons) and submerged in seawater tanks on the deck of the boat, which is continually refreshed with

running seawater pumped aboard from the surface waters of the sea. The trawling grounds for both boats are located in the Clyde sea area inside the Campbeltown peninsula; however the exact location is very variable. At Carradale the catch from both trawlers is transported on flat bed trucks (during this stage the Prawn tubes are open to the elements, only covered by Hessian sacks that have been soaked in seawater. At Campbeltown they are transferred to refrigerated trucks and transported to Scotprime Ltd, Ayr.

At the processing plant, *N. norvegicus* are held in "prawn tubes" in circulating filtered seawater tanks (6°C, 33 ppt salinity). 24 h after arrival the landings are sorted and classified for export. *N. norvegicus* are initially classified as small, medium or large, and then categorised into "live" (sent for live export) or "dead" (rejected for the live trade and sold as whole animals or tailed for "scampi"). *N. norvegicus* rejected for the live export market were classified as weak, moribund or dead. The total number of rejected *N. norvegicus* were divided by the total number of lobsters to give a combined percentage of morbidity and mortality (M + M%). This method was used by Goodrick *et al.* (1993).

The meteorological data was provided by Prestwick weather station (55.50°N, 4.50°W), the nearest weather station to the trawling locality. The wind speed, maximum gust speed, mean temperature and hours of sunshine on the day of landing were correlated with the percentage of *N. norvegicus* rejected from the live trade and classified "dead" (M+M%), for each size class or from the total landings, for the corresponding day.

The meteorological data was not normally distributed and this distribution could not be normalised using the usual transformations. Pearson's correlation analysis could not be performed as the basic assumptions were not met, therefore Spearman's rank-order correlation analysis was performed using MINITAB^{TM} statistical software. All the meteorological variables and the M+M% were therefore ranked and subjected to

Spearman's rank-order analysis. Further analysis could be performed through regression analysis as the assumption that both variables be normally distributed is lifted (Dytham, 1999).

For regression analysis a number of assumptions have to be met, specifically that the dependant (y) variable, the M+M%, is normally distributed. Percentages tend not to be normally distributed (Hampton, 1994), requiring transformations that help to improve the normality of the distribution. In past studies an angular transformation of the M + M percentage was performed, with the transformed variable (Y) defined as arcsine$\sqrt{}$ (M+M %) (Dagnelie, 1975; Spanoghe & Bourne, 1997). However, following this method in the present study failed to result in normally distributed variables, therefore a logarithmic transformation of the data was conducted termed log(M+M%).

5.3 RESULTS

5.3.1 Impact of meteorological factors on the suitability of *Nephrops norvegicus* for export

Throughout the study period the catches from 137 days at sea were monitored, 99 from the *Dunan Star* and 38 from the *Avalon*. The median percentage of morbidity and mortality (M+M %) from the *Dunan Star* (36.33%) 24 h after arrival at the processing factory was significantly higher than that of the *Avalon* (31.31%) (Kruskal-Wallis H value 8.40, p 0.004, d.f. 136) and the mean amount received by the processing factory, before sorting, from the *Avalon* (70.9 kg) was significantly higher than the *Dunan Star* (44.9 kg).

The results (Table 5.1) indicate that for *N. norvegicus* captured by the *Avalon*, the meteorological factors analysed demonstrated no significant correlation with the log(M+M %). However, for *N. norvegicus* caught by the *Dunan Star*, both mean and maximum

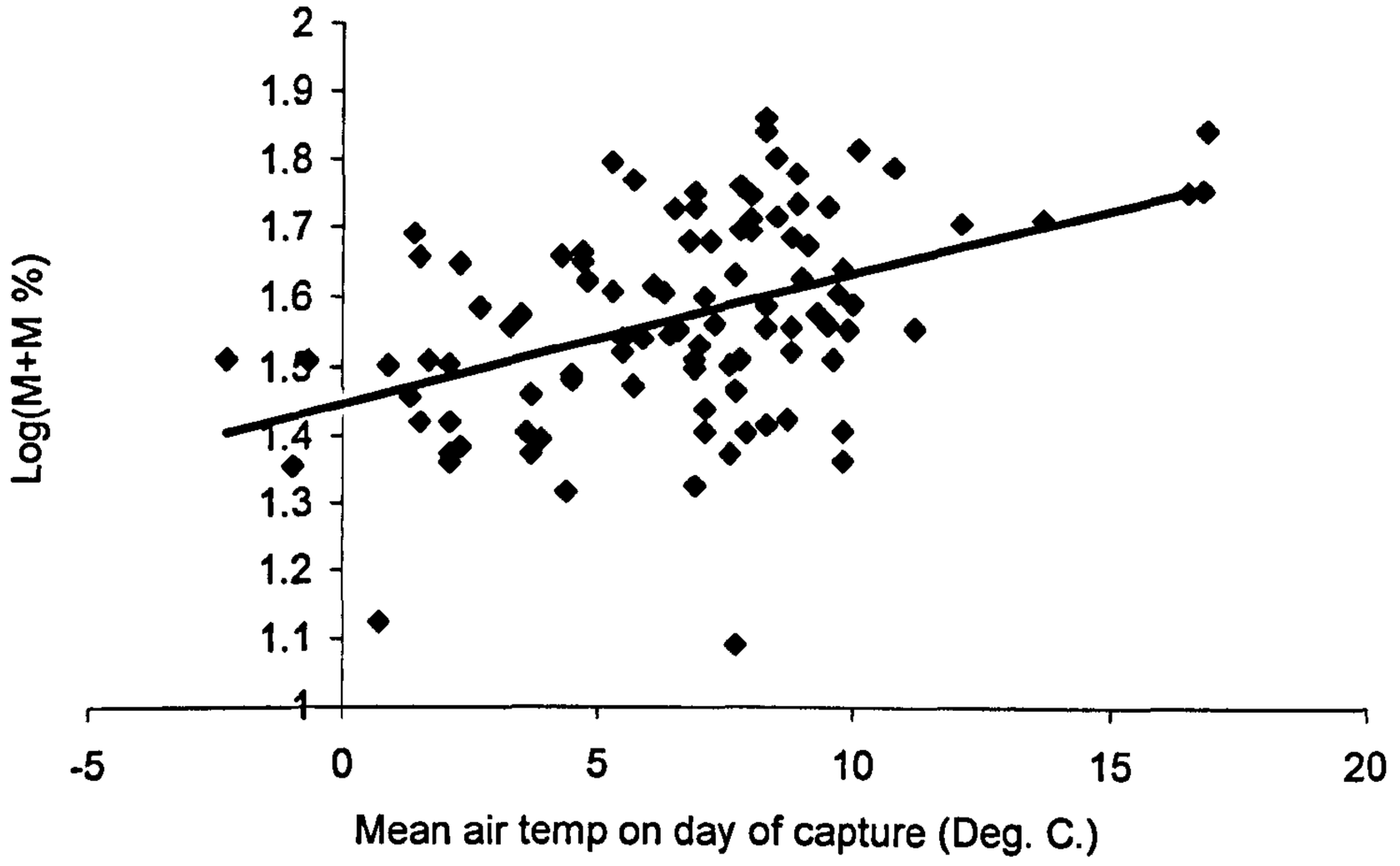
temperature on the day of trawling demonstrated a significant correlation with the log(M+M %).

Table 5.1: The results of the correlation analysis between the meteorological variables and the percentage of morbidity and mortality log(M+M%). The above value is the Spearman's Correlation coefficient and below the associated probability, <0.05 indicates a significant correlation between the two variables, as indicated by an asterisk (*Dunan Star* d.f. 98, *Avalon* d.f. 37).

	Mean wind speed	**Max gusts**	**Mean air temp.**	**h of sunshine**
Avalon	-0.122	-0.138	0.167	0.130
	0.465	0.409	0.317	0.436
Dunan Star	0.194	0.148	0.408	-0.092
	0.055	0.143	<0.001*	0.365

Figure 5.1 indicate the relationship following regression analysis between the mean temperature on the day of capture and the $\log_{10}$ of percentage morbidity and mortality (M+M %) observed, indicating that the rate of increase of M+M % is greater at the higher mean temperatures. Interestingly, the low R^2 value associated with the regression analysis indicates that the response of the y variable (M+M %), although significantly dependant on the x variable (mean temperature), is also affected by other factors, such as the duration of the trawl, and the post capture treatment.

Figure 5.1: Regression analysis of $\log_{10}$ M+M % observed following capture on the *Dunan Star* and the mean temperature on the day of capture (°C). The regression equation is: Log_{10}(M+M %) = 1.44739 + 0.0185618 Mean air temp. R^2 (adj.) = 18.3 %. (F value 23.00, p <0.001, d.f. 98).



5.3.2 Impact of meteorological factors on different sized *Nephrops norvegicus*

The effect of the size of *N. norvegicus* on the percentage of morbidity and mortality observed after trawling was analysed for each boat separately. Table 5.2 details the median M+M % for each boat in each size class. Large and medium sized *N. norvegicus* caught on the *Avalon* have significantly higher M+M% than the small animals. However, there are significant differences between all of the size classes caught on the *Dunan Star*, with the small animals again having the lowest M+M %, but the medium sized *N. norvegicus* having a significantly higher M+M % than the large animals.

Table 5.2: The median percentage of morbidity and mortality experienced by different sized *N. norvegicus* from each boat, the *Avalon* and the *Dunan Star*. Significant differences between each size class from the same boat is indicated by a different letter, as indicated by Mann-Whitney non-parametric test.

	Avalon	*Dunan Star*
Large M+M %	38.61 A	40.53 A
Medium M+M%	38.41 A	46.94 B
Small M+M %	26.16 B	32.58 C

The correlations between the M+M% of large, medium and small *N. norvegicus* and the mean air temperature on the day of capture for each boat are presented in Table 5.3. This analysis suggests that the differences observed in Table 5.3 may be attributed to factors other than the mean air temperature on the day of capture. The *N. norvegicus* classified as large from the *Avalon* exhibit a significant correlation with the mean air temperature; however, this is not the case for the medium sized class which experienced a similar M+M%, suggesting that another factor may be responsible for the high M+M % observed. The landings from the *Dunan Star* demonstrate different relationships, with both the medium and small proportions of the catch, but not the large, exhibiting significant positive correlations with the mean air temperature on the day of landing. These findings may illustrate differences in the post capture handling of *N. norvegicus* on each boat according to size.

Table 5.3: The results of correlation analysis between the percentage of morbidity and mortality observed in large, medium and small *N. norvegicus* landed on each boat and the mean air temperature. The value above is Spearman's Correlation coefficient, and below the associated probability, a probability <0.05 indicates a significant correlation between the two variables, and is indicated by an asterisk.

	Mean air temperature (*Avalon* catch)	**Mean air temperature (*Dunan Star* catch)**
Large M+M %	0.313	-0.074
	0.056	0.493
Medium M+M%	0.058	0.483
	0.730	<0.001*
Small M+M %	0.052	0.327
	0.756	0.001*

To summarise, the results have demonstrated that only on the *Dunan Star* did the temperature on the day of capture have significant affects on the M+M % of the catch. Interestingly the correlation analysis suggests that only the small and medium proportions of the catch were affected.

The data corresponding to capture by the *Avalon* inform that over the whole catch combined, M+M% was not significantly affected by the aerial temperature, however on further analysis the M+M% of the large sized proportion of the catch demonstrated the greatest positive correlation, although this was not quite significant (0.056), with the aerial temperature on the day of capture.

5.4 DISCUSSION

There was a large degree of variability in the condition of *Nephrops norvegicus* on arrival at a processing plant. However, on one of the boats, the *Dunan Star,* the mean air temperature on the day of capture had a significant negative impact on the health of the catch. No other meteorological factors other than air temperature appeared to have an impact on the morbidity and mortality of the catch. The data from the two boats were not pooled as the catch pertaining to the *Avalon* were far less numerous than those of the *Dunan Star* and any correlations would be masked by the greater number of observations from the *Dunan Star.*

A similar study by Spanoghe & Bourne (1997) was performed to ascertain the affect of a range of environmental factors on the morbidity and mortality of the western rock lobster, *Panulirus cygnus*. It was found that holding time in the export cartons, ambient temperature within the export cartons, and chilling period before packing the lobsters had the greatest impact on the morbidity and mortality observed.

It is well established that the duration of which crustaceans can be exposed to air for varies greatly amongst species (Taylor & Whiteley, 1989; de Fur *et al.,* 1988; Zainal *et al.,* 1992; Goodrick *et al.,* 1993; Paterson *et al.,* 1997), but these studies have suggested that the environmental factors, particularly temperature, play an important role in survival during live transport. There have been few studies on the tolerance of *N. norvegicus* to a range of temperatures during aerial exposure. In this thesis (chapter 2) it has been demonstrated that survival of *N. norvegicus* is greatly reduced once air temperatures get above 15°C. Studies on other crustaceans have also indicated that survival is improved if the temperature of aerial exposure is reduced. Whiteley & Taylor (1990, 1992) and Whiteley *et al. (*1990) reported increased survival rates of *Homarus gammarus* when a temperature of 5-10°C

was maintained in the export cartons, while Goodrick *et al.* (1993) and Paterson (1993) reported that 12-15°C was the optimum temperature range for the live export of *Penaeus japonicus*.

It was observed that on both vessels the smaller *N. norvegicus* exhibited the lowest M+M%. Interestingly, when analysing the catches separately the morbidity and mortality rate of the *N. norvegicus* classified as large from the *Avalon* exhibited a significant positive correlation with the mean air temperature on the day of capture. It has been suggested that crustaceans have the ability to cope with the increased concentrations of L-lactate which accumulates in the haemolymph because of the anaerobic metabolism required during periods of aerial exposure (Burnett, 1988). There have been few studies on the ability of different sized crustaceans to compensate for increased concentrations of L-lactate, but DeFur & McMahon (1984) reported that the degree of compensation was greater in smaller *Cancer productus*. However, on the *Dunan Star* the large sized *N. norvegicus* were the only category where the mean air temperature on the day of capture did not have a negative impact. This may reflect preferential treatment by the crew of the larger animals, as these animals obtain a higher market value.

The large variability observed in the data is likely to be a result of other variables. The trawling grounds that each boat uses varies largely on a daily basis and so the steaming time back to harbour may vary greatly resulting in a longer recovery time in the onboard seawater tanks before further stress during the road transport stage (handling and further aerial exposure). The oxygen debt and respiratory acidosis (following trawling and subsequent aerial exposure) are reduced during the periods that *N. norvegicus* are submerged in the seawater tanks. However, if lobsters are further stressed, by re-handling or aerial exposure before this recovery is complete, the acidiotic stress is compounded.

Following this treatment acid-base stress is consequently more severe and survival times in air potentially reduced (Taylor *et al.*, 1997).

Interestingly, Spanoghe & Bourne (1997) also reported that survival was greater if lobsters were packaged at the processing units during the day compared to the evenings. This was attributed to the peak foraging time of *P. cygnus* being in the evenings which is reflected in the circadian rhythm of the crustacean hyperglycaemic hormone (CHH) (Kallen *et al.*, 1990).

The study period was from November, 2002, until April 2003. During the March and April months there is a peak in the occurrence of *Hematodinium* in *N. norvegicus* (see Field *et al.*, 1992; Appleton *et al.*, 1997; Field *et al.*, 1998; Stentiford *et al.*, 2001a), which coincides with the peak temperatures observed during the study period. *Hematodinium* is a motile protozoan parasite which infects *N. norvegicus* rendering them moribund, exhibiting an opaque appearance and white haemolymph. This poor appearance and condition causes *N. norvegicus* to be rejected for the live transport market. It could be suggested that the high degree of morbidity and mortality observed during the spring periods, when air temperature would be at its highest, could be due to *Hematodinium* infection, however if this was the case then the catch from both boats would exhibit similar morbidity and mortality rates. As this does not occur this hypothesis is unlikely.

Other factors are likely to result in the observed variations in M+M. In this thesis, chapter 3 has reported that increasing the duration of the trawl results in higher stress levels and increased physical damage. In chapter 3 it was also found that greater catches were obtained in the spring which would results in greater crush forces in the net, possible causing more damage. Sea temperature varies little through the seasons, but surface waters vary in terms of temperature and salinity, as it is this water which is used to submerge the

export cartons, higher aerial temperatures resulting in higher surface temperatures may have a negative impact on the health status of the catch. Interestingly the boat with the lowest rate of morbidity and mortality also had one more crew member, which may result in faster sorting times, reducing the periods of aerial exposure.

Symonds & Simpson (1971) studied that survival of small discarded *N. norvegicus* and hypothesised that during severe weather (when the boat might roll excessively during hauling) the mortality of rejected animals would be increased. In the present study there was no correlation observed between the degree of mortality observed and the wind speed on the day of capture.

This study has demonstrated the need for a standard post capture protocol in the Clyde Seafishery to reduce the variability observed in the M+M% of the catch. A larger study, with more detailed information on the post capture treatment, as done by Spanoghe & Bourne (1997), including surface seawater temperature, estimates of economic losses, and the negative effect of prolonged sorting times will aid in optimising the returns from the fishery. The Clyde Sea fishery and related industries have become increasingly reliant on the *Nephrops* fishery, through optimising catch condition and survival, reducing the amount of rejected *N. norvegicus*, the impact of the fishery on the population can be minimised resulting in a more sustainable fishery.

Chapter 6

Idiopathic muscle necrosis in the Norway lobster *Nephrops norvegicus*: progression, aetiology and pathology.

6.1 INTRODUCTION

6.1.1 *Nephrops* fisheries

In recent times there has been increasing demand to provide high quality seafood, and research into the post capture treatment of *N. norvegicus* is attempting to achieve a better quality of meat for consumers. Traditionally, vivier transportation of *N. norvegicus* has been based on animals captured by creeling, since this capture process provides animals that are in good condition and are able to survive the stresses of transportation. Recently, however, attempts have been made to transport trawl-captured *N. norvegicus* alive to continental markets, since profit margins can potentially be greater from trawled animals. However, since trawling is considered a more stressful method of capture, post capture holding conditions and treatment become even more crucial for animals captured in this way.

In the summer and autumn of 1999 *N. norvegicus* caught in the sound of Jura and other locations on the West Coast of Scotland were reported to be dying or moribund during vivier transport, with some catches being refused at market because of the opaque appearance of their tail musculature. Stentiford & Neil (2000) described this condition as a rapid-onset post capture muscle necrosis. Through a detailed study of the rejected animals they concluded that the condition most resembled idiopathic muscle necrosis (IMN). They reported that affected animals exhibited a characteristic whitening of individual muscle

fibres and fibre bundles of the abdomen within h of capture. The pathology caused loss of normal function of the abdomen, preventing the "tail-flip" action. Histological analysis failed to reveal any causative agent but indicated a progressive disruption of the muscle structure, with complete loss of the sarcomeric structure. A reduction of the major contractile proteins was revealed through SDS-PAGE analysis, with the myosin heavy chain being completely absent. It was concluded that the probable causes of the observed pathology were integumental damage in conjunction with exposure to stressors associated with the capture and post capture fishery processes.

6.1.2 Idiopathic muscle necrosis (IMN)

Idiopathic muscle necrosis, also known as 'tail rot', muscle opacity, idiopathic myopathy, muscle necrosis, and spontaneous muscle necrosis, has been reported to occur in wild stocks and under aquaculture situations. The condition has been reported in a number of crustacean species, including the freshwater prawn *Macrobrachium rosenbergii* (Fujimura & Okamato, 1972 Sandifer *et al.,* 1975; Sindermann, 1977; Akiyama *et al.,* 1982; Anderson *et al.,* 1990); *Penaeus* spp. (Rigdon & Baxter, 1970; Venkataramaiah, 1971; Lightner, 1977; Lakshmi *et al.,* 1978; Momoyama & Matsuzato, 1987), the freshwater crayfish *Cherax tenuimanus* (Evans *et al.,* 1998), *Procambarus clarkii* (Lindqvist & Mikkola, 1978; Lowery & Mendes, 1977) and most recently *N. norvegicus* (Stentiford & Neil, 2000).

Previous research into the occurrence of IMN has failed to discover the aetiology of the condition, although several authors have reported its occurrence following exposure to various stressors such as trawl capture (Rigdon & Baxter, 1970; Stentiford & Neil, 2000), temperature and salinity changes (Lakshmi *et al.,* 1978), handling and air exposure (Venkataramaiah, 1971), and starvation (Lindqvist & Mikkola, 1978). Prevalence has

ranged from 11% (Venkataramaiah, 1971, following handling and air exposure) to 85% (Rigdon & Baxter, 1970, following trawl capture), presumably related to the severity of the stresses to which the crustacean was exposed.

The histopathological descriptions of these various cases of IMN in different crustaceans have been very similar, with the pathology being restricted to the abdominal musculature, where there was extensive loss of abdominal muscle structure (eg. Stentiford & Neil, 2000), pathological damage of other tissues, such as the hepatopancreas (eg. Lindqvist & Mikkola, 1978), and an absence of any causative pathogen (eg. Lakshmi *et al.*, 1978). Rigdon & Baxter (1970) did report the presence of bacteria, apparently a secondary feature of the condition, although they were rarely observed in the degenerated tissue.

Stentiford & Neil (2000) also reported that the *N. norvegicus* which developed IMN died within 24-48 h of capture, which is in agreement with previous studies (Overstreet, 1973; Venkataramaiah, 1971). A number of previous studies have also reported that following removal of the stressors, IMN was reversible (Rigdon & Baxter, 1970; Venkataramaiah, 1971).

The aim of this chapter is to further investigate the pathological condition of IMN in *N. norvegicus* by examining its histopathology and progression, and to examine possible aetiologies. Following the fishery process the progression of IMN will be followed and the prevalence recorded. To inform whether IMN is initiated during the capture or post capture periods progression of IMN will be followed immediately after capture and following a period of aerial exposure, once the individuals have recovered from the fishery process. The affect of IMN on the abdominal muscle structure and other organ systems will be observed through histological analysis at the light micrograph level.

6.2 MATERIALS AND METHODS

Experiments to investigate the progression and pathology of IMN were conducted over the course of one year from December 2001 to December 2002. *Nephrops norvegicus* were caught using a standard otter trawl (70 mm mesh size) of 2 h duration from the Hunterston Channel south of the Isle of Cumbrae in the Clyde Sea area, Scotland, UK (55.41° N, 04.56° W). All the animals were in the intermoult stage as defined by Aiken (1980).

6.2.1 Progression

Following capture and any further post-treatments, lobsters were submersed in tanks of aerated seawater and transferred to the University of Glasgow aquaria. In the aquaria animals were placed in tanks holding circulating seawater (10°C, salinity 33ppt) within which they were isolated in specially constructed holding cages (tanks sectioned into 9 compartments using modified commercial "prawn tubes"), in order to prevent misidentification and damage caused by inter-animal conflicts. On arrival, and every 24 h thereafter, for 96 h, the development of IMN on the abdomen of *N. norvegicus* was assessed. Using a score chart representing the segmental division of the abdomen, the location and extent of IMN on the underside of the abdomen was recorded and measured. The number of hemisegments that displayed a white appearance was scored (i.e. one whole white segment was scored as 2/12, and total coverage was scored as 12/12). Selected specimens were photographed using a Canon PowerShot A70 digital camera (Canon, UK) to illustrate the distribution and progression of IMN.

6.2.2 Field prevalence

In a number of initial field trials, 100 *N. norvegicus* were exposed to air for 1 h following trawling before being transferred to aquaria at the University of Glasgow in "Prawn-

tubes". Forty-eight h after trawl capture the percentage of *N. norvegicus* experiencing IMN (of any severity) was recorded. An individual was classified as experiencing IMN if there was any incidence of IMN in the abdomen, no matter how comprehensive the coverage.

6.2.3 Effect of capture and post capture processes on the progression of IMN

In later trials a more systematic study of the progression of IMN was undertaken. The objective was to separately examine the effects of capture and post capture stresses on the progression of IMN. Animals were captured with a standard 2 h otter trawl at the same location as previously detailed. Following landing, groups of 25 animals were then subjected to different periods of aerial exposure (0, 30, 60 and 120 min). Following transportation to the aquaria, lobsters were held, and the extent of IMN was measured as described in section 6.2.1.

The remainder of the catch was transferred to the aquaria and maintained under standard holding conditions for a 3-week acclimatisation period (to recover from the stress effects of the capture and post capture period). After this time, groups of 25 animals were handled and subjected to 0, 60 or 120 min of aerial exposure. The progression of IMN was recorded over the following 96 h, as detailed in section 6.2.1.

6.2.4 Histology

Throughout the experiments detailed above, *N. norvegicus* were euthanised and tissue samples were collected for histological analysis. This was to enable a detailed understanding of the pathological progression of IMN and to confirm that the pathology was restricted to the abdominal musculature. Animals were fixed whole using Davidson's seawater fixative (330 ml ethyl alcohol, 220 ml 100 % formalin, 115 ml glacial acetic acid, and 335 ml distilled water). Approximately 5 ml of the fixative was injected directly into

the haemolymph (throughout the body) to ensure rapid euthanasia and tissue fixation. Once injected, specimens were placed into 250 ml of the same fixative for 24 h (See Hopwood 1996; Bell & Lightner, 1988) before being transferred to 70 % industrial methylated spirit (IMS) for storage and transportation. The various tissues (abdominal and claw muscle, gut, hepatopancreas, heart, gonad) were dissected from fixed specimens, dehydrated in ethanol and embedded in paraffin wax at the CEFAS Laboratory, Weymouth. Sections were cut (3-5 μm thickness) and stained with haematoxylin and Eosin (H&E), and assessed using a Nikon Eclipse E800 microscope fitted with a DXM 2000 digital camera and the LuciaG™ image capture software (all Nikon, UK).

6.3 RESULTS

6.3.1 IMN pathogenesis

Figure 6.1 depicts the progression of IMN in the abdomen of *Nephrops norvegicus* through a series of photographs. Figure 6.1a shows an animal without IMN, the ventral abdomen of which has a translucent appearance (IMN score 0/12). IMN often developed initially as isolated streaks in a segment of the abdomen (eg. Fig. 6.1b) (IMN score 1.5/12), with the pathology restricted to individual muscle fibre bundles. Over time, however, this progressed along the fibres (eg. Fig. 6.1c) (IMN 5/12). By the final stages of the condition, the entire abdomen had become an opaque whitish colour, although even in these advanced cases some segments might still remain unaffected (eg. Fig 6.1d) (IMN score 10/12).

6.3.2 Patterns of progression of IMN following capture

In initial trials during the winter of 2001-2, the progression of IMN in individual *Nephrops norvegicus* was determined following 3 trawls (see appendix 1). From the scores obtained (detailed in Appendix 1), four main patterns of progression could be discerned:

- Necrosis, once started, progressed rapidly. Death occurred within 24-48 h.
- Necrotic regions developed but remained restricted to the areas initially affected.
- Necrotic regions expanded steadily over a protracted period.
- Few, if any, signs of necrosis in initial period followed by initiation and rapid progression in the following days.

In an attempt to clarify these various responses, similar experiments were carried out under more controlled conditions. Here, the segment in which the IMN initiated was recorded (Fig. 6.2). In 52% of the cases it was initiated in the proximal segments (segments 1-3) and in 48% of the cases in the distal segments (segments 4-6). IMN most commonly occurred in segments 3, 4 and 5. During progression experiments there was no evidence of any regression of the IMN condition to the pre-exposed state.

6.3.3 Prevalence

Idiopathic muscle necrosis occurred in 38% of *N. norvegicus* within 48 h of capture, and visible damage to the abdomen was recorded in 28% of those affected.

6.3.4 Effect of air exposure following capture on the progression of IMN

Following trawl capture, *N. norvegicus* were subjected to periods of aerial exposure lasting for 0, 30, 60 or 120 min and the progression of IMN monitored over the successive 96 h (Fig. 6.3). The animals that were exposed to air for only 30 min exhibited a very similar pattern of IMN progression to those submerged in seawater immediately upon landing. In both groups the mean IMN coverage of the ventral surface of the abdomen was around 5/12. Increasing the duration of aerial exposure to 60 then 120 min resulted in successive increases in the coverage of IMN.

6.3.5 Effect of air exposure after post capture recovery on the progression of IMN

Exposing *N. norvegicus* to air after they had recovered from the effects of the capture procedure through acclimatising in seawater aquaria for 3 weeks resulted in a similar pattern as that observed immediately post trawl (Fig. 6.4). When the duration of aerial exposure was 2 h, before returning the animals to seawater, IMN scores of up to 9/12 were recorded at 96 h, thus indicating that aerial exposure alone is sufficient to initiate onset of IMN.

Induction of IMN (at an approximately equivalent level to that induced by air exposure for 1 h) occurred in the group of animals that were not exposed to air, but which were only handled underwater within their holding cages. This indicates that handling alone is also sufficient to initiate onset of (albeit limited) IMN.

6.3.6 Histology of organ systems

Histological analysis was performed on a range of tissues and organs from animals that were exhibiting extensive signs of IMN. In all cases it was found that IMN pathology was restricted to the abdominal musculature, with no other muscle systems apparently affected. As an example, the claw musculature exhibited no signs of necrosis or associated pathologies (Fig. 6.5a), with muscle bundles retaining their structure and sheathed by abundant reserve inclusion (RI) cells. There was no evidence of any epibionts such as bacteria or fungi on the gill filaments (Fig. 6.5b). The hepatopancreas appeared histologically normal, with tubules containing small lumens indicating that epithelial reserves were intact (Fig. 6.5b).. Light micrographs of the nerve cord (Fig. 6.5d) failed to provide any evidence of denervation to the affected areas. Figure 6.7e is a cross section of gut wall taken from a specimen of *N. norvegicus* with IMN. There appears to be some

necrotic progression in the circular muscles surrounding the gut, but the longitudinal muscles and other structures (peritrophic membrane, and epithelial cells) of the gut remain unaffected.

6.3.7 Histology of deep abdominal flexor muscle systems (DAF)

In the early stages of IMN the muscle fibres of the deep abdominal flexor muscles (DAF) appear to separate with an amorphous fluid matrix filling the intercellular space (Fig. 6.5f). The series of light micrographs in Figure 6.5 depict the progression of IMN from separation of the myofibres to the complete loss of sarcomeric structure. The focal area of necrosis depicted in Figure 6.6a shows the separation of myofibres, this often accompanied by pyknosis and karyorrhexis of the basophilic muscle nuclei. Necrosis of the muscle fibre appears progressive along its length. The normal striated appearance of the muscle, produced by registration of the sarcomeric structure of the muscle, is shown in the same section as a zone of necrosis (Fig 6.6b). As the IMN progresses, all structure in the muscle fibre is lost (Figs. 6.6c and 6.6d). In advanced lesions, fragments of myofibrils and pyknotic nuclei are scattered throughout the necrotic debris, and the area appears to be infiltrated by elements of connective tissue. Little inflammatory response from the host is observed to such pathologies (see Figures 6.6d and 6.6e). The final stages of IMN are characterised by condensation of the myofibrillar fragments and necrotic debris to form densely stained eosinophilic masses (Figures 6.6e and 6.6f).

6.3.8 Other histological observations

Throughout the histological study of IMN in *Nephrops norvegicus* other pathologies were also observed. Figure 6.7a is a transverse section of the midgut passing through the hepatopancreas; the gut wall is encapsulated by flattened haemocytes that are responding

to the presence of marker proteins (cell adhesion proteins) on the surface of the inner haemocytes. This could be an immune response in the animal to prevent invasion of bacteria and other pathogens present in the gut. Figure 6.7b depicts the haematopoietic tissue in the process of releasing haemocytes into the haemolymph; the eosinophilic stained haemocyanin is visible within the haemal space. Another encapsulation response, this time in the gill filaments, is captured in Figure 6.7c: the centres of the granulomas are darkly stained as a result of melanisation. During the study very few parasites were observed, but in a number of specimens commensal parasites, possibly gregarines, were observed attached to gut wall (Fig. 6.7d and 6.7e). Figure 6.7f is of a section of hepatopancreas containing a large granulomatous lesion, possibly comprising several fused smaller granuloma foci. The central areas are necrotic and contain pigment (including melanin, a downstream product from the ProPO cascade), and possibly necrotic remnants of the cause of the response. Outside the central area (blue-ish band) are whorls of flattened haemocytes. This region could be the site of a necrotic hepatopancreatic tubule but is more likely an inflammatory reaction to foreign bodies (such as bacteria) within the haemal sinus.

6.4 DISCUSSION

This chapter has progressed earlier studies on idiopathic muscle necrosis (IMN) carried out by Stentiford & Neil (2000), and reaffirms the suggestion that the condition observed in *Nephrops norvegicus* during the post capture period most closely resembles IMN.

6.4.1 Progression and initiation of IMN

There have been conflicting findings in the literature on the localities in the abdomen affected by IMN lesions. Momoyama & Matsuzato (1987) most commonly observed

lesions in segments 3-5, while both Stentiford & Neil (2000) and Evans *et al.* (1999) concluded that lesions most commonly occurred in those segments adjacent to the cephalothorax. Others reported IMN most commonly in the distal segments (Rigdon & Baxter, 1970; Lakshmi *et al.*, 1978); Akiyama *et al.*, 1982). In the present study the position from which IMN initiated was investigated. It was concluded that IMN could be initially observed in any of the abdominal segments but most commonly in segments 2-5.

Various researchers have suggested that IMN is reversible (Lakshmi *et al.*, 1978; Rigdon & Baxter, 1970; Venkataramaiah, 1971). Rigdon & Baxter (1970) reported the diminution of the number and size of the white foci within 24 h of shrimp being removed from overcrowding situations. Lakshmi *et al.* (1978) found that reversal of muscular lesions occurred faster in purely oxygenated water (aerated with oxygen) than in normoxic water (aerated with air). In the present study there was no evidence of reversal of the condition, however the condition was never followed for more than 1 week. Due to the pathological affects of the condition it would be expected that repair and regeneration of the muscular lesions would occur over a longer period (weeks to months) than that studied here. It is possible that condition could regress with cessation of the progression, including infiltration of the lesions by other tissues, such as collagen, could occur over a shorter time period, although regeneration will be a more prolonged process. Akiyama *et al.* (1982) noted that after several days, muscle lesions in *Macrobrachium rosenbergii* typically show a proliferation of sarcolemmal nuclei, which Rigdon & Baxter (1970) claimed indicates regeneration in the necrotic foci. Fontaine & Lightner (1973) investigated the histological responses of *Penaeus aztecus* to wounding using a pin, and discovered that it was not until 16 days post insertion that wound repair was essentially complete and the tissue had regained its previous structure. In the vivier transport industry, holding the animals for any

longer than a week is not economically viable. It is the author's view that regressing the condition is not a viable solution to reducing economic losses associated with IMN.

We have demonstrated several different patterns of IMN progression in *N. norvegicus*. In the literature it has generally been reported that most animals affected with the condition die within 24 h and a few of them within 2-4 days (Rigdon & Baxter, 1970; Venkataramaiah, 1971; Overstreet, 1973; Akiyama *et al.*, 1982). The speed at which IMN progresses will be affected by a number of factors; these include the duration spent in the trawl net, the length of time exposed to air on the deck and the degree of physical damage caused by the capture and post capture processes.

In the present study a number of animals exhibited initiation and rapid progression of IMN several days after the initial stress of capture. The reason for this is unknown, however there are two possibilities: that the necrosis observed after 48 h could be attributed to the systemic bacteraemia reported in chapter 7; or, that poor water quality caused by the decay of deceased animals in the tanks, not removed rapidly enough following death, reduced water quality sufficiently to cause further stress and re-commence the necrosis.

Prevalence of the condition 48 h following trawl capture was estimated to be 38%. The original study by Stentiford & Neil (2000) reported prevalence to be slightly lower at 29% but they assessed prevalence at only 4 h post trawling. Previous IMN prevalence studies on other crustaceans has ranged from 85% (Rigdon & Baxter, 1970, following trawl capture) to 11% (Venkataramaiah, 1971, following handling and air exposure), presumably related to the severity of the stress the crustacean was exposed to.

The fishery processes (trawl capture, aerial exposure, and handling) readily initiated IMN in *N. norvegicus*, however we were also able to initiate the condition through periods of

aerial exposure (in absence of trawling). Prolonging the duration of aerial exposure, both immediately after trawling and after a period of acclimatisation, resulted in increased severity of the necrotic lesions in the abdominal musculature of *N. norvegicus.* This suggests that although IMN is inevitable in trawl caught *N. norvegicus*, the severity of the condition, and therefore economic losses, can be minimised by careful post capture handling and treatments. Sindermann (1977) reported that the only treatment for IMN in crustaceans is to reduce the stress-inducing environmental factors quickly when the first signs of it appear.

According to Stentiford & Neil (2000), it was perhaps significant that physical damage was observed in 46% of *N. norvegicus* affected with IMN. The present study found this value to be slightly lower (28%) although due to the spiny nature of *N. norvegicus* and other by-catch species, such as the velvet swimming crab (*Necora puber*), minor puncture wounds may have been undetectable.

6.4.2 Pathological affects of IMN

Histological analysis of the necrotic lesions observed in *N. norvegicus* closely correlated with studies of IMN in other species. The present findings closely matched those of Momoyama & Matsuzato (1987) who classified the histopathological change in the skeletal muscle into three stages. Stage 1: muscle fibres are necrotic with fusion and cross splitting of myofibrils or separating from myoseptum. Haemocytic infiltration at the necrotic foci is observed. Stage 2: the necrotic fibres are diminished. Proliferation of fibrocytes and production of collagenous fibres are extensive. Stage 3: the necrotic muscle fibres have disappeared and are replaced by connective tissue. Generally the literature has detailed similar pathology associated with the condition: a loss of sarcomeric structure with necrotic lesions containing pyknotic nuclei, fragments of myofibrils and connective tissue elements

and eventual condensation of these elements into separate islands of heavily stained eosinophilic material (Rigdon & Baxter, 1970; Nash *et al.,* 1987; Stentiford & Neil, 2000).

Stentiford & Neil (2000) concentrated their pathological study on the abdominal musculature of *N. norvegicus*. The present study has investigated other major organs and tissues of *N. norvegicus* and reports no other significant effect of IMN on other tissues and organs (including the skeletal musculature of the claws). Akiyama *et al.* (1982) also reported that no lesions were observed in the gill, hepatopancreas, heart, or green gland. Lindqvist & Mikkola (1978) described a similar abdominal muscle wasting condition in the freshwater crayfish, *Procambarus clarkii,* and reported that although the claw muscle appeared normal the hepatopancreas demonstrated a lack of structure, with thin tubule walls, a reduction in overall size and contained no carbohydrates. They attributed this pathology to internal hypoxia caused by lesions and a heavy growth of bacteria and fungi on the gills, the reduced oxygen uptake and continued hypoxia lead to starvation and hence a wasted abdominal muscle and hepatopancreas.

Although there was some evidence of small-scale haemocytic infiltration into the necrotic lesions, the condition affecting *N. norvegicus* appeared to be largely non-inflammatory. Evans *et al.* (1999) reviewed the literature and stated that it was apparent that there were two forms of muscle necrosis observed in decapod crustaceans: one which involved extensive haemocytic infiltration, tissue destruction regeneration (Momoyama & Matsuzato, 1987; Nash *et al.,* 1987; Cheng & Chen, 1998); and the other comprising a non-inflammatory necrosis, in which a weak immune reaction is observed, such as only a few infiltrating haemocytes (Rigdon & Baxter, 1970, Lakshmi *et al.,* 1978; Akiyama *et al.,* 1982).

Holloway & Smith (1982), investigating a myopathy in North Dakota walleye (*Stizostedion vitreon*), also realised that there were two degenerative processes occurring. The first and most pronounced lesion consisted of coagulation necrosis of muscle fibres followed by an immune response and the formation of granulomas. They reported that coagulation necrosis was characterised by destruction of muscle fibres and loss of cellular detail, but retention of the basic shape. The second lesion was non-inflammatory and characterised by focal areas of necrosis, fragments of myofibrils and nuclear debris were scattered throughout the affected area.

Holloway & Smith (1982) offered no explanation for the two conditions; however, Evans *et al.* (1999) suggested that the haemocytic infiltration might occur in response to tissue injury caused by secondary pathogens, such as bacteria. The current study (and allied chapters 2, 3 and 7 in this thesis) support the suggestion of Evans *et al.* (1999) that further studies are required to investigate the relationship between immune responses and necrosis in decapod crustaceans. Specifically, why immune reactions to necrotic tissue sometimes occur, when other times there is no immune response by the individual.

During the present study there was no evidence of any pathogen involvement with IMN lesions of the abdominal musculature. The only parasite observed was a commensal gregarine parasite attached to the gut wall, however these are rarely associated with disease and are not likely implicated in IMN pathogenesis. A number of previous investigations into IMN in decapod crustaceans have reported bacteria present in the necrotic lesions. Rigdon & Baxter (1970) stated that bacteria were rarely observed in degenerated tissue and when present appeared to be a secondary factor and not the primary etiological agent. Nash *et al.* (1987) discovered that *Macrobrachium rosenbergii* larvae affected with IMN lesions in the abdominal musculature also had separate bacterial necrosis lesions, and suggested

that there may be some environmental condition predisposing larvae to both conditions. Stentiford & Neil (2000) and Akiyama *et al.* (1982) noted a lack of any pathogens associated with IMN.

During the present study a bacterial necrosis of the abdominal musculature was observed which is described in the following chapter; the condition, a bacterial septicemia, termed vibriosis, is characterised by high levels of gram negative bacteria spoiling the abdominal musculature and causing death in 24-48h. It is still unclear whether the bacterial necrosis is a secondary factor associated with the IMN or a separate condition that is stress induced as observed by Nash *et al.* (1987), or that following trawl capture, *N. norvegicus* are predisposed to both conditions. One hypothesis is that IMN results in the necrosis of the muscle surrounding the gut allowing gut bacteria to contaminate and proliferate within the abdominal musculature resulting in a second type of necrosis with a bacterial aetiology.

6.4.3 Possible aetiologies

Large economic losses as a result of IMN in decapod crustaceans have been occurring for over 30 years (Rigdon & Baxter, 1970; Momoyama & Matsuzato, 1987; Stentiford & Neil, 2000), and yet the aetiology and pathogenesis of the condition is still unknown. So far it is thought that the condition is non-infectious in nature (Venkataramaiah, 1971; Rigdon & Baxter, 1970; Lakshmi *et al.,* 1978) and that periods of stress-induced hyperactivity are instrumental in the causation of IMN (Lakshmi *et al.,* 1978; Nash *et al.,* 1987; Momoyama & Matsuzato, 1987).

During early studies into IMN high mortality rates coincided with sub-optimal and supra-optimal salinity levels, leading Venkataramaiah (1971) to suggest that necrosis may occur as a result of some osmoregulatory problems. However, necrosis was initiated by physical

handling, hypoxia, and overcrowding (Venkataramaiah, 1971), all of which caused hyperactivity in the near sedentary *Penaeus aztecus* (Lakshmi *et al.*, 1978). Hyperactivity was also associated with occurrence of spontaneous muscle necrosis in penaeid shrimp treated with the anaesthetic Quinaldine (see section 1.6.1). Momoyama & Matsuzato (1987), following a histological and etiological investigation of muscle necrosis in *Penaeus japonicus,* concluded that the possible cause of the condition was related to the violent movement for escaping from nets, or overcrowding in the nets used for harvest.

Stentiford & Neil (2000) summarised possible factors that induce the onset of necrosis. During the capture and post capture processes *N. norvegicus* are exposed to an array of stressors such as crushing, physical damage, overcrowding, and aerial exposure which causes considerable disruption to the immune health and physiology of the animal (chapters 3 and 4). Nash *et al.* (1987) considered that stress induced hyperactivity leading to rapid development of muscle hypoxia and accumulation of L-lactate during anaerobic glycolysis were the most likely steps in the pathogenesis of IMN.

It has been reported that exertional myopathies reported in mammals are initiated by intensive or exhaustive activity primarily involving strongly glycolytic fibres (Hulland, 1985). Extreme exertion in these strongly glycolytic muscles causes rapid utilisation of glycogen generating heat and L-lactate, both of which induce degenerative changes in these and adjacent muscle fibres (Hulland, 1985). Stentiford & Neil (2000) hypothesised that repetitive tail flipping by *N. norvegicus* during capture caused this type of affect, in the deep abdominal muscles and the condition may be exacerbated by further post capture conditions.

Exertional or capture myopathies are non-infectious diseases of the muscle brought about by physiological changes, usually following extreme exertion, struggle and/or stress. They have been reported in River otters (*Lutra canadensis*) (Hartup *et al.*, 1999). Both mammals

and birds of all ages and sexes are susceptible to this disease and species differ in their susceptibility to the disease because of physical and behavioural attributes, but is most commonly seen in wild ungulates in British Columbia (Williams & Thorne, 1996; Elkin & Zambke, 2001). Interestingly hot weather appears to exacerbate the condition, and the condition has the potential to occur anywhere wild animals are trapped or pursued (Williams & Thorne, 1996). IMN in *N. norvegicus* could possibly be a capture or exertional myopathy.

In the marine environment Munday *et al.* (2003) described a condition known as "burnt tuna", which is mainly associated with the yellowfin tuna, *Thunnus albacares*, handline fishery (Watson, 1995) and occasionally southern bluefin tuna, *Thunnus thynnus* (Williams, 1986). Affected muscles are pale watery and soft instead of being red, translucent and firm. The most plausible explanation for this condition is that it is a form of calpain proteolosis (Watson, 1995). Stress involved in handline fishing causes the release of catecholamine which promotes glycogenolysis. In fish with low glycogen reserves, low intracellular ATP concentrations and elevated muscle pH are likely to occur. Low intracellular ATP concentrations lead to the breakdown of calcium homeostasis with an increase in cytosolic calcium, which activates calpain. Calpain is an enzyme which attacks non-contractile proteins, such as the Z-discs of muscle. Munday *et al.* (2003) suggested that more efficient catching methods such as polling for wild fish should prevent the problem.

Interestingly hyperactivity was not observed in freshwater crayfish (*Cherax tenuimanus*) exhibiting muscle necrosis (Evans *et al.*, 1999). Evans *et al.* (1999) suggested that muscle necrosis in *Cherax tenuimanus* is initiated by other factors other than excessive muscular contraction and that the necrosis may result from an aberrant effect of an immune process

on muscle fibres. It has been suggested that the rapid progression of the necrosis may be linked to haemocytic infiltration of the muscle and subsequent production of super oxide radicals (Fridovich, 1978; Thornqvist & Söderhall, 1997) which cause peroxidation of membrane lipids and damage to protein molecules leading to cellular injury in muscle (Hulland, 1985). Indeed nutritional myopathy syndrome of fish is known to be associated with free radical production (Miyazaki, 1995) (see section 1.6.1).

Interestingly, the loss of Z-line material, a prominent feature of IMN in *N. norvegicus* (Stentiford & Neil, 2000), represents an early step in pre-moult atrophy in crustaceans (Mykles & Skinner, 1990a). Studies of land crab chela muscle have indicated the importance of calcium-dependent proteases (CDPs), localised in the sarcoplasm, in mediating muscle protein degradation (Mykles, 1990). Mykles & Skinner (1990b) also concluded that compounds that elevate intracellular Ca^{2+} concentrations, such as caffeine, stimulate proteolysis. Interestingly, the Ca^{2+} ion concentration changes during buffering of acidosis (Mercaldo-Allen, 1991). Stentiford & Neil (2000) stated that it is conceivable that IMN in *N. norvegicus* involves activation of these proteases, leading to the initial breakdown of Z-line material.

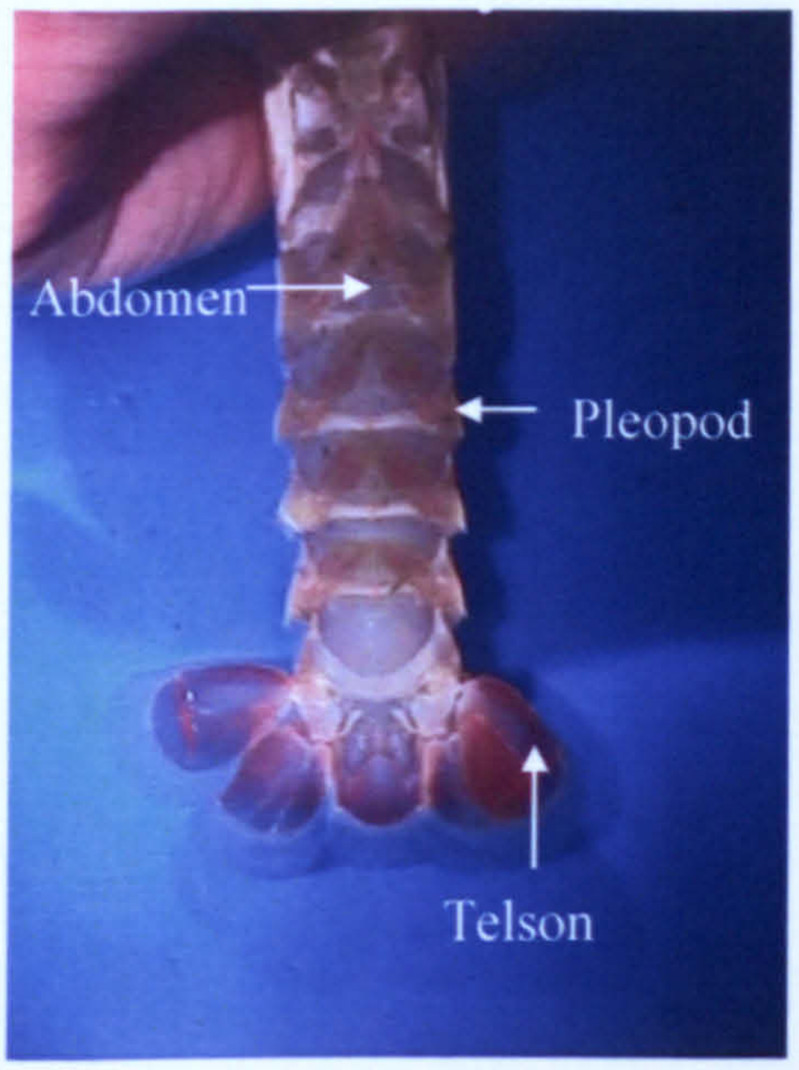
Stentiford & Neil (2000) originally stated that the condition in *N. norvegicus* most closely resembled IMN. Since there has been no evidence of pathogenic implication in the current study, it is still believed to be an idiopathic condition. However, it is yet to be ascertained whether the degradation of the muscle is through necrosis or apoptosis (programmed cell death which results in necrosis). It has been observed that despite severe tissue damage by yellow head virus in *Penaeus monodon*, no inflammatory response has ever been detected in the tissues (Flegel & Pasharawipas, 1998), which suggests that cell death may be due to apoptosis rather than oncosis (which refers to cell swelling, rather than contraction prior to

necrosis) (Khanobdee *et al.*, 2002). Further studies by Khanobdee *et al.* (2002) demonstrated that apoptosis correlated with mortality in *Penaeus monodon.* Apoptosis can be detected through TUNEL staining using the methods of Khanobdee *et al.* (2002) and it is suggested that the type of cell death in IMN by fully investigated in further studies.

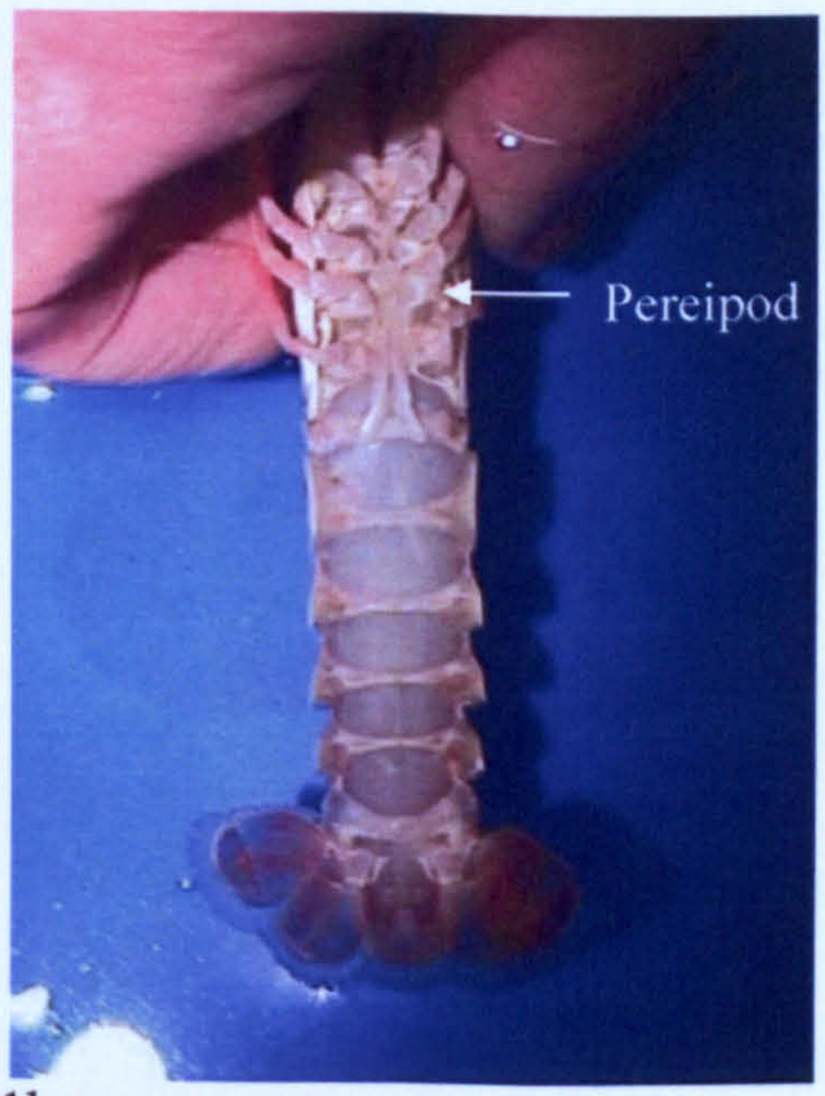
6.4.4 Conclusions

This chapter has furthered our understanding of the pathology associated with IMN and what periods of the fishery process are likely to initiate the condition. The findings support the conclusion of Stentiford & Neil (2000) that the pathological damage and increased mortality observed is the result a stress induced idiopathic muscle necrosis. The actual aetiology is still to be ascertained, but it is evident that the condition is induced through the stressors associated with fishery-related processes. Following trawling, IMN is likely to occur, but the severity of the condition, and therefore economic losses, can be reduced by minimising periods of post capture handling and aerial exposure. Ensuring lobsters are removed from situations likely to cause further stress will prevent the condition from progressing further.

These results suggested that many factors were controlling the rate at which IMN progressed through the abdominal musculature.

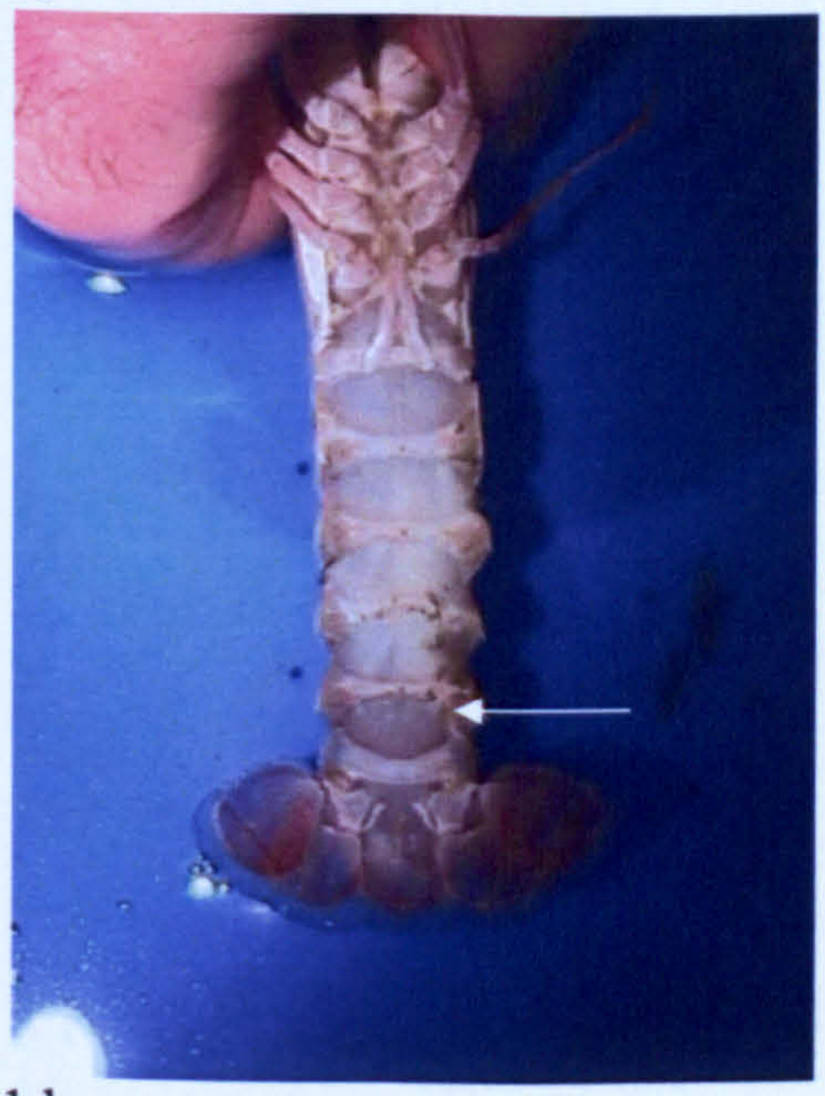


6.1a



6.1b

6.1c



6.1d

Figure 6.1: Series of photographs illustrating the progression of idiopathic muscle necrosis (IMN) in the abdomen of *Nephrops norvegicus*. The abdomen of a healthy individual (a) has a translucent appearance (IMN score 0/12). IMN develops initially as isolated streaks in a segment of the abdomen (b-pleopods removed) (IMN score 1.5/12), initially the pathology is restricted to muscle fibre bundles, and over time progresses along the fibres (c) (IMN 5/12). By the final stages of the condition (d) almost the entire abdomen has a dense opaque whitish appearance, however even in these advanced cases some segments may still remain unaffected (arrow) (IMN score 10/12).

Figure 6.2: Chart demonstrating which abdominal segments the idiopathic muscle necrosis (IMN) initiated from in *Nephrops norvegicus* during the experimental period (expressed as percentage of total *N. norvegicus* monitored).

Figure 6.3: The mean development of idiopathic muscle necrosis (IMN) in groups of *Nephrops norvegicus* exposure to air for 0, 30, 60 or 120 minutes following trawl capture (n=25).

Figure 6.4: The mean development of idiopathic muscle necrosis (IMN) in groups of *Nephrops norvegicus* exposure to air for 0, 60 or 120 minutes following an acclimatisation period in the aquaria (n=25).

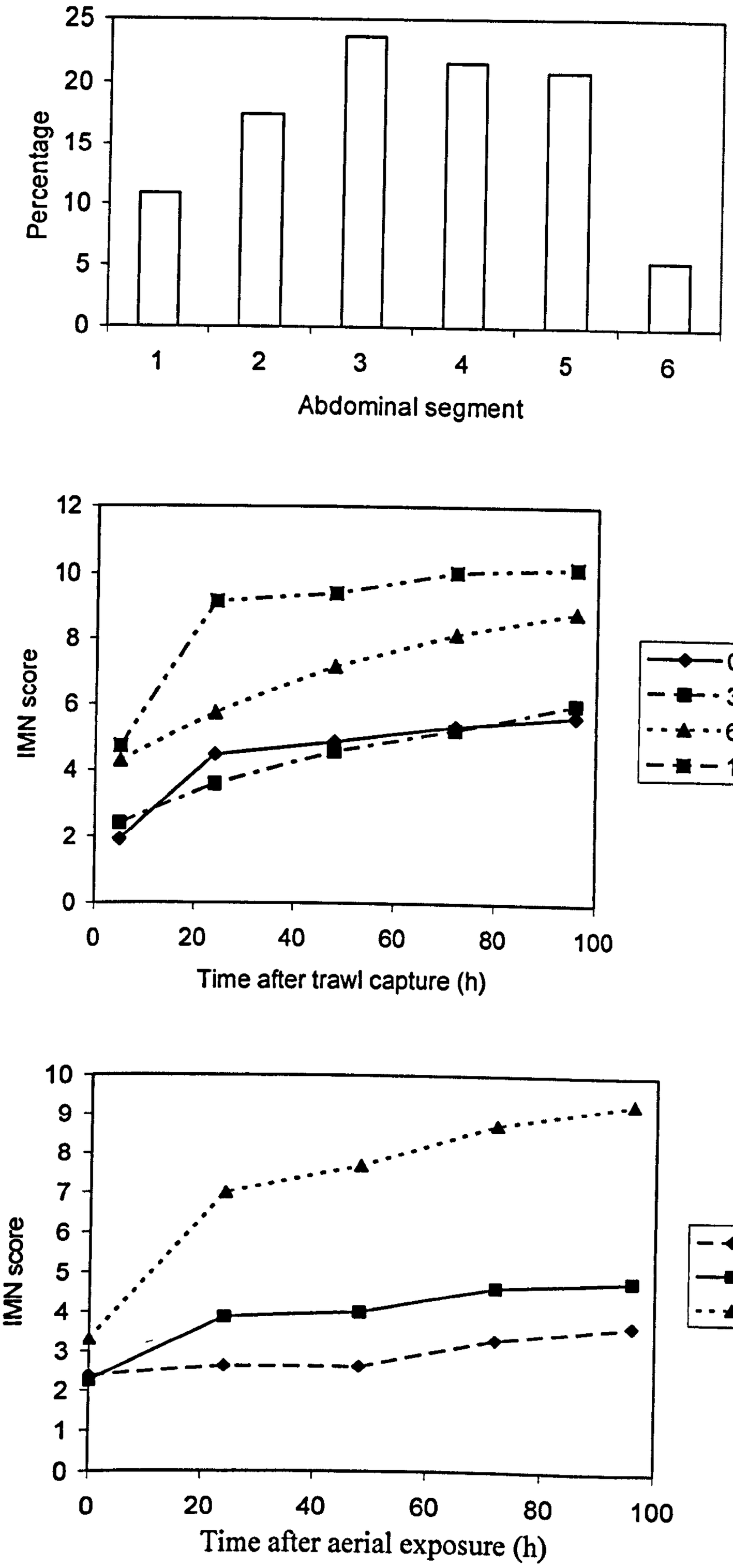
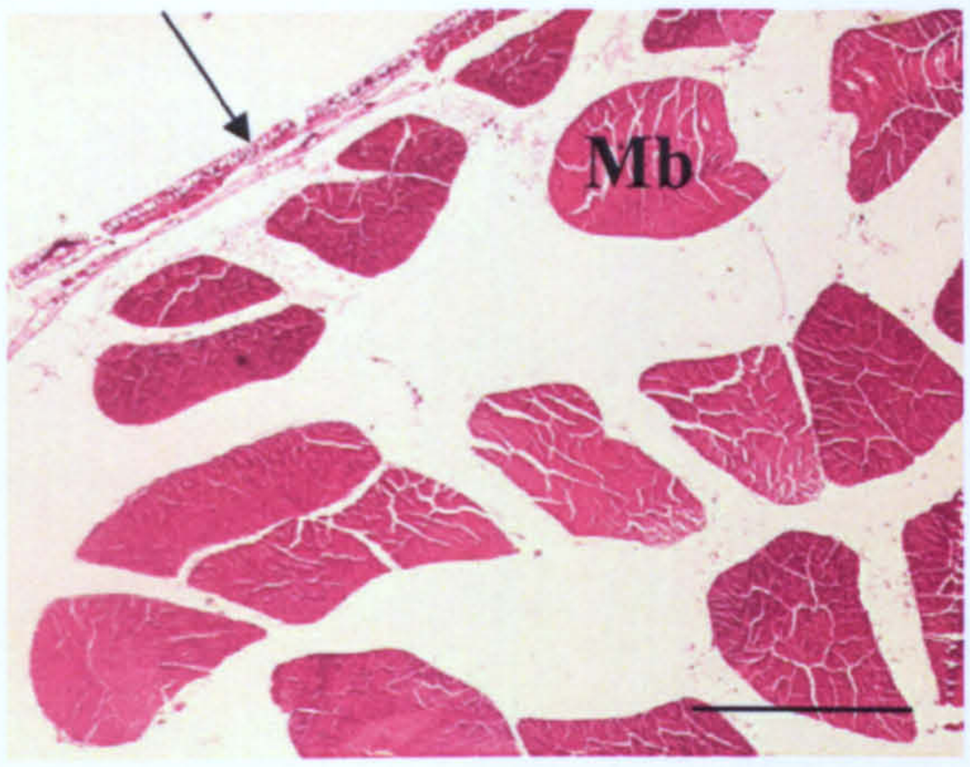

Percentage
25
20
15
10
5
0
1
2
3
4
5
6
Abdominal segment
IMN score
12
10
8
6
4
2
0
0
20
40
60
80
100
Time after trawl capture (h)
0 min
30 min
60 min
120 min
IMN score
10
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
Time after aerial exposure (h)
0 min
60 min
120 min

Figure 6.5: Light micrographs of various tissues removed from *Nephrops norvegicus* experiencing idiopathic muscle necrosis (IMN). 6.5a: The claw muscle showed no signs of any pathology, muscle bundles (Mb) demonstrate good structure and the storage cells can be observed (arrow) indicating the tissue is unaffected by any pathology (scale bar 100μm). 6.5b: In the gill there was no fouling of the filaments (F) (scale bar 100μm). 6.5c: R cells (storage) (R) in the hepatopancreas (Hpc) showed no sloughing of cells, or any other indication of necrosis, and the lumen (L) were small indicating the animal was well fed (scale bar 100μm). 6.5d: Nerve chord (Nc) showing no unusual pathology (scale bar 100μm). 6.5e: Gut wall from a *N. norvegicus* with advanced IMN indicating possible necrosis of the circular (Cm) and the longitudinal muscle (Lm), peritrophic membrane (Prm) (thin membrane on the edge of the gut) and epithelial cells (Ec) show no pathology (scale bar 50 μm). 6.5f: Early signs of IMN, observed in the ventro-lateral muscles of the abdomen, necrosis (N) and separation (S) of the muscle fibres, with necrosis appearing to progress longitudinally not laterally along muscle fibres (scale bar 50μm).



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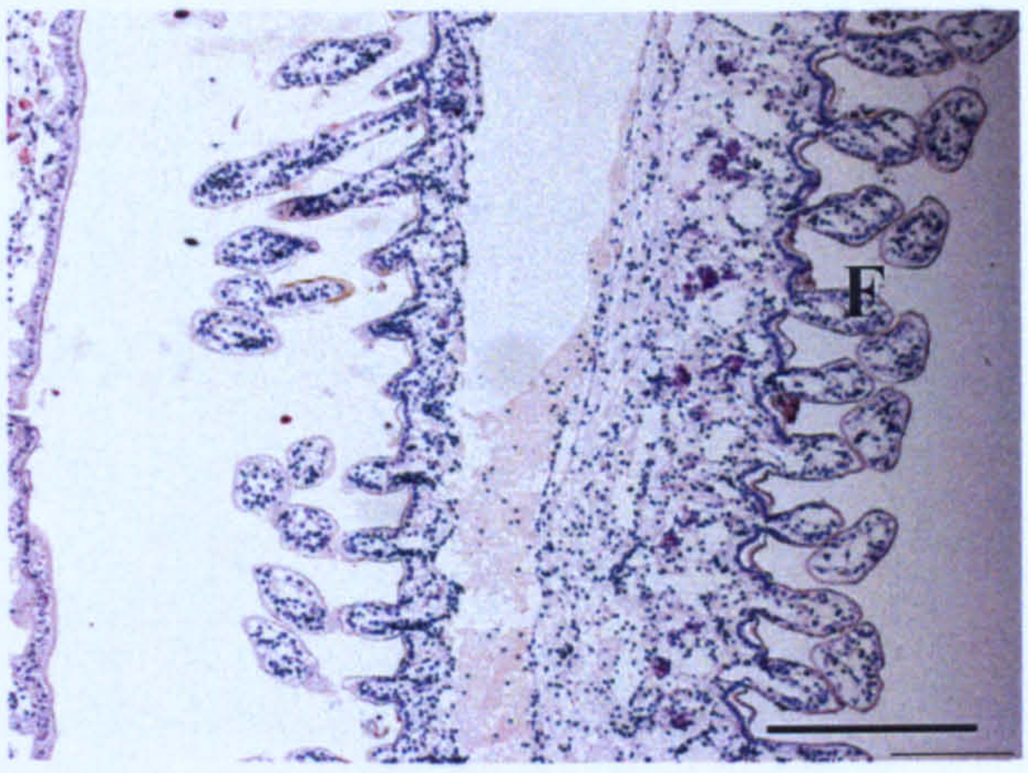

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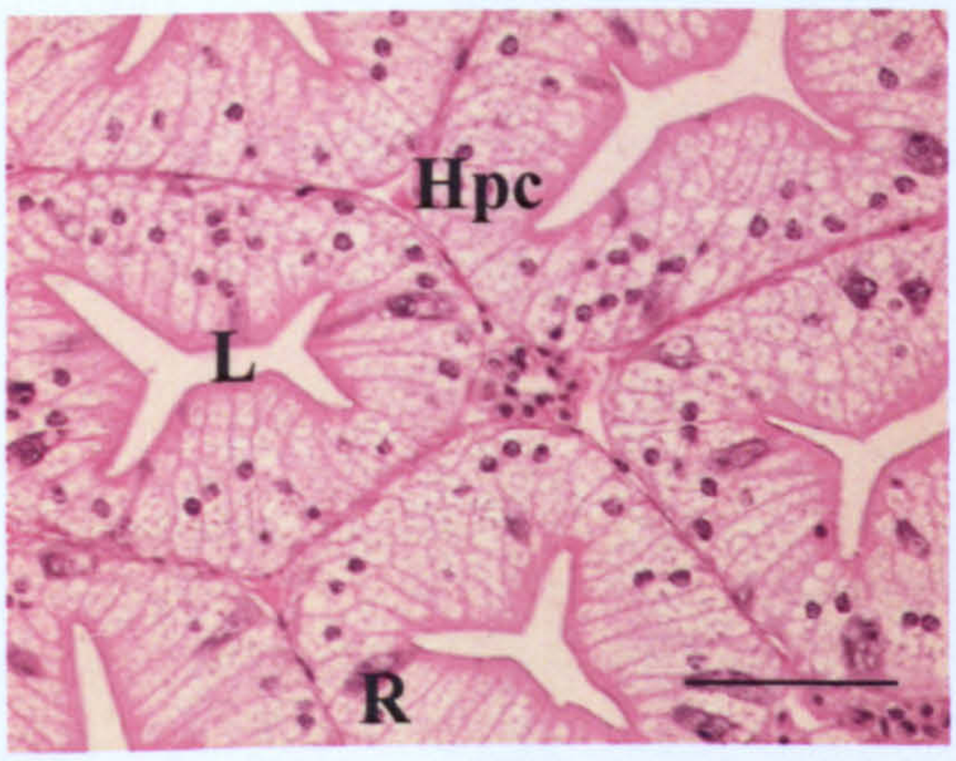

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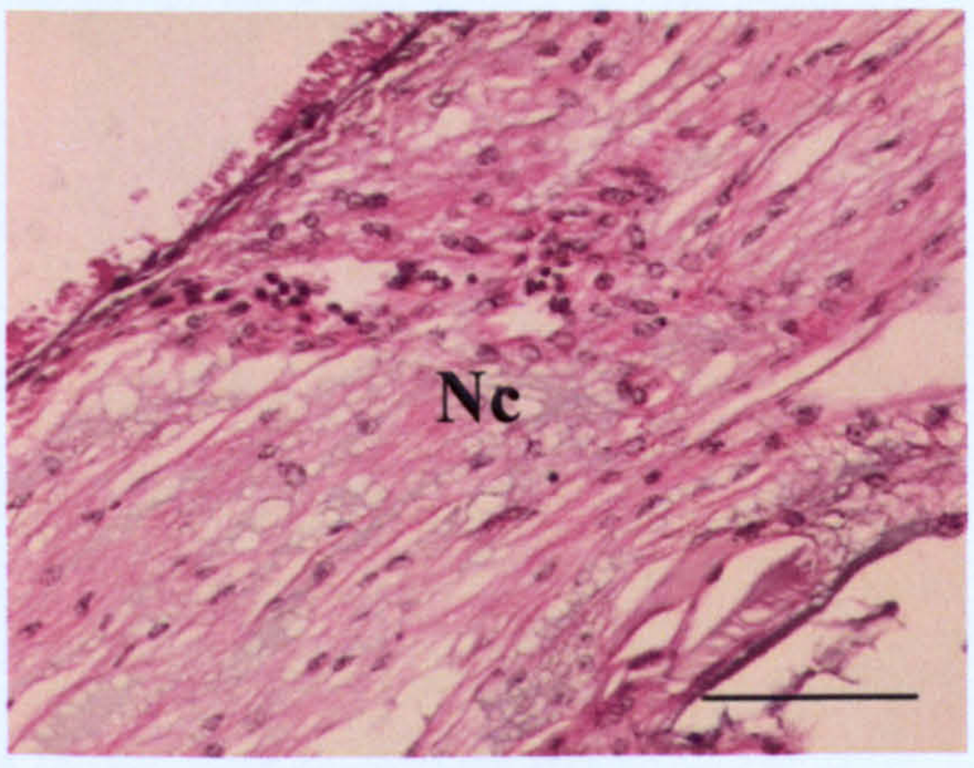

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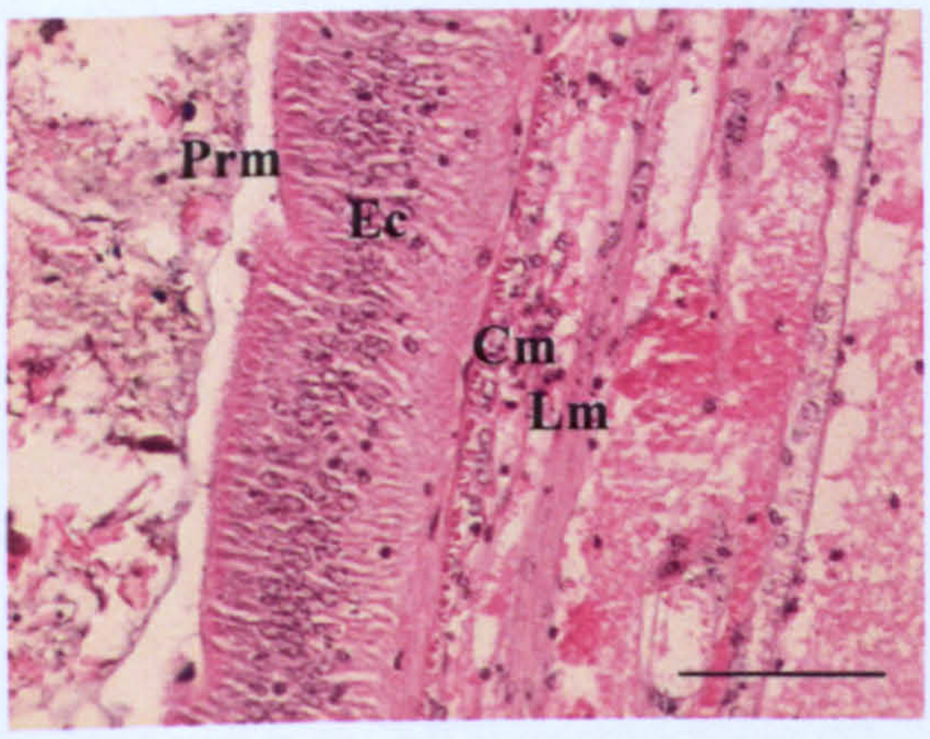

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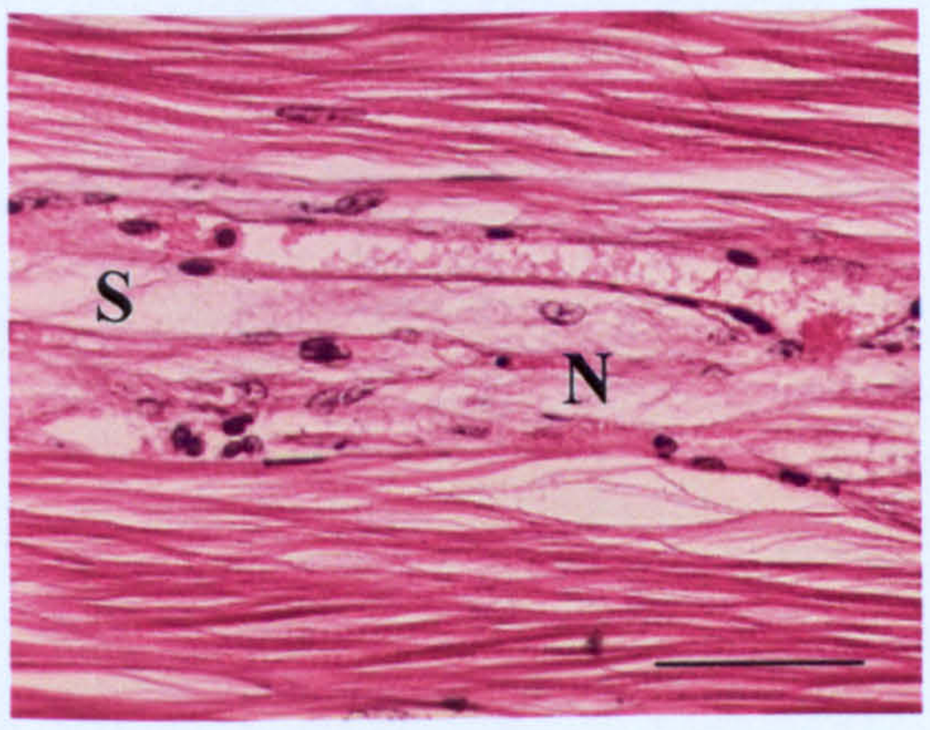
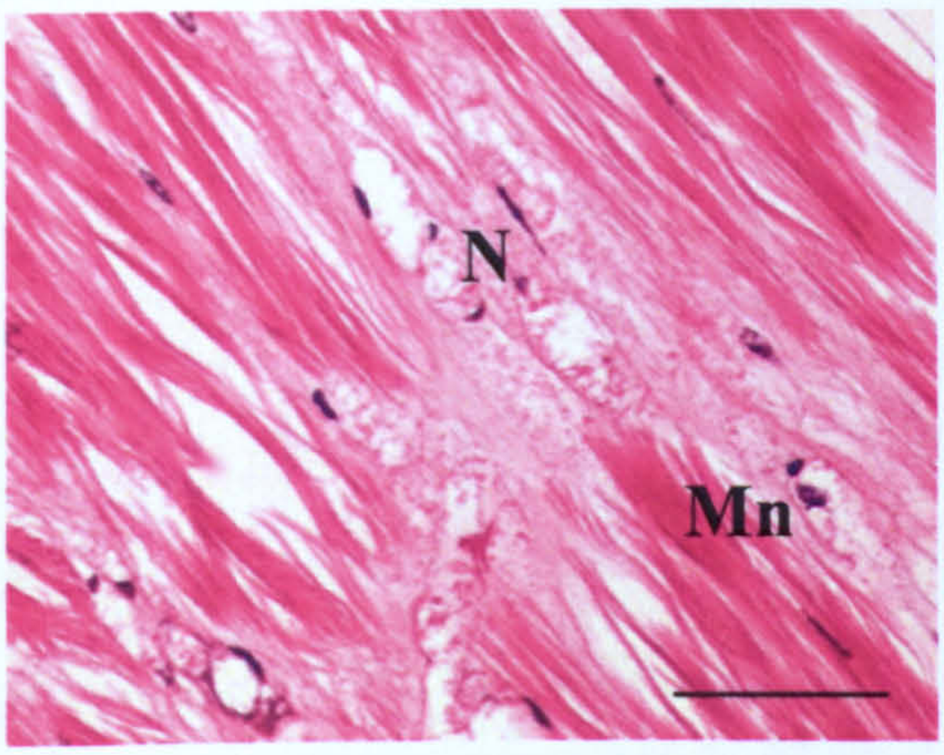
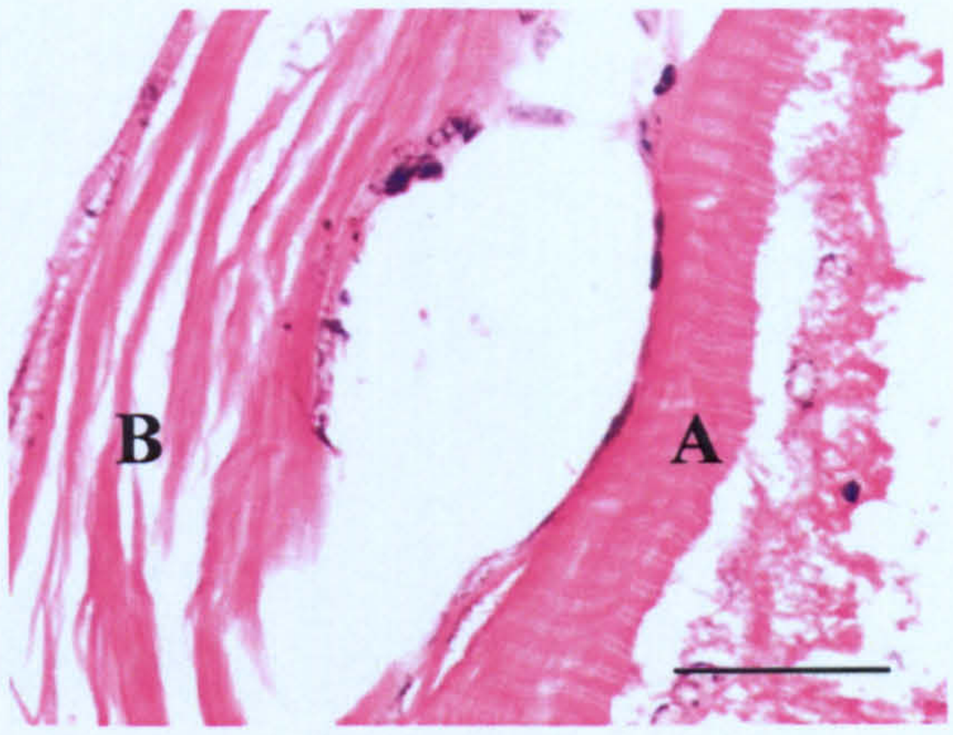


6.5f

Figure 6.6: Histological images detailing the progression of idiopathic muscle necrosis (IMN) in the deep abdominal flexor muscles of *Nephrops norvegicus* (in all images scale bar 50μm) 6.6a: Focal area of necrosis (N) illustrating splitting of the myofibrils and occasional pyknosis and karyorrhexis of the basophilic muscle nuclei (Mn), with IMN progressing along the muscle fibres. 6.6b: Two contrasting sections of muscle, a band of muscle with normal sarcomeric structure (A) and a section of muscle where the myofibrils are separating resulting in a loss of muscle structure (B). 6.6c: "Wave" of the necrotic foci moving in the direction of the arrow, the area behind the arrow has lost all sarcomeric structure, in the area of the necrosis (N) there is an increase in myofibrillar fragments and connective tissue elements. 6.6d: Haemocytic infiltration (arrow) and early attempts at encapsulation, again the area is infiltrated with connective tissue elements. 6.6e: Late stage IMN, some haemocytic infiltration (H) representing failed attempt at encapsulation, the myofibrillar fragments are condensing together to form densely stained eosinophilic (E) material. 6.6f: End stage separating islands (I) of condensed myofibrillar bundles.





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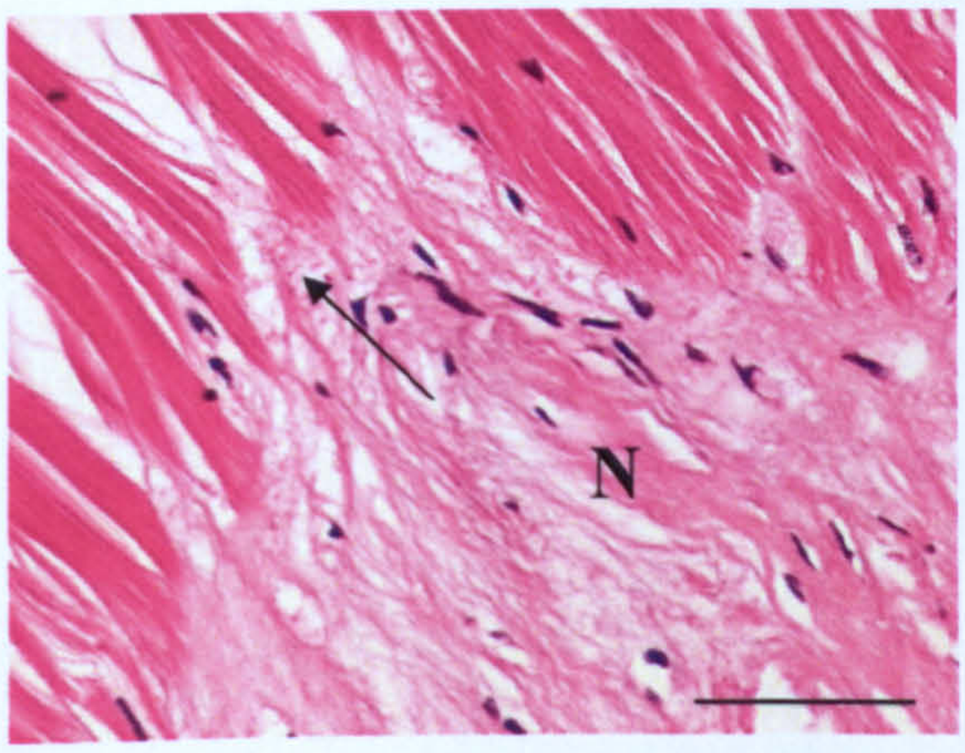

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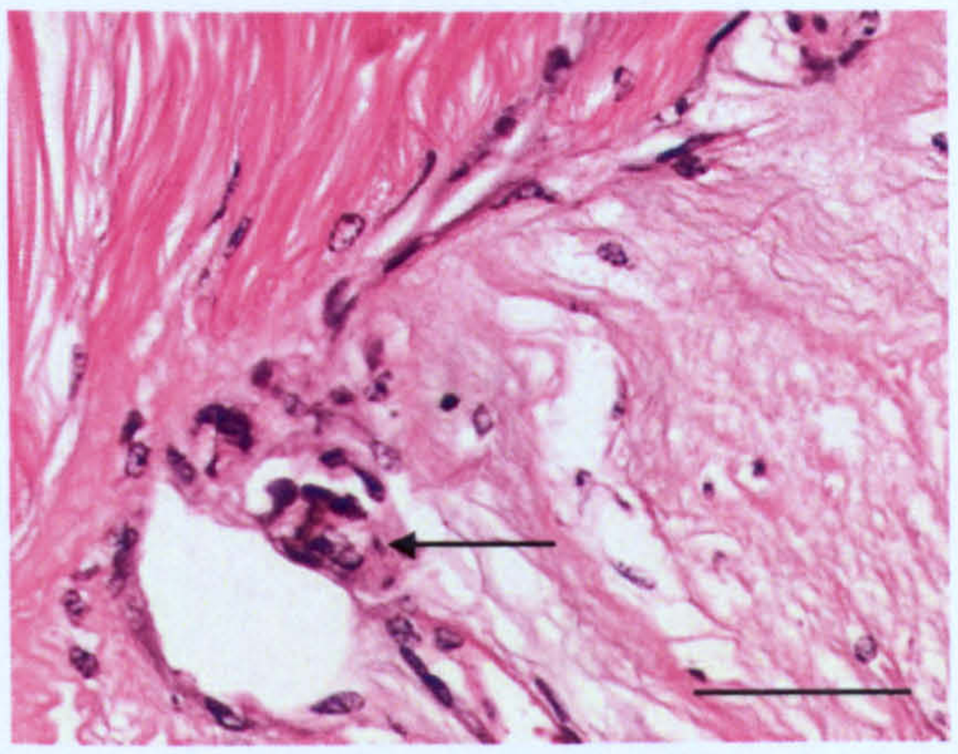

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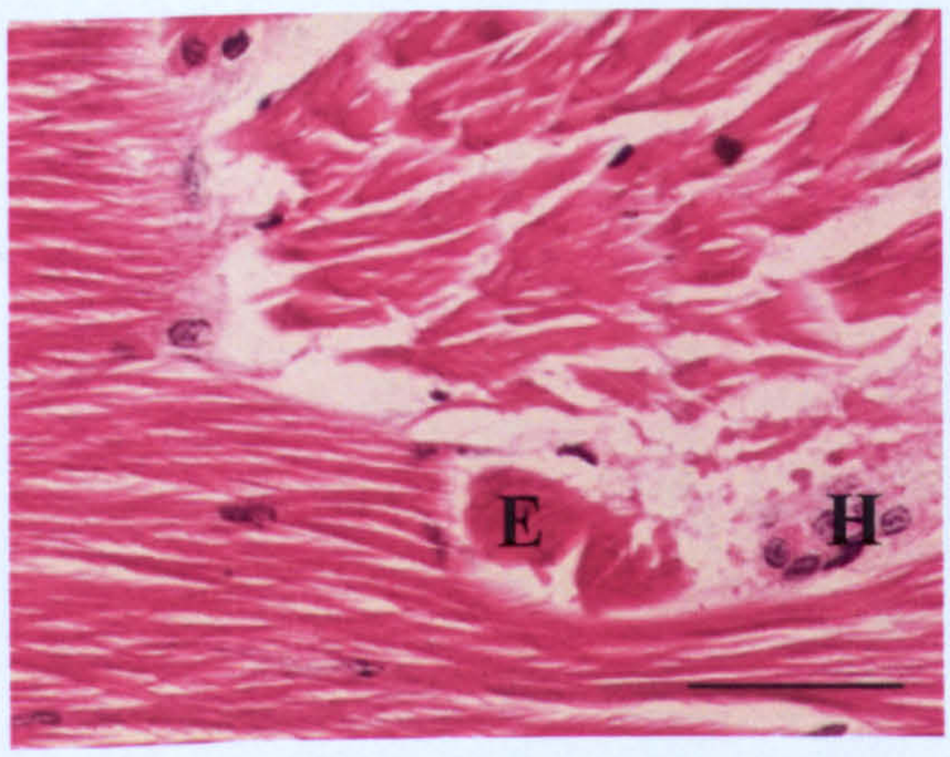

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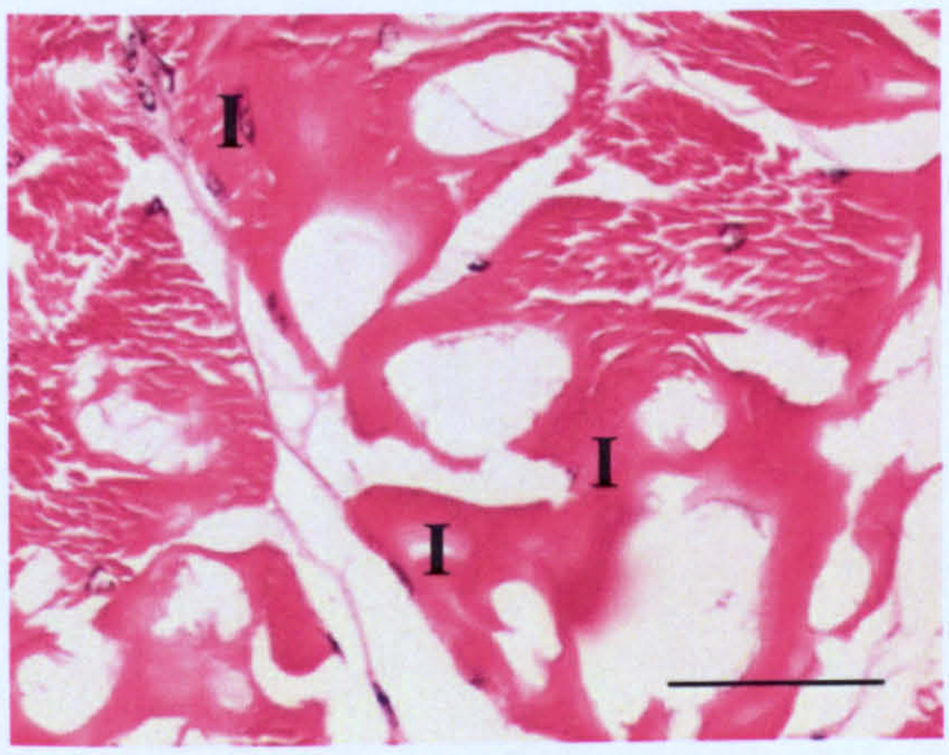
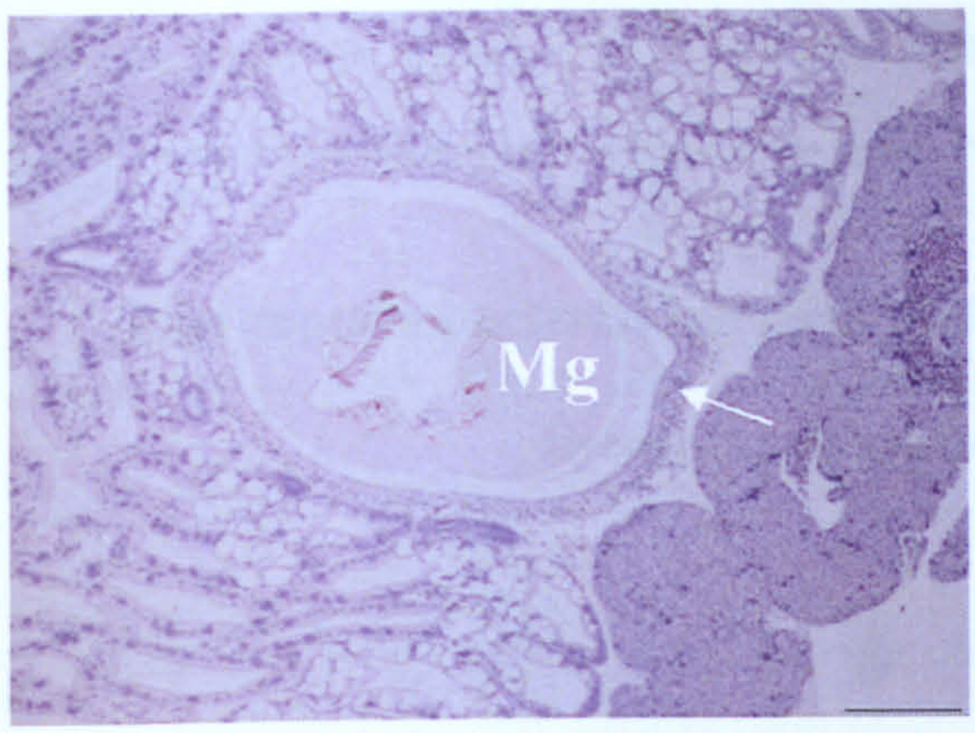


6.6e

6.6f

Figure 6.7: Throughout the histological study of *Nephrops norvegicus* other pathologies were observed. 6.7a: Transverse section of the midgut (Mg) passing through the hepatopancreas, the gut wall is encapsulated by haemocytes (arrow) (scale bar 100µm). 6.7b: Proliferating haematopoietic tissue (Hpt) in the process of releasing haemocytes into the haemolymph containing haemocyanin (Hc) (scale bar 50µm). 6.7c: Encapsulated foreign (Ef) material in the gill filaments following melanisation reaction by the surrounding haemocytes (arrow) (scale bar 50µm). 6.7d: Gregarine parasites (Gg) attached to the gut wall (Gw) (scale bar 50µm). 6.7e: Higher magnification light micrograph of a gregarine (Gg) attached to the gut wall (Gw) (scale bar 25µm). 6.7f: Large granulomatous lesion, possibly comprising several fused smaller granuloma foci. The central (colourful) areas are necrotic (Nc) and contain pigment (including melanin (downstream effect from ProPO), and possibly remnants of whatever caused the response (bacteria?). Outside are whorls of flattened haemocytes (arrow), this could be a necrotic tubule/tubules but more likely an inflammatory reaction within the haemal sinus (scale bar 100µm).



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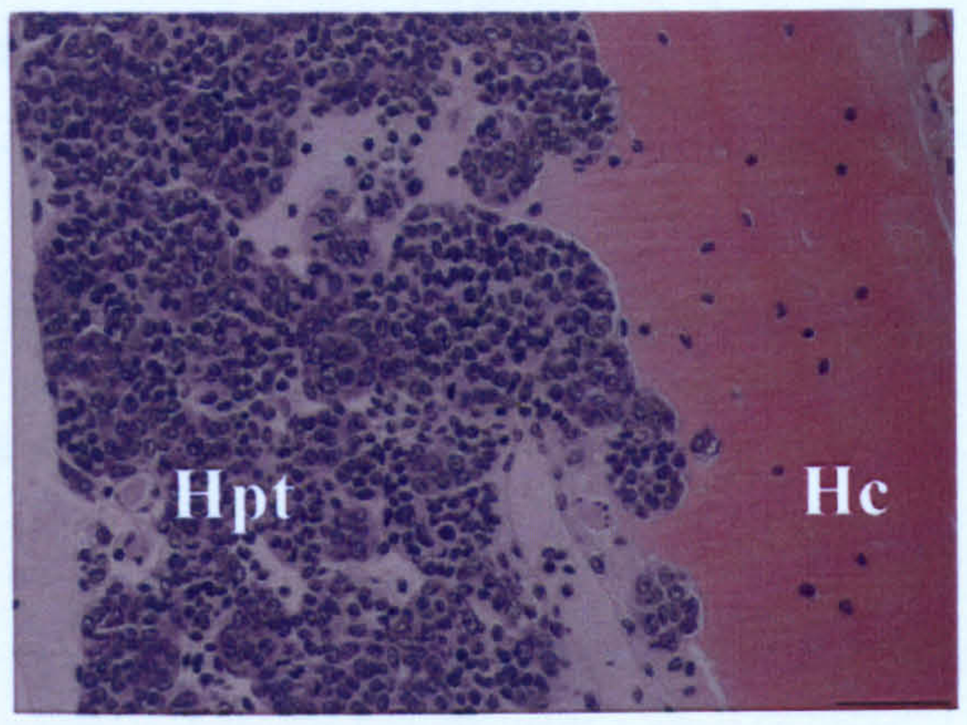

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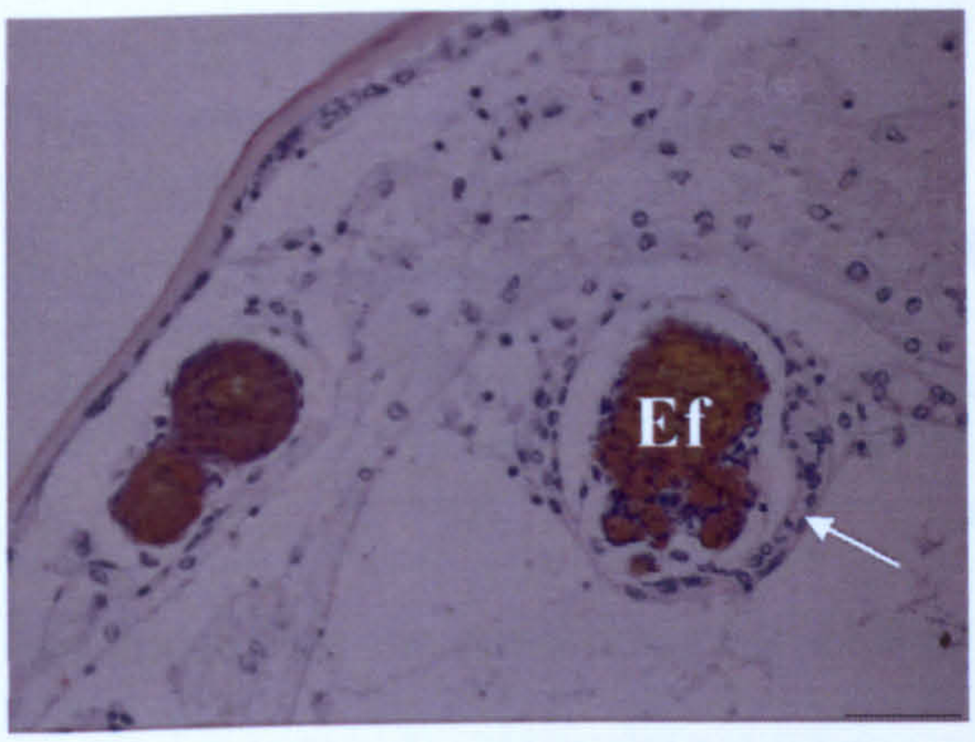

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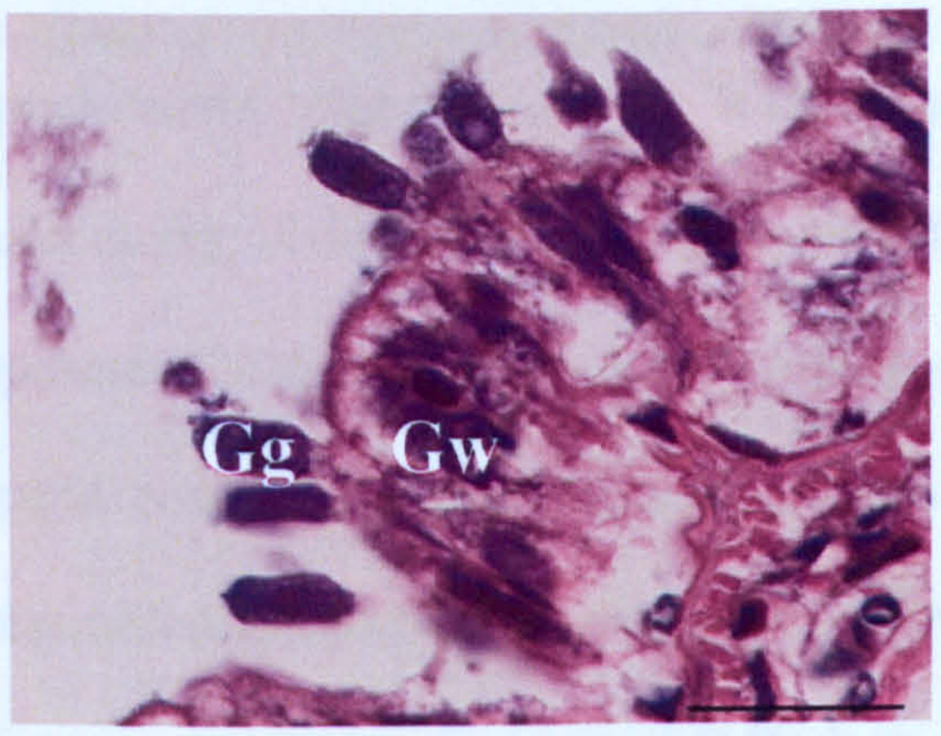

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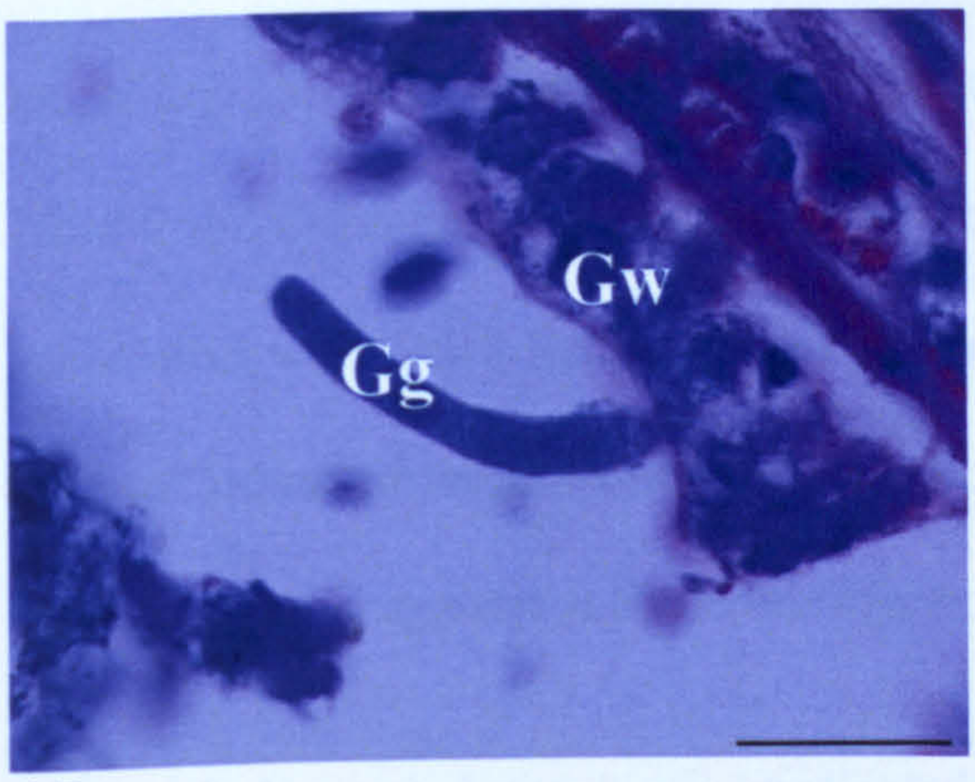

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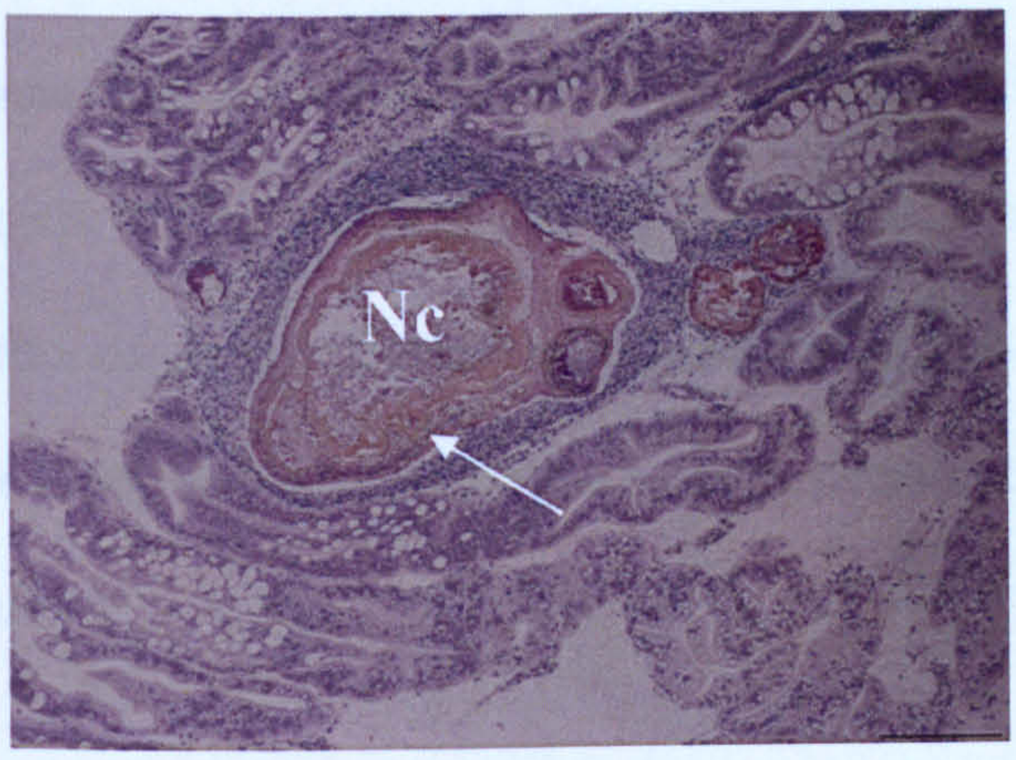


6.7f

Chapter 7

Post capture bacterial infection of the Norway lobster, *Nephrops norvegicus*: discovery, characterisation and pathology.

7.1 INTRODUCTION

During the summer and autumn of 1999, creel-caught lobsters from the West Coast of Scotland were reported to be dying or moribund during vivier transport, with some catches being refused at market due to the opaque appearance of the abdomen. A preliminary study by Stentiford & Neil (2000) discovered a rapid onset post capture muscle necrosis and concluded that the condition most closely resembled Idiopathic Muscle Necrosis (IMN). During further research by this group into progression and pathology of IMN it became evident that another condition, in addition to IMN, was responsible. Lobsters were exhibiting different clinical symptoms to those animals classified as suffering from IMN Briefly the different clinical symptoms were the totally white abdomen, not the streaking associated with IMN, reddening of the pleopods, dorsal flexion of the abdomen and a "rotten" odour to the muscle. Further histological analysis, in addition to behavioural and clinical signs, suggested a bacterial infection.

Bacterial diseases have been implicated as major causes of mortality in crustaceans (Sparks, 1985). The capture and retention of wild shrimp frequently results in catastrophic epizootics caused by vibriosis (Sparks, 1985). Sparks (1985) reported that bacteraemias of penaeid shrimp have been attributed to bacteria from the genus *Vibrio*; however, there is evidence that an *Aeromonas* sp. may also be implicated. Cheng & Chen (1998) also reported a bacterial necrosis in *Macrobrachium rosenbergii* associated with poor culture

facilities, and identified a gram-positive *Enterococcus*-like bacterium as the causative pathogen.

Vibriosis has been reported in cultured shrimp, *Penaeus monodon* (De la Peńa *et al.*, 2001) the American lobster, *Homarus americanus*, (Bowser *et al.*, 1981), and the blue crab, *Callinectes sapidus*, (Johnson, 1976). Diggles *et al.* (2000) also reported luminous vibriosis in phyllosoma larvae of the rock lobster, *Janus verreauxi*, reared in an experimental culture facility. To date, with the exception of an experimental infection with *V. harveyi* (Montero, 1998), there have been no reports of bacterial infections in *N. norvegicus*. Typically, these have been attributed to stressful conditions often associated with poor water quality and aquaculture management (Lightner, 1996). Coincidentally, many reports suggest that *Vibrio* spp. are opportunistic rather than primary pathogens (Song *et al.*, 1993; Chou *et al.*, 1995).

Vibrio spp. are pathogenic bacteria capable of overcoming the host immune system and then multiplying in the body fluids and internal organs, causing inflammation and necrosis of many organs (Aguirre Guzmān & Ascencio Valle, 2000), and thus having a major impact on meat quality and survival post capture. Once a virulent bacterial strain has gained access into the host tissue, then disease and death are normally imminent. Virulence factors, such as cytolytic and haemolytic toxins, have been described for *V. damsela* (Kreger, 1984; Kreger *et al.*, 1987; Toranzo *et al.*, 1993).

Diagnosis of bacteraemia is dependent on the demonstration of bacteria in the haemolymph, as gross clinical signs common to bacteraemia are non-specific and could indicate one or more other disease conditions. Such symptoms include lethargy, white discoloration of the abdomen, and redness of the pleopods (Sparks, 1985). Further

histology and the use of selective growth media will confirm the epidemiology of the condition.

This study was undertaken to ascertain the epidemiology of this condition affecting *N. norvegicus* and to describe its pathology and the effect of the condition on the muscle structure. Abdominal muscle, removed under sterile conditions, from *N. norvegicus* displaying the gross clinical signs of bacteraemia will be plated out on media plates to quantify the extent of bacterial involvement. Isolates removed from necrotic and healthy abdominal muscle tissue will be characterised through a range of behavioural, biochemical (Biolog) and molecular techniques (16s rRNA sequencing). The pathology of the bacteraemia on the abdominal muscle structure will be studied through histological (at the light and electron micrograph level) and SDS-PAGE analysis.

7.2 MATERIALS AND METHODS

Collection of lobsters

Norway lobsters, *Nephrops norvegicus* were obtained by an otter trawl (70 mm mesh size) of 3 h duration from the Hunterston Channel south of the Isle of Cumbrae in the Clyde Sea area, Scotland, UK (55.41° N, 04.56° W); all lobsters were transferred to seawater tanks within 30 min of landing.

7.2.1 Experimental design

On landing, the catch was transferred to the University of Glasgow in seawater tanks, covered by sacks. Upon arrival in Glasgow 12 *Nephrops norvegicus* were sacrificed for microbiological analysis. The remainder of the catch was maintained in aquaria (10°C, salinity 33ppt) for 48 h before further sampling, when 12 apparently healthy and 12 *N.*

norvegicus exhibiting clinical signs of bacteraemia were sacrificed for microbiological analysis. Only male *N. norvegicus* (size range of 30 – 40 mm carapace length) were used for the experiments. All the animals were in the intermoult stage as defined by Aiken (1980).

A further group of animals from the catch (48 h after landing) showing the gross clinical signs of bacteraemia was sacrificed for further histological analysis at the light and electron micrograph level.

Storage of bacteria

10 isolates from each group of animals (healthy at time of capture, necrotic after 48 h, and healthy after 48 h) were grown up 2-3 times on marine agar plates to provide isolated colonies. Isolates were stab inoculated into the Protect system (Technical Services Consultants, Lancaster, UK), 100 µl of 30% NaCl was added to raise the salinity to 3%, and the isolates were stored at -70°C until required for further analysis.

7.2.2 Gross clinical signs of the condition

Before being sacrificed for microbiological and histological analysis, the gross clinical signs exhibited by *N. norvegicus* suspected of having high bacteraemia levels were recorded.

7.2.3 Quantification of bacteria

Marine agar (1.5% (w/v) technical agar (Difco), 29.92 g marine broth (Difco), 800 ml single distilled H_2O) and Thiosulphate-Citrate-Bile salts-Sucrose agar (TCBS) (a medium selective for *Vibrio* spp.) plates were prepared as growth media.

Tissue (~1 mg) was removed using sterile techniques from the deep abdominal flexor muscles below the gut (to prevent contamination by gut bacteria), and was weighed. The tissue was homogenised with 10ml sterile seawater, and 100µl of the suspension was spread over marine agar plates. Further 10^{-1} and 10^{-2} dilutions of this suspension were also plated out.

The plates were incubated at 20 °C for 48 h, and then the colonies were counted. The results were adjusted to take into account dilution factors, and the final counts were expressed as colony forming units per gram (CFU g^{-1}) of tissue.

7.2.4 Characterisation of bacteria

7.2.4.1 Biochemical characteristics

Colony characteristics and morphology

Pigmentation, morphology and growth characteristics on fresh nutrient medium constitute relatively simple though important taxonomic features. Colony size, morphology, colour and texture were visually assessed and recorded after the colonies had been grown for five days at 20°C.

Low power microscopy was used to indicate whether the surfaces were smooth (shiny glistening surface), rough (dull, bumpy, granular or matt surface) or mucoid (slimy or gummy appearance). The opacity (transparent, opaque and translucent), texture when tested with a needle (butyrous, viscous or dry), form, elevation, size, and margin were all recorded. Table 1 lists the main characteristics of each isolates colony.

Oxidase test

The oxidase test was carried out using Kovac's method (Cowan, 1974). This assay tests for the enzyme oxidoreductase, indicating the presence of bacterial cytochrome c. In facultative bacteria, which are capable of living in aerobic and anaerobic environments, cytochrome c is often not found. A positive result is detected in the presence of the enzyme which rapidly catalyses the oxidation of tetramethyl-p-phenylenediamine into a coloured product. The test should be performed on a culture grown on nutrient agar and not on one grown on a medium containing fermentable carbohydrate such as TCBS.

Individual colonies of all the isolates were removed from the nutrient agar using thin wooden sticks and placed on filter paper which had been previously soaked in a 1 % solution of tetramethyl-p-phenylenediamine, prepared immediately beforehand.

Reactions for oxidase test.

Colour	Reaction
Purple, immediately or <10 sec.	+
No colour or after > 10 sec.	-

Luminescence test

The 30 isolates (10 from each group as detailed in section 7.2.1) were grown on marine agar at 20°C for a five-day period. Luminescence was checked daily by examining the plate in the dark. Luminescence is most marked in young cultures and may be lost on further incubation. It is most important that time is allowed for the eyes to become fully adapted to the dark, (at least 5 min) before examining for luminescence.

Gram staining

There are two major types of cell wall, gram-negative and gram-positive. The type of cell wall can generally be determined following reaction to crystal violet dye. Bacteria that retain the dye are called gram-positive, and those that decolourise are gram-negative. Gram's method of staining is probably the most important in bacteriology. It is a differential stain, which divides bacteria into two categories. Gram-positive bacteria stain purple or blue black, and gram-negative stain pink. Additionally, it also allows classification as a rod shaped bacillus or spherical coccus (Atlas, 1986).

A colony was taken from each original plate and grown for 24 h on fresh marine agar plates at 20°C. A loop of each colony was mixed with a drop of distilled water. The bacteria were heat fixed by passing them through a Bunsen flame three times. A drop of filtered, 0.5% crystal violet solution was placed on the colony for 1 min. After rinsing in tap water a drop of iodine solution (20g potassium iodide dissolved in 250 ml distilled water to which 10 g iodine was added before making up to 1 litre with distilled water), applied for 1 minute, rinsed again, and followed by a brief exposure (1 sec) to a decolourising solution (50 % absolute alcohol and 50 % acetone). Finally, the slide was treated for 30 sec with a carbol fuschin solution (1 g basic fuschol dissolved in 10 ml absolute alcohol mixed with 100 ml 5 % phenol solution in distilled water and used as a 1:9 dilution with distilled water).

Controls of each type of cell wall were also carried out on each slide. The positive control used was *Staphylococcus aureus* and the negative control *Escherichia coli.*

The slides were examined using light microscopy with a x100 oil-emersion objective, and the bacterial colour and shape were recorded.

Colour	Gram Stain
Blue / Purple	+
Pink	-

Motility

Isolates were transferred aseptically into 10 ml bijoux bottles containing 5ml of DIFCO™ marine broth, incubated overnight at 20°C. A loop of broth was collected, placed on a cover slip and inverted over a microscope cavity slide. The liquid was examined under x40 objective and motility was recorded.

Oxidative / fermentative

Bacterial metabolism may occur in the presence or absence of oxygen. The metabolism of oxidative bacteria, or aerobes, which involves oxidative phosphorylation, is dependent on the presence of oxygen. Some anaerobic bacteria, facultative anaerobes, are capable of both oxidative and fermentative metabolism, the latter occurring in the absence of oxygen. Obligate anaerobes employ only fermentative metabolism and are inhibited by the presence of oxygen. This test uses a chemical indicator to determine the broad nature of bacterial metabolism.

Difco™ Hugh and Leifson medium (M O/F medium; 200g O/F medium, 1.88g Basal medium, 5g NaCl) was prepared according to the manufacturer's instructions (Difco™, BD Diagnostic systems, New Jersey, U.S.). A glucose solution was added aseptically to the medium via 0.2 μm acrodisc filter to produce a total glucose concentration of 15%. The

prepared medium was then dispensed into capped glass tubes, 3 ml per tube and 2 tubes per isolate. Both tubes were stab inoculated with each isolate and 1ml liquid paraffin added to one of the tubes (anaerobic condition). The tubes were incubated at 20°C, for 14 days, although regular checks were made throughout this period. After this time the reaction was recorded.

Tube 1 (aerobic)	Tube 2 (anaerobic)	Designation
Yellow	Yellow	Fermentative
Yellow	No reaction	Oxidative
Blue	No reaction	Alkaline
No reaction	No reaction	Inert

Catalase

This assay tests for the presence of the enzyme catalase which catalyses the decomposition of hydrogen peroxide into water and oxygen. Hydrogen peroxide is potentially toxic to microorganisms on account of its high oxidative capacity leading to disruption of phospholipid membranes. Consequently, bacterial groups have defensive mechanisms to break down such oxidisers, with catalase being present in aerobes and facultative bacteria.

Isolates were transferred aseptically onto Difco™ 2216 agar slopes prepared in Bijoux bottles and incubated at 20°C for 24 h. After this time, 1-2 drops of hydrogen peroxide were added to the colony and the presence or absence of gas bubbles was noted. If gas bubbles were produced then the isolate was considered catalase positive.

Acid and gas from glucose

Energy production from carbohydrate results in acid products, which may be buffered depending on the metabolism of the bacteria. Some bacterial groups are capable of further catabolism of the acid products into similar compounds including carbon dioxide and hydrogen. Bubbles of gas produced as a result of these additional reactions allow further division of the bacteria into acid or acid and gas producers.

Phenol red broth was prepared by dissolving 10g peptone (protease or pancreatic digest of casein) and 0.018g of phenol red indicator in 1l distilled water. NaCl was added to produce a solution of 2% NaCl and pH adjusted to 7.4.

The phenol red broth was autoclaved in capped glass tubes, which contained small, inverted Durham tubes (care was taken to ensure that no air was trapped in these smaller tubes), at 15 p.s.i. for 15 min and allowed to cool slightly. A 10% glucose solution (or other carbohydrate) was then sterilised by injection through a 0.2 μm acrodisc filter into the phenol broth to produce a final concentration of 1% glucose. The tubes were aseptically inoculated with isolates and incubated at 20°C for 14 days. During this time the tubes were checked regularly and the medium colour and presence or absence of gas within the Durham tubes was recorded.

Colour	Reaction
Pink	Alkali
Yellow	Acid Production

7.2.4.2 Identification and clustering of bacterial isolates with Biolog

10 ml of Sterile Marine Broth (Difco™) was inoculated with isolated colonies grown on marine agar and incubated overnight at 20°C at 100 rpm in a shaking incubator.

The following day the growth cultures were centrifuged at 4000 rpm for 10 min, the supernatant removed, 10 ml sterile seawater was added and centrifuged again at 4000 rpm, and exactly 10 ml sterile seawater was added. 5 ml of the bacterial suspension was transferred to glass tubes, and optical density (OD 600) readings were taken. The suspension was centrifuged again at 4000 rpm and exactly 10 ml Biolog medium was added and mixed well.

The suspension was diluted to obtain an OD 600 of 0.1 with a final volume of 20 ml; 150 µl of this suspension was distributed into each of the 96 wells of a Biolog plate which was then incubated overnight at 20°C. The Biolog system is based on a 96 well plate of 95 different bacterial growth substrates and a control well. It is a colorimetric test and the intensity of the resulting purple colour is scored as 0, 2, 5, and 10. The reaction patterns were then compared with those of known isolates using a cluster analysis package (Clustan, Edinburgh).

7.2.4.3 16S rRNA sequencing

Amplification of the 16S rRNA of the selected bacteria was undertaken using the Polymerase Chain Reaction (PCR), following which restriction enzymes were used to digest the amplicons, and the fragments viewed by electrophoresis.

DNA template extraction and preparation

Template DNA was prepared using a commercial kit (Instagene Matrix, BIORAD), and the manufacturer's protocol was followed with minor modifications. A single isolated colony from a desired isolate was suspended in 1 ml single distilled H_2O in a microfuge tube and centrifuged at 1200 x g. The supernatant was discarded, 200µl of Instagene matrix was added, and the mixture incubated for 15-30 min at 56°C. The mixture was vortexed at high speed for 10 sec and boiled 8 min at 100°C, before being vortexed for another 10 sec and centrifuged at 1200 x g for 3 min.

Polymerase chain reaction

The respective DNA template preparations were subjected to PCR amplification using the universal eubacterial primers 27F and 1522R. For PCR the method of Suzuki *et al.* (1997) was followed. PCR reactions were performed in 25µl total reaction volumes by adding 2.5µl of 10 x reaction buffer (100 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 500 mM KCl, 0.1 % Gelatin (w/v), 1 % Triton X-100 (v/v)), 0.5 µl dNTP's (final concentration 50 mM), 0.12 µl *Taq* Hotstart polymerase (1 unit/µl), 17.88 µl ultrapure H_2O, 1 µl forward primer (27F), 1 µl reverse primer (1522R), and 2 µl target DNA. Following an initial denaturation at 95°C for 15 min thermal cycling conditions were as followed: denaturation at 94°C for 1 min; primer annealing at 55°C for 1 min; chain extension at 72°C for 1 min; repeated for 35 cycles, with a final cycle incorporating a 10 min extension. Amplified products were run on 0.8% (w/v) agarose gels, stained with ethidium bromide and viewed under UV illumination. Images were captured on a UPV gel documentation system.

Cloning and sequencing

Fresh amplification products were ligated into the TOPO® cloning vector and transformed into TOP10 One shot® Chemically Competent *Escherichia coli* by heat shock according to the manufacturer's guidelines (Invitrogen TOPO TA Cloning® kit for sequencing). 100 µl and 50 µl of the transformed *E. coli* suspension were plated onto Luria-Bertani (LB) agar (15 g l^{-1} agar, 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, pH 7.0), containing ampicillin (50 µg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (40 mg ml^{-1}) and incubated overnight at 37°C.

The following day 3 colonies were then cultured overnight at 37°C in 3 ml LB media (15 g l^{-1} agar, 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, pH 7.0), containing ampicillin (50 µg ml^{-1}). The plasmids were then analysed using restriction analysis, 5 µl of the plasmid preparation was added to 1 µl buffer H (Restriction Enzyme 10 x Buffer, Promega), 0.5 µl *Eco*R I (Restriction Enzyme, Promega), and 3.5 µl H_2O and incubated at 37°C for 2 h, and electrophoresis of the products was conducted through submarine agarose gels. Sequencing of selected plasmids was performed by the School of Biological and Biomedical Sciences, University of Durham. Sequences were compared for similarity with those of other bacteria in Genbank using the programme BLAST.

7.2.5 Histology

For electron microscopy 1mm cube tissue sections of deep abdominal flexor (DAF) muscle were fixed for 2 h in a solution containing 2.5% Glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4. Fixed tissue samples were rinsed in 0.1 M sodium cacodylate buffer with 1.75% sodium chloride (pH 7.4) and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1-2 h. Tissue sections were washed for 3 x 10 min in 0.1

M sodium cacodylate buffer and block stained in 0.5% uranyl acetate for 1 h. Following dehydration through the acetone series, resin was infiltrated with a 50:50 solution of 100% acetone: resin for 1 h, sections were then embedded in 100% epoxy resin 812 (Agar Scientific-pre-mix) for 1 h. Specimens were then placed in BEEM capsules with fresh resin and polymerised in oven at 60°C overnight (until polymerisation, all the above was carried out at room temperature). Thick sections (1μm) were stained with toluidine blue and viewed with a light microscope. Suitable areas were identified and thin sections (60-70nm) were cut and mounted on uncoated copper/palladium grids before staining with uranyl acetate and Reynolds lead citrate (Reynolds, 1963). Examination was performed using a JEOL 1210 transmission electron microscope.

For light microscopy *N. norvegicus* were fixed whole using Davidson's seawater fixative (330 ml ethyl alcohol, 220 ml 100% formalin, 115 ml glacial acetic acid, and 335 ml distilled water). Approximately 5 ml of the fixative was injected directly into the haemolymph to ensure rapid euthanasia and tissue fixation. Once injected, specimens were placed into 250ml of the same fixative for 24 h (see Hopwood 1996; Bell & Lightner, 1988), before being transferred to 70% industrial methylated spirit (IMS) for storage and transportation. The various tissues (abdominal and claw muscle, gut, hepatopancreas, heart, gonad) were dissected from fixed specimens, dehydrated in ethanol and embedded in paraffin wax at the CEFAS Laboratory, Weymouth. Sections were cut (3-5 μm thickness) and stained with Haematoxylin and Eosin (H&E), and assessed using a Nikon Eclipse E800 microscope fitted with a DXM 2000 digital camera and the LuciaG™ image capture software (all Nikon, UK).

7.2.6 SDS-PAGE

The muscle protein finger print from the deep abdominal flexor muscle showing the two different types of necrosis were analysed using SDS-PAGE. Sections of affected muscle blocks (~20 mg) were dissected out under *Nephrops* physiological saline and placed into 200 µl of SDS-sample buffer, denatured at 95 °C for 1 minute and stored at –20 °C until electrophoretic separation (see Neil *et al.*, 1993). Discontinuous SDS-PAGE was performed using the technique of Laemelli (1970) with gels containing 5% and 10% acrylamide separating gel and 4% acrylamide stacking gel. The gels were run under the conditions described by Neil *et al.* (1993) using a BioRad mini-Protean system, and stained for 30 min with Coomassie-blue solution (0.1% Coomassie blue). Muscle tissue from healthy DAF muscle was run as a control.

7.3 RESULTS

7.3.1 Gross clinical symptoms

The first recognisable sign of bacteraemia was a gradual change in the appearance of the abdomen, from a translucent colour in the abdominal musculature of healthy individuals to a white opaque colour in necrotic *N. norvegicus* (Fig. 7.1). Melanisation and pigmentation occurred on the ventrolateral edges of the carapace in some individuals. Expansion of integumental erythrophores produces a reddening of the pleopods of moribund or recently dead animals. In some moribund animals dorsal flexion of the abdomen was observed, but this was not observed in all cases. As the condition developed swimming was retarded, and near-moribund animals became lethargic and rested on the bottom, often upright. The condition progressed rapidly, with death generally occurring within 24-48 h after capture.

It was also evident that a "rotten" odour was released when dissecting necrotic *N. norvegicus.*

7.3.2 Quantification of bacteria

The mean bacterial concentration in the abdominal musculature of apparently healthy *N. norvegicus* shortly after capture was 2.4 x 10^4 cfu.g^{-1}; 48 h later this remained at a similar value of 2.9 x 10^4 cfu.g^{-1}. In *N. norvegicus* exhibiting gross clinical signs of bacteraemia the mean bacterial concentration of the abdominal muscle tissue plated onto marine agar had increased 100 fold to 6.1 x 10^6 cfu.g^{-1}, and when plated onto TCBS agar this value was a factor of 10 lower, at 4.4 x 10^5 cfu.g^{-1} (Fig. 7.2).

7.3.3 Taxonomic classification depending on biochemical and morphological characteristics

Following biochemical and morphological analysis of the isolates, the Buchanan & Gibbons (1974), Oliver (1982), and Muroga *et al.* (1987) identification schemes for bacterial isolates were used to designate the isolates to their most likely genera. The results of the biochemical and morphological analysis are summarised in Table 7.1.

All isolates cultured were classified as gram negative rod shaped, motile, oxidase positive bacteria belonging to *Vibrio/Aeromonas or Pseudomonas*-like genera. Following this analysis it is evident that bacteria in each of the groups (A, B and N) were of similar genera, although in group N *Vibrio/Aeromonas* were the predominant genera.

7.3.4 Biolog analysis

Following Biolog colormetric testing the results were analysed using the Clustan™ statistical package. This clustered the isolates on the basis of the growth patterns

demonstrated on the 95 substrates. The growth patterns of a number of *Vibrio* spp. are included as reference.

The Clustan™ analysis separated the data into 5 distinct clusters (Clusters 1-6) (Fig. 7.3). Of the 8 isolates of cluster 1, 6 were from group A and 2 were known *Vibrio* spp. (*V. tapetis* and *V. fischeri*). The next 3 clusters (2, 3 and 4) contained a mixture of 4 isolates from group A, and 6 isolates of group N, and 5 known *Vibrio* spp. (2 isolates of *V. splendidus* and *V. salmonicida.* and 1 isolate of *V. tubiashii*). The 5th cluster contained 4 group N isolates and all 10 isolates from group B. The last cluster contained only known *Vibrio* spp. (*V. parahaemolyticus, V. anguillarum, V. alginolyticus*).

7.3.5 16s rRNA gene sequencing

5 isolates were subjected to 16s rRNA sequencing. Following extraction, amplification and cloning the selected plasmids were sequenced. BLAST searches of GenBank revealed that isolate A6 demonstrated 99% homology with *Pseudoalteromonas issachenkonii* (GenBank accession number AF316144); N3 demonstrated 99% homology with *Psychrobacter* spp. (AY443042); N6 demonstrated 99% homology with *Pseudoalteromonas* spp. (AF539775); N8 demonstrated 99% homology with *Vibrio* spp. (AY262019); N10 demonstrated 99% homology with *Pseudoalteromonas* spp. (AY040229).

7.3.6 Histology

Light microscopy revealed that there was a loss of muscle fibre structure and an expansion of the sarcomeric space (Fig. 7.4). However, some areas contained intact myofibrillar structure, with the damage extending into these undamaged areas. Figure 7.5 is a high power light micrograph of an area of necrotic deep abdominal flexor (DAF) muscle tissue. There is a total loss of muscle structure, and the basophilic muscle nuclei have become

hypertrophied. However in both these light micrographs there is no evidence of a host immune response to the observed pathology and no bacteria can be observed.

Examination of the tissue by electron microscopy revealed that the normal sarcomeric structure of the muscle is totally lost. Throughout the areas of necrotic DAF muscle, gram negative (Fig. 7.6.) and rod shaped bacteria were observed (Fig. 7.7). A group of gram negative rod shaped flagellated bacteria varying in size from 1-2 μm in length was also observed (Fig. 7.8), and a bacterial cell in the process of division (indicated with an arrow) was also viewed. The clear zones around the bacterial cells indicate proteolytic activity (Fig. 7.9).

7.3.7 SDS-PAGE analysis

SDS-PAGE analysis of the necrotic DAF muscle revealed major disruptions in the patterns caused by the electrophoresis of the contractile proteins (Fig. 7.10). Gel A reveals numerous extra bands in the region of 100-50 kDa and below the band of the myosin heavy chain in gel B. The smaller structural proteins, such as actin, tropomysosin and the troponins appeared to be present, and not reduced in quantity. In gel B (5% SDS-PAGE gel) the myosin heavy chain in each lane is of a similar intensity. Another marked difference is the total absence of any haemolymph protein (haemocyanin) in the infected animal.

7.4 DISCUSSION

The chapter has described a post capture bacterial infection in *Nephrops norvegicus.* This is the first reported case of bacteraemia or bacterial septicaemia in this species. Preliminary investigations have indicated that *Vibrio* spp. are the predominant pathogens, allowing the infection to be termed Vibriosis. Initial results suggest that this is an opportunistic bacterial

infection associated with the stressful conditions post capture. Histological studies and SDS-PAGE analysis of muscle tissue have indicated substantial breakdown of the muscle structure.

The plate counts indicate that bacteria are present in *N. norvegicus* that are exhibiting no clinical signs of infection. Similar findings were also made by Collwell *et al.* (1975) and by Lightner (1977), indicating that the haemolymph is not a sterile environment and that the host immune system is able to control low-level bacterial infection (Brock & Lightner, 1990). However, other researchers (Bang, 1970; Lee & Pfeiffer, 1975; Johnson, 1976) have reported that bacteria are not present in the haemolymph of healthy crustaceans. While there has been some debate as to the sterility of muscle tissue, the relative ease with which sterile muscle tissue can be obtained (Gill, 1983) suggests that if present, populations of bacteria in healthy muscle tissue are extremely low (Ingram & Simonsen, 1980). Previous studies with freshly caught *N. norvegicus* have reported the presence of *Achromobacter, Acinetobacter, Cytophaga, Micrococcus, Pseudomonas, Alteromonas* and *Vibrio* species (Cann, 1970; Hobbs, 1971, Montero, 1998). The sterility of crustacean muscle is an important point in the study of stress management in crustaceans. An external stressor (such as elevated temperatures) results in an increase in haemolymph bacteria (chapter 2), if abdominal muscle is sterile particular attention should be focussed on preventing entry of pathogens (wounds etc). Research so far suggests that this is not the case and bacteria are present in low numbers in the haemolymph of *N. norvegicus*.

A number of causative agents have been reported to be responsible for bacterial septicaemia (termed vibriosis if *Vibrio* species are the predominant pathogens) including both gram negative (*Acinetobacter, Aeromonas, Citrobacter, Flavobacterium, Pseudomonas* and *Vibrio*) and gram positive (*Enterococcus, Micrococcus, Bacillus,*

Staphylococcus and *Corynebacterium*) species (Sparks, 1985; Cheng & Chen, 1998; Evans *et al.*, 1998).

Bacterial septicaemia is frequently described as an opportunistic bacterial infection attributed to stressful conditions (Lightner, 1996) and many reports suggest that *Vibrio* spp. are more often opportunistic rather than primary pathogens (Song *et al.*, 1993; Chou *et al.*, 1995). Typically, mildly pathogenic strains of ubiquitous bacteria gain entry into the host, proliferate in the haemolymph and then multiply in the body tissues (Vey *et al.*, 1975; Johnson, 1983; Alderman & Polglase, 1988). The route of entry is still to be ascertained but there are three possibilities. Although wounds were not always noticeable on *N. norvegicus* experiencing bacteraemia in the present study, small wounds undoubtedly occur in the handling, capture and retention of wild caught crustaceans, which provide a portal of entry for potentially pathogenic bacteria that are part of the normal microbial flora of crustaceans (Vanderzvant *et al.*, 1970, 1971). Another route for entry is via the gut, and in marine shellfish and crustaceans facultative anaerobes belonging to the genera *Aeromonas, Pseudoalteromonas, Pseudomonas* and *Vibrio* are usually present, with *Vibrio* being the dominant genus (Suzuki, 1977; Rodriguez & Hofer, 1986; Oxley *et al.*, 2003). The last possibility is that the bacteria resident in healthy animals, which normally are controlled to a low level infection (Brock & Lightner, 1990), exploit the window of opportunity provided by periods of immuno-suppression attributed to the stress of capture.

In the present study 5 of the 8 isolates were classified through biochemical tests as belonging to the *Vibrio / Aeromonas* genera. These data, in addition to the evidence obtained through the TCBS plate counts, suggest that the bacteria causing the infection are predominantly belonging to the *Vibrionaceae*. In previous studies a range of different species of bacteria have been isolated from a diseased animal (Edgerton *et al.*, 1995, *C.*

quadricarinatus; Thune *et al.*, 1991, *Procambarus clarkii*). The dominance of gram negative rods reported in the present study is common to all previous studies in marine crustaceans (Yasuda & Kitao, 1980; Dempsey & Kitting, 1987; Hameed, 1993).

The results highlight a discrepancy in the results of the different methods of taxonomic classification. Biochemical tests allow the classification of bacterial isolates into likely genera, but due to the similarities in reaction patterns of marine gram negative bacteria these methods are not as robust as 16s rRNA gene sequencing. The latter molecular method is a more robust method of taxonomic classification.

In addition to mortalities attributed to outbreaks of bacterial septicaemia, ranging from inconsequential to 100% of the infected population (Lightner, 1988; Rosemark & Fisher, 1988), muscle quality is also severely affected as significant necrosis occurs. The poor external appearance of the animal also makes it unsuitable for market (Bowser, 1997).

Histological examination, a diagnostic method for bacterial septicaemia, reveals histopathological lesions such as small granulomas, haemocyte aggregations, encapsulation and necrosis of the tissues (Edgerton *et al.*, 1995; Jiravanichpaisal *et al.*, 1995; Camus *et al.*, 1998). Montero (1998) reported that following the experimental infection of *N. norvegicus* with *Vibrio harveyi,* histopathological analysis of the muscle demonstrated a dystrophy and necrosis around the lesion, in addition to the destruction of muscle tissue due to the proteolytic activity of the bacteria. In the present study there appeared to be very little if any immune reaction from the host. This is possibly connected with the immuno-suppression experienced by *N. norvegicus* following exposure to capture and post capture stressors, as detailed in chapters 2 and 3.

Jackson *et al.* (1997) defined muscle foods as spoiled when organoleptic changes made them unacceptable to the consumer, and that organoleptically detectable spoilage is usually a result of decomposition and the formation of metabolites resulting from the growth of microorganisms. It is generally considered that spoilage occurs when the bacteria present in the muscle exceed 10^7 cfu.g^{-1} (Jackson *et al.*, 1997); however in situations where there is reduced glucose in the tissues, as occurs after periods of excessive stress, spoilage will occur at lower bacterial densities (<10^7 cfu.g^{-1}). This occurs because glucose is unavailable and there is little delay in degradation of amino acids by pseudomonads and other spoilage bacteria (Newton & Gill, 1980).

The extent of bacterial spoilage is dependent on a number of factors (Frazier & Westhoff, 1978): host immune response, host condition, tissue type (shellfish deteriorate quickly due to the rapid oxidation of unsaturated fats) and temperature (most temperate bacteria exhibit optimum growth at 20-25°C) (Anon, 1981).

The hydrolysis of macromolecules such as proteins and lipids, which originate from organic sources (Davis *et al.*, 1980), is the primary means by which heterotrophic bacteria obtain nutrients from their environment. Gram-negative bacteria accomplish hydrolysis by the secretion of exoenzymes (proteins and lipases) into the periplasmic space. In addition to autolysis by the host, these enzymes act to break down proteins into peptides and amino acids, and possibly further to ammonia.

Glycogen and other carbohydrates are preferentially utilised by microbes, since their hydrolysis usually results in readily usable monosaccharide sugars (Frazier & Westhoff, 1978). In the abdominal muscle of *N. norvegicus,* glycogen and other sugars are found in large quantities and, therefore, represent a potential nutrient source if invasion of the tissue occurs.

Crustacean shellfish contains higher quantities of free amino acids and other soluble compounds than do finfish (Feiger & Novak, 1961). This facilitates rapid bacterial spoilage, accompanied by a production of large amounts of volatile base nitrogen (Feiger & Novak, 1961; Jay, 1992). The phospholipid component of muscle tissue membranes that are rich in unsaturated fatty acids, and the highly unsaturated lipids of shellfish meat (Kraft, 1992) are susceptible to auto oxidation. This process results in the production of stable compounds such as aldehydes, ketones and short chain fatty acids, which produce the rancid odours (Gill, 1982) reported as a gross clinical symptom of the present condition. When bacteria are involved in fat breakdown the released lipases cause hydrolytic rancidity, with the production of the breakdown products of fatty acids, glycerol and other alcohols that represent the characteristic odours of decaying shellfish. These end products result in osmotic and pH imbalance, leading to further necrosis and subsequent bacterial invasion.

The SDS page analysis indicated extra bands around 100 KDa. A similar pattern of tissue breakdown was also demonstrated by Martinez *et al.* (2001). They observed the cross-reaction of these bands with anti-MHC antiserum, and a concomitant decrease in the intensity of the MHC band. In the present study, antisera were not available to investigate cross reactions and so it is possible that these extra bands come from proteolysis of minor muscle proteins (such as titin ~3600 kDa) which act as a spring to keep the thick filament in the centre of the sarcomere. Titin was only recently discovered as it does not enter polyacrylamide gels (Voet & Voet, 1995). Martinez *et al.* (2001) attributed the proteolytic degradation observed in their study to the Ca^{2+} dependent proteases contained in crustacean muscle tissue (Beyette *et al.*, 1993; Mykles & Skinner, 1986), a different process to that believed to be occurring in the present case.

Spoilage processes are generally brought about by aerobic organisms, but many marine bacteria are facultative anaerobes or fermenters whose action results in putrefaction and the associated foul odours (Jackson *et al.*, 1997). *Vibrio* and *Aeromonas* species are particularly important in this respect. Whether acting alone or in mixed populations, motile aeromonads are responsible for significant financial losses annually (Camus *et al.*, 1998). They are considered to be opportunistic pathogens capable of producing disease only in weakened populations or as secondary invaders of hosts with other diseases (Camus *et al.*, 1998).

16s rRNA sequencing suggested that *Pseudoalteromonas* spp., in addition to *Vibrio* spp. are present in healthy and diseased *N. norvegicus*. *Pseudoalteromonas* is a marine genus, created in 1995 by Gauthier *et al.* (1995) which included most of the previous *Alteromonas* genus. *Pseudoalteromonas* spp. are antagonistic and produce bacteriostatic and bacteriolytic compounds (Nair & Simidu, 1987; Holmström & Kjellberg, 1999). Such properties greatly benefit *Pseudoalteromonas* cells in their competition for nutrients and colonisation of surfaces, and Paarup *et al.* (2002) suggested these reasons for the observed dominance of *Pseudoalteromonas* in whole squid during iced storage. However, they also reported a complex microbiological picture in gutted individuals which they could not explain. The spoilage of iced squid described by Paarup *et al.* (2002) was the first report linking *Pseudoalteromonas* to spoilage of marine seafood. Costa-Ramos & Rowley (2003) studied the effects of extracellular products of *Pseudoalteromonas atlantica* (isolated from shell-disease infected edible crabs, *Cancer pagurus*) on the edible crab and reported rapid death and destruction of tissues.

Particular importance has been placed on *Vibrio* species for two reasons. Firstly their influence on the initial stages of soft tissue breakdown and resultant bacterial proliferation

is important (Duncan, 1993). *Vibrio* spp. are pathogenic bacteria capable of overcoming the host immune system and then multiplying in the body fluids and internal organs. Once a virulent strain of bacterium has gained access into the host tissue, then disease and death are normally imminent. Virulence factors, such as cytolytic and haemolytic toxins, have been described for *V. damsela* (Kreger, 1984; Kreger *et al.,* 1987; Toranzo *et al.,* 1993).

In addition to the detrimental effects of bacteria on their host, some microorganisms or their metabolic products can be important in terms of human health. Of the 34 currently recognised *Vibrio* spp., at least 20 have been associated with human infection (Kaysner, 2000, Thompson *et al.,* 2004). Most notably *Vibrio cholerae* is the cause of epidemic cholera (Tison & Kelly, 1984), *Vibrio parahaemolyticus* accounts for almost 70% of all acute gastroenteritis in Japan (Sakazaki, 1965) and *V. vulnificus* is considered to be one of the most invasive and rapidly lethal of human pathogens described (Oliver, 1989). All 3 species have been isolated from shellfish, which are considered to be important vectors for the bacteria (West, 1989).

In 1989, *Vibrio* infections in the US were estimated to have been responsible for 400 deaths at an estimated cost of 239 million US$ (Roberts, 1989). In 1999 the *Vibrio* Surveillance System (Anon, 1999) reported that of the persons with gastroenteritis or septicaemia who consumed a single seafood item in the 7 days before the onset of illness, 21% consumed cooked crustacean meat. Although the risk of illness is greater from eating raw oysters, these results suggest that there is potential risk to consumers if control methods are not applied.

Aeromonas species also cause infections in humans (Camus *et al.,* 1998), generally gastrointestinal illnesses (West, 1989). *A. hydrophila* have been reported to cause

infections in humans, although often associated with the consumption of oysters (Abeyta *et al.*, 1986).

Spoilage organisms, such as *Pseudomonas*, may cause human illness through the ingestion of significant quantities of bacterial toxins. Interestingly, the number of aerobic bacteria present in *N. norvegicus* experiencing bacterial septicaemia exceeds that allowed in cooked whole crustaceans by EC community legislation (Decision 93/51/EEC) (Anon, 2002).

This condition will not only affect the meat quality and mortality of *N. norvegicus* at the processing plants, but will also affect the shelf life of the product. The presence of elevated bacterial numbers on products before storage results in a shorter shelf life (Jackson *et al.*, 1997). If microbial populations are sufficiently high, spoilage microorganisms and even pathogens may survive processing treatments and, if storage conditions are suitable, proliferation and further product defects will occur (Jackson *et al.*, 1997).

Dainty (1971) reviewed the literature on controlling spoilage and concluded that a combination of three approaches could be utilised. Strategies could be focused on the prevention of the initial bacterial contamination, inactivation of bacteria which are present or the use of storage conditions to prevent or slow the growth of bacteria present. In the case of the present infection the first two approaches are most valid. For opportunistic bacterial infections, such as vibriosis, the infection is a pathological consequence of stress. The most important factor is good handling and storage. At processing plants *N. norvegicus* destined for live transport may be held for up to 4 days. Therefore at these locations it is important to maintain adequate water quality with low bacterial biomass, to sterilise or filter the recirculated water, and to avoid extremes or rapid variations in temperature, handling, overcrowding and other stressors (Bowser, 1997).

Reducing the temperature of the holding water at processing plants will reduce bacterial growth, as temperature is the most important factor influencing the growth of bacteria (Jackson *et al.*, 1997). Since most pathogenic bacteria are mesophiles, chilling shellfish prevents their growth. However, altering the temperature between -1 and +7 °C often results in influencing the dominant organism, with pseudomonads typically dominating at lower temperatures (Tompkin, 1973); such organisms can produce proteases down to 2°C (Alford *et al.*, 1971; Juffs, 1976).

Probiotics (Rengpipat *et al.*, 1998) and immuno-stimulants (Sung *et al.*, 1994) in the feed have both improved survival of shrimp challenged with *Vibrio* species. In aquaculture settings the use of probiotic bacterium in shrimp feed has resulted in significantly increased survival of *Penaeus monodon* when challenged with *V. harveyi* (Rengpipat *et al.*, 1998). Immune-stimulants, such as β-1,3/1,6-Glucans, are primarily prophylactic agents which should be used to elevate the general defence barrier of the organism and hence to reduce the risk of disease, but not as a curative medicine (Raa, 1996). However the practicality of both these treatments in the live transport scenario remains unascertained, and above all any treatment must be economically viable.

Future studies should concentrate on the seasonal prevalence of the condition, furthering our knowledge into bacterial pathogens responsible for this infection, possibly confirming vibriosis. Routine sampling should screen for incidences of bacterial strains pathogenic to humans present in the tissue, although at present there is no evidence suggesting they are present.

To conclude, this chapter has described a post capture bacterial septicaemia in *N. norvegicus,* which is probably caused by opportunistic bacteria exploiting periods of stress-induced immuno-suppression. These bacteria are either part of the resident bacterial

fauna or have gained entry through physical damage incurred during trawl capture. Treatments have been discussed, but the most effective solution is likely to be reducing the degree of physiological stress and physical damage incurred during the fishery process, thus reducing the opportunity for bacteria to exploit the situation.

Table 7.1: The taxonomic classification following analysis of visual colony characteristics and various biochemical and taxonomic tests of the 3 different groups of isolates from homogenised *Nephrops norvegicus* abdominal muscle. A1-A10 isolated from animals at capture, B1-B10 isolated from healthy animals 48 h after capture, and N1-N10 isolated from necrotic animals 48 h after capture. All isolate colonies had a rough surface, opaque in colour and butyrous in texture and were gram negative, rod shaped, oxidase positive, motile and non luminescent. (+) symbol refers to a positive result, (-) refers to a negative result and (o) refers to an inert reaction. (*Vib/Aer* means *Vibrio/Aeromonas*). The final column is the likely genera of the isolates according to the schemes of Buchanan & Gibbons (1974), Muroga *et al.* 1987) and Oliver (1982).

Colony	Size	Form	Elevation	Margin	Catalase	Acid	Alkali	Oxidative	Fermentative	Likely Genus
A1	2-3	Punctiform	Convex	Undulate	+	-	+	+	-	*Pseudomonas*-like
A2	3-4	Punctiform	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like
A3	2-3	Irregular	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like
A4	1-2	Irregular	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like
A5	1-2	Irregular	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like
A6	2-3	Irregular	Convex	Undulate	-	-	+	-	-	*Vib/Aer*
A7	1-2	Irregular	Convex	Undulate	+	-	+	-	+	*Pseudomonas*-like
A8	1-2	Irregular	Convex	Undulate	-	+	-	-	-	*Pseudomonas*-like
A9	1-2	Irregular	Convex	Undulate	-	+	-	-	-	*Pseudomonas*-like
A10	1-2	Irregular	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like
B1	1	Irregular	Convex	Undulate	+	+	-	-	-	*Pseudomonas*-like
B2	1-3	Irregular	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like
B3	1-3	Irregular	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like
B4	2-3	Irregular	Convex	Undulate	-	+	-	-	+	*Vib/Aer*
B5	2-3	Irregular	Convex	Undulate	-	-	+	-	-	*Pseudomonas*-like
B6	2-3	Irregular	Convex	Undulate	+	+	-	-	-	*Pseudomonas*-like
B7	2	Irregular	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like
B8	1-3	Irregular	Convex	Undulate	-	+	-	-	+	*Vib/Aer*
B9	1-2	Punctiform	Pulvinate	Undulate	-	-	+	-	-	*Pseudomonas*-like
B10	1-2	Punctiform	Pulvinate	Undulate	+	-	+	-	+	*Vib/Aer*
N1	2-3	Irregular	Convex	Undulate						
N2	1-3	Irregular	Convex	Undulate	-	+	-	-	+	*Vib/Aer*
N3	1	Irregular	Convex	Undulate	+	+	-	-	+	*Vib/Aer*
N5	1-2	Irregular	Convex	Undulate	+	+	-	+	-	*Pseudomonas*-like
N6	1-2	Irregular	Convex	Undulate	-	-	+	-	-	*Pseudomonas*-like
N7	1-2	Irregular	Convex	Undulate	+	+	-	-	+	*Vib/Aer*
N8	2	Filamentous	Convex	Filamentous	+	+	-	-	+	*Vib/Aer*
N9	1-3	Irregular	Convex	Undulate	+	+	-	-	+	*Vib/Aer*
N10	1-3	Irregular	Convex	Undulate	+	-	+	-	-	*Pseudomonas*-like

Figure 7.1: *Nephrops norvegicus* (A) exhibiting the gross clinical signs of the condition; white abdominal musculature, and reddening of the pleopods. Healthy *N. norvegicus* (B) is also demonstrated for comparison.

Figure 7.2: The mean bacterial count (cfu.g^{-1} muscle tissue) of the abdominal musculature of apparently healthy and necrotic *Nephrops norvegicus* at time of capture and 48 h after capture. For necrotic *N. norvegicus* 48 h after capture counts TCBS counts for presumptive *Vibrios* are also included. Error bars represent standard deviations.


B
A

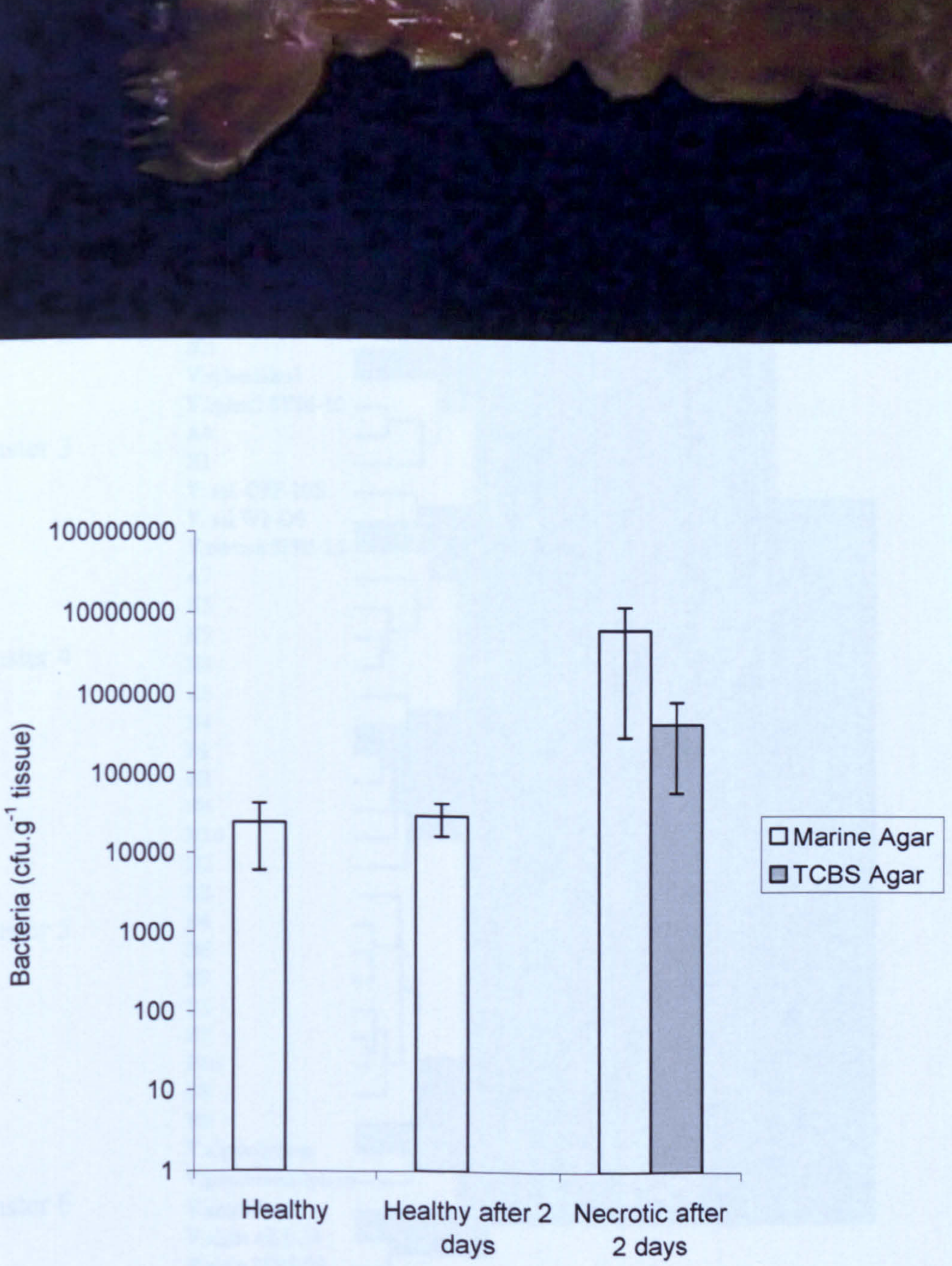

100000000
10000000
1000000
100000
10000
1000
100
10
1
Bacteria (cfu.g-1 tissue)
Healthy
Healthy after 2 days
Necrotic after 2 days
Marine Agar
TCBS Agar

Figure 7.3: The results of the cluster analysis following the Biolog colorimetric analyses on the bacterial isolates obtained from the DAF muscle of *Nephrops norvegicus*. Isolates A1-A10 were obtained from healthy *N. norvegicus* at the time of capture, B1-B10 were obtained from healthy *N. norvegicus* 48 h following landing and N1-N10 were obtained from *N. norvegicus* exhibiting the gross clinical symptoms of bacteraemia. The data from other *Vibrio* spp. (*Vibrio tapetis, V. splendidus, V. fischeri, V. salmonicida, V. tubiashii, V. parahaemolyticus, V. anguillarum, V. alginolyticus*) are also included.

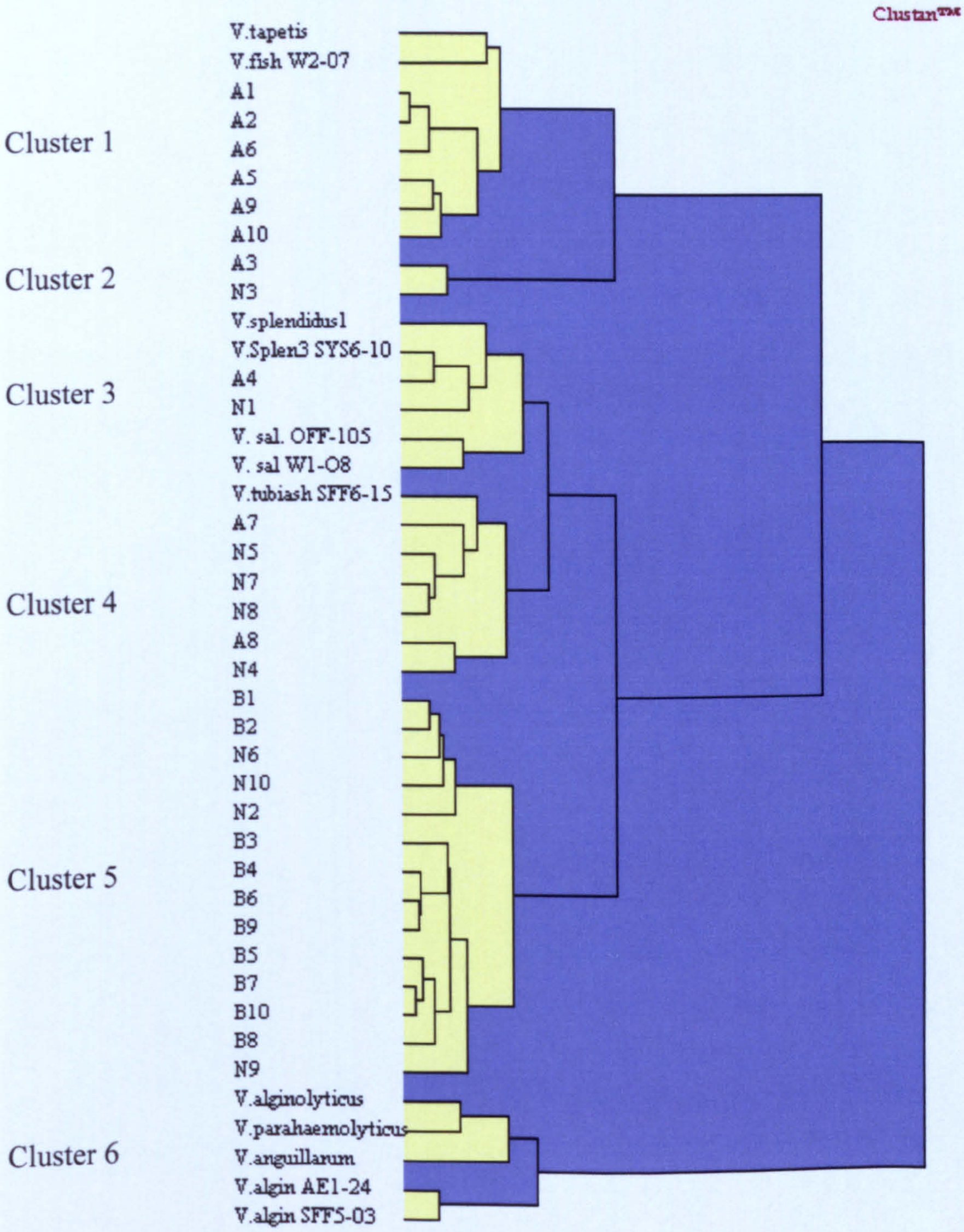
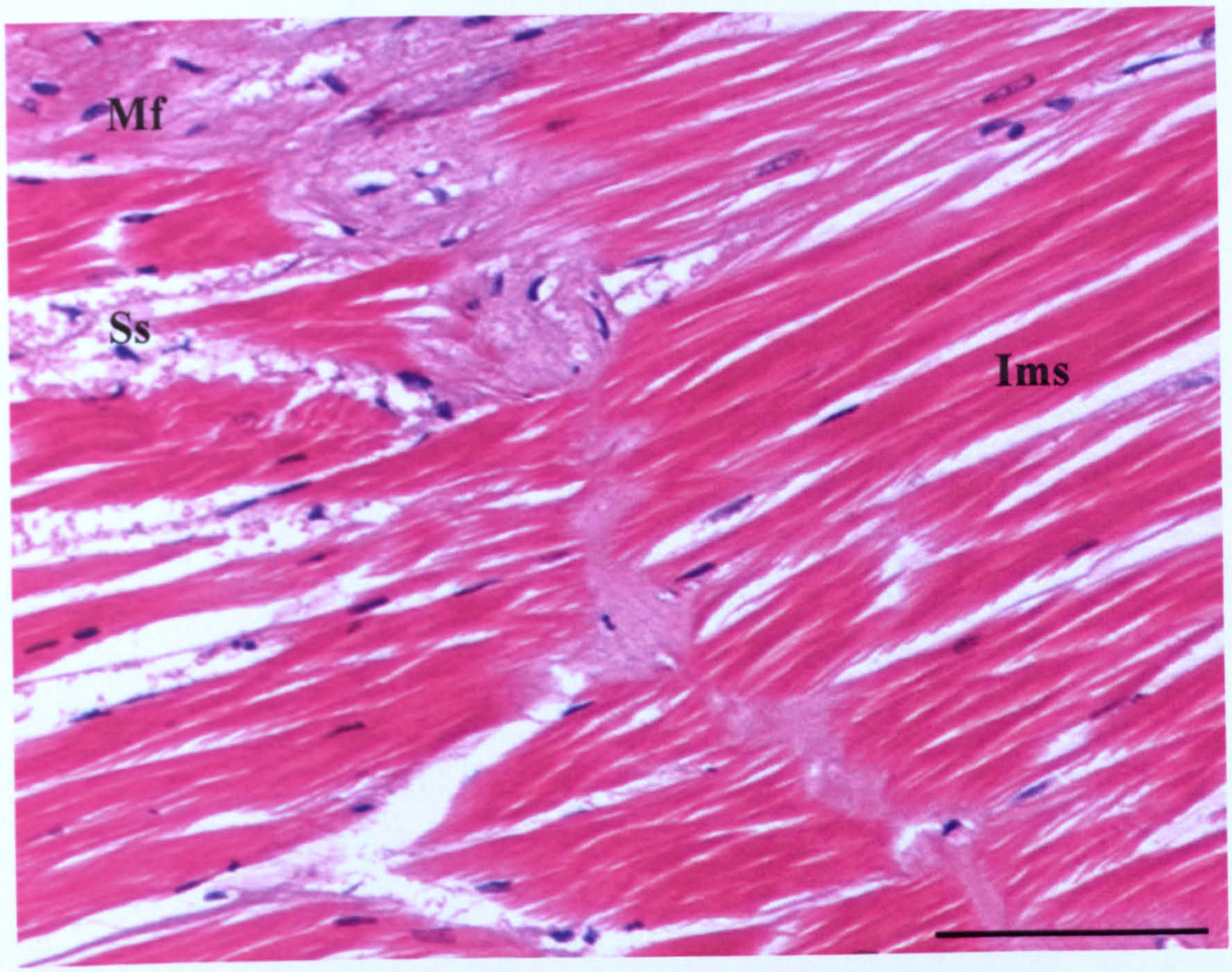


Figure 7.4: Light micrograph of a section of deep abdominal flexor (DAF) muscle illustrating loss of muscle fibre structure (Mf) and an expansion of the sarcomeric space (Ss). However, some areas contain intact myofibrillar structure (Ims), with the damage extending into these undamaged areas. Scale bar is 100 μm.

Figure 7.5: Light Micrograph of a section of deep abdominal flexor (DAF) muscle from *N. norvegicus* exhibiting the gross clinical symptoms of bacteraemia. Note the total lack of structure of the striated muscle (Sm) and the basophilic muscle nuclei (Mn), which are hypertrophied. Scale bar 25μm.


Mf
Ss
Ims

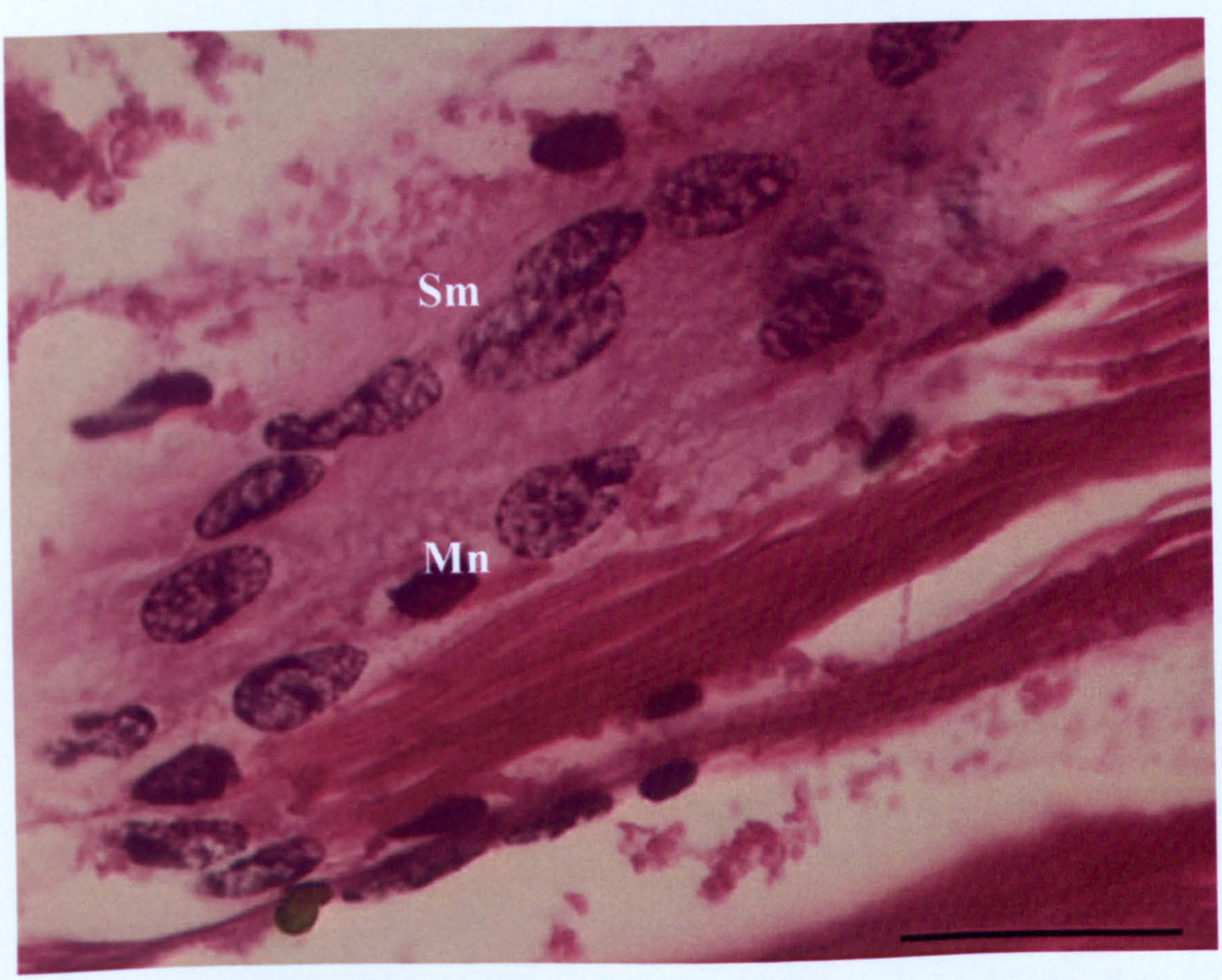
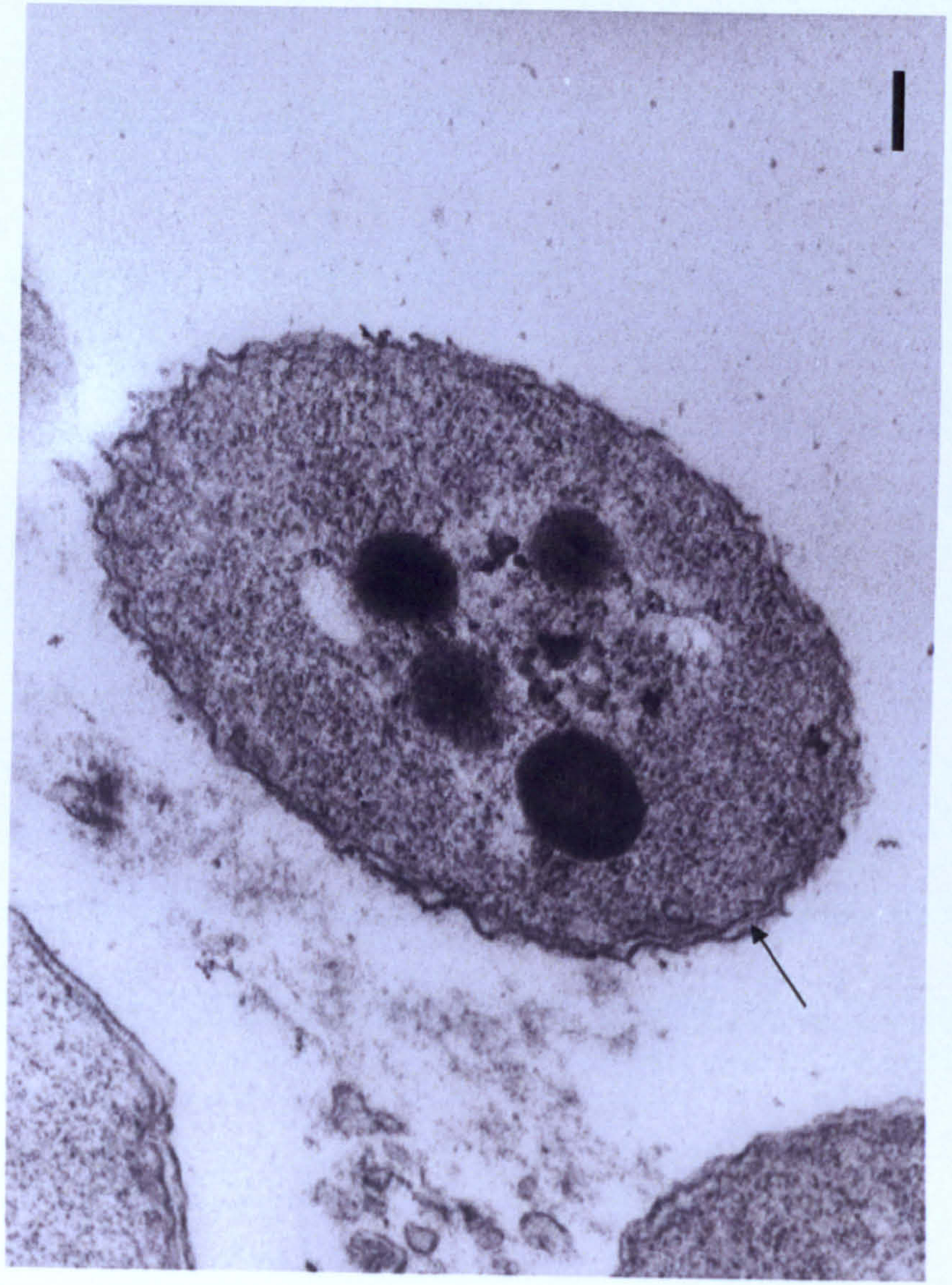

Sm
Mn

Figure 7.6: Transmission electron micrograph of a bacterial cell in the DAF of *N. norvegicus.* Scale bar is 100 nm. The bacteria cell appears to have a gram-negative type cell wall (arrow).

Figure 7.7: Transmission electron micrograph of another type of gram-negative rod shaped bacteria cell in the necrotic DAF of *N. norvegicus.* Note the total lack of structure in the surrounding muscle. Scale bar is 500 nm.



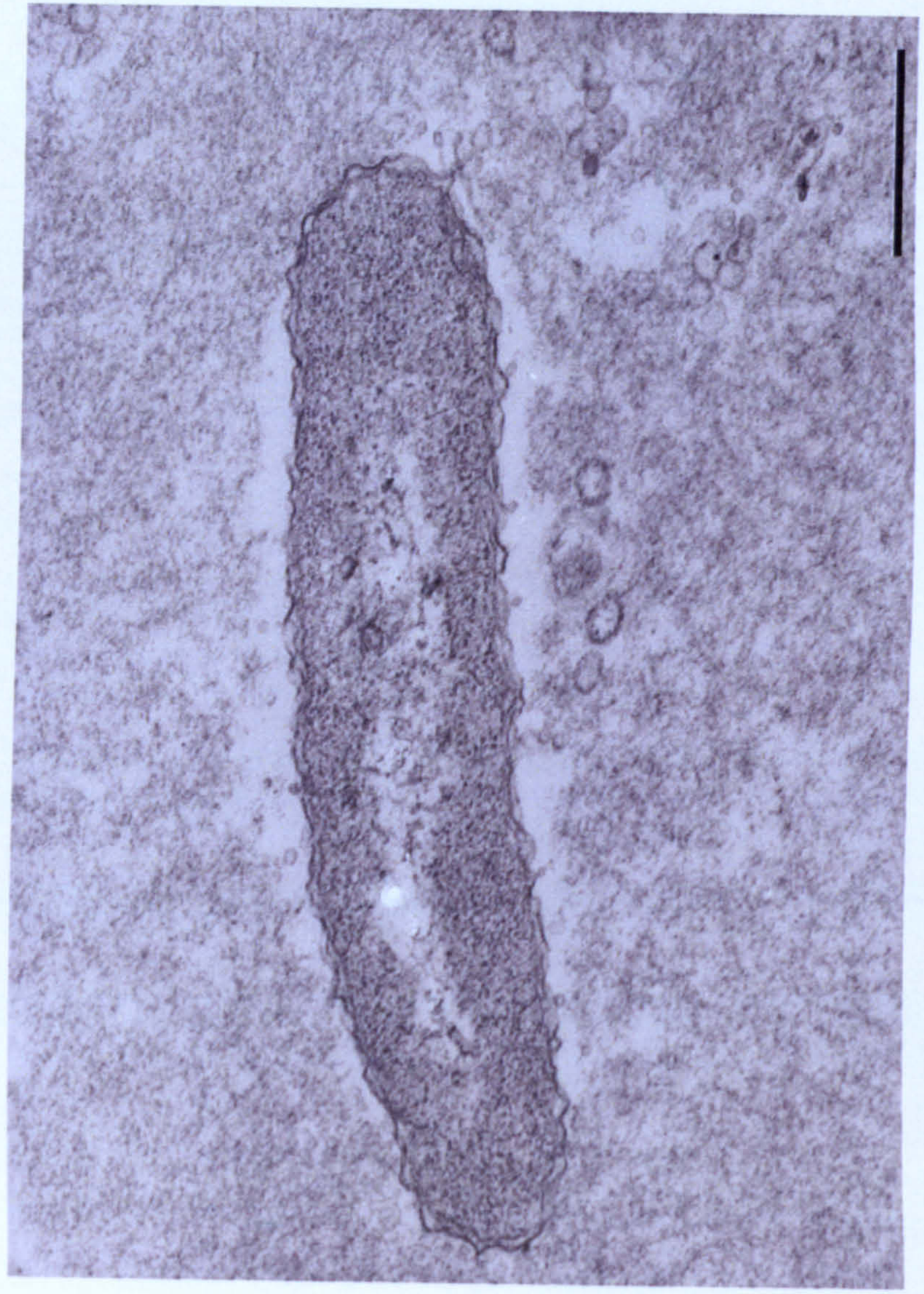
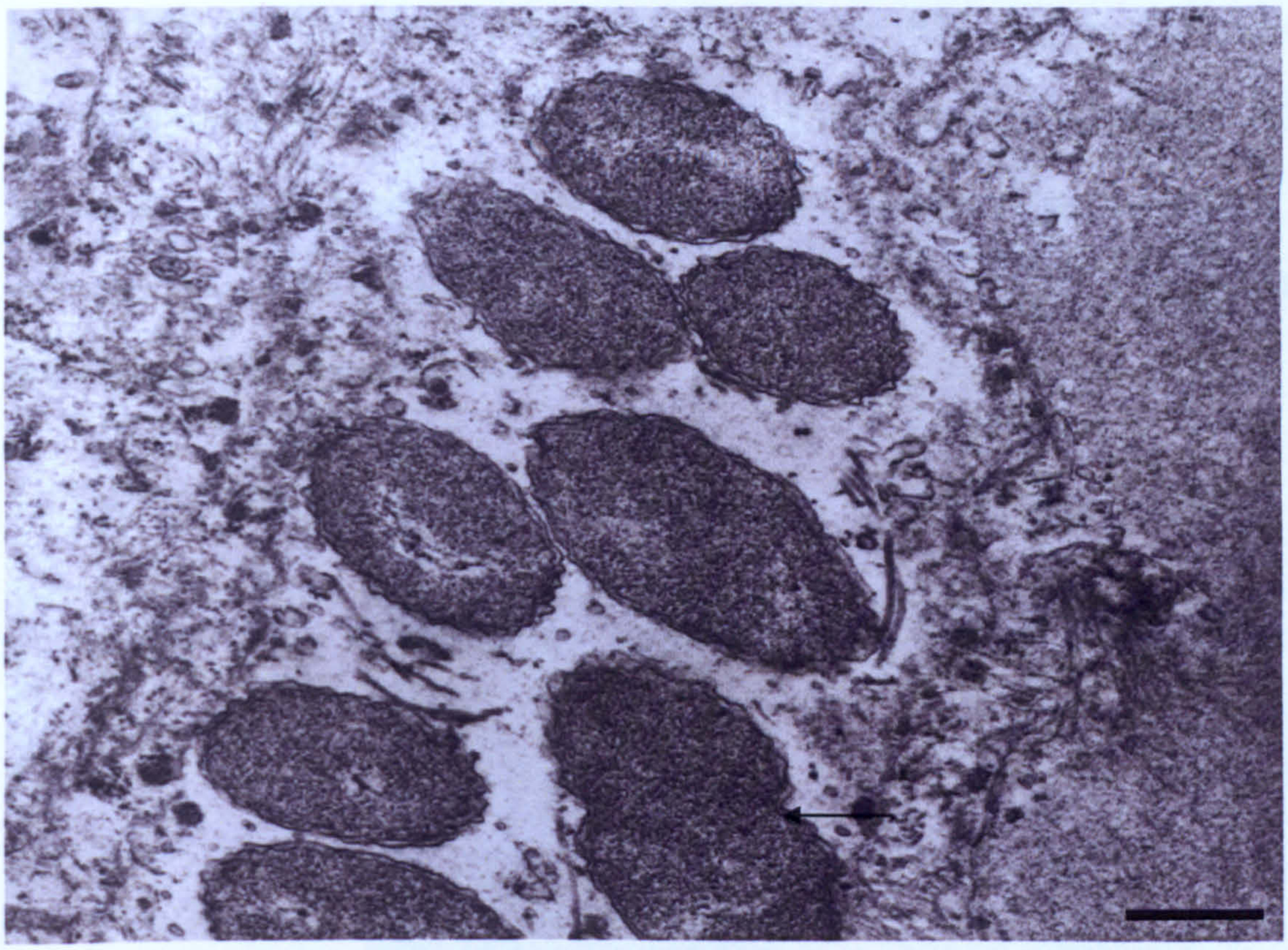


Figure 7.8: Transmission electron micrograph of a group of gram-negative, rod shaped, flagellated bacteria, varying in size from 1-2 μm in length, in the necrotic, DAF of *N. norvegicus*. An arrow indicates a bacterial cell in the process of division. Scale bar is 500 nm.

Figure 7.9: Transmission electron micrograph of a group of bacterial cells in the necrotic DAF of *N. norvegicus*. Note again the total lack of structure in the muscle and the clear zone around each of the bacteria cells suggesting the bacteria would be releasing tissue degrading enzymes. Scale bar is 1μm.



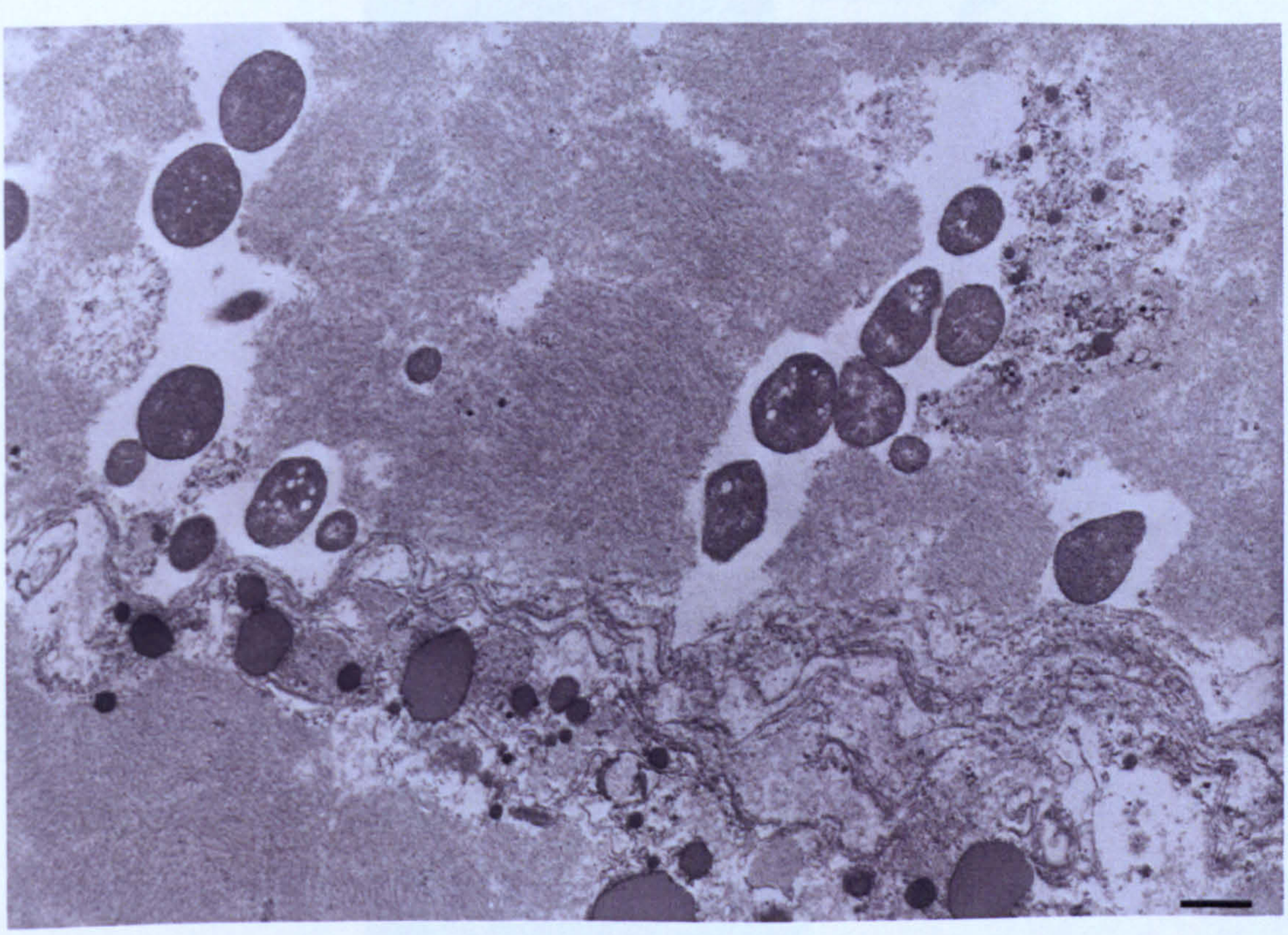
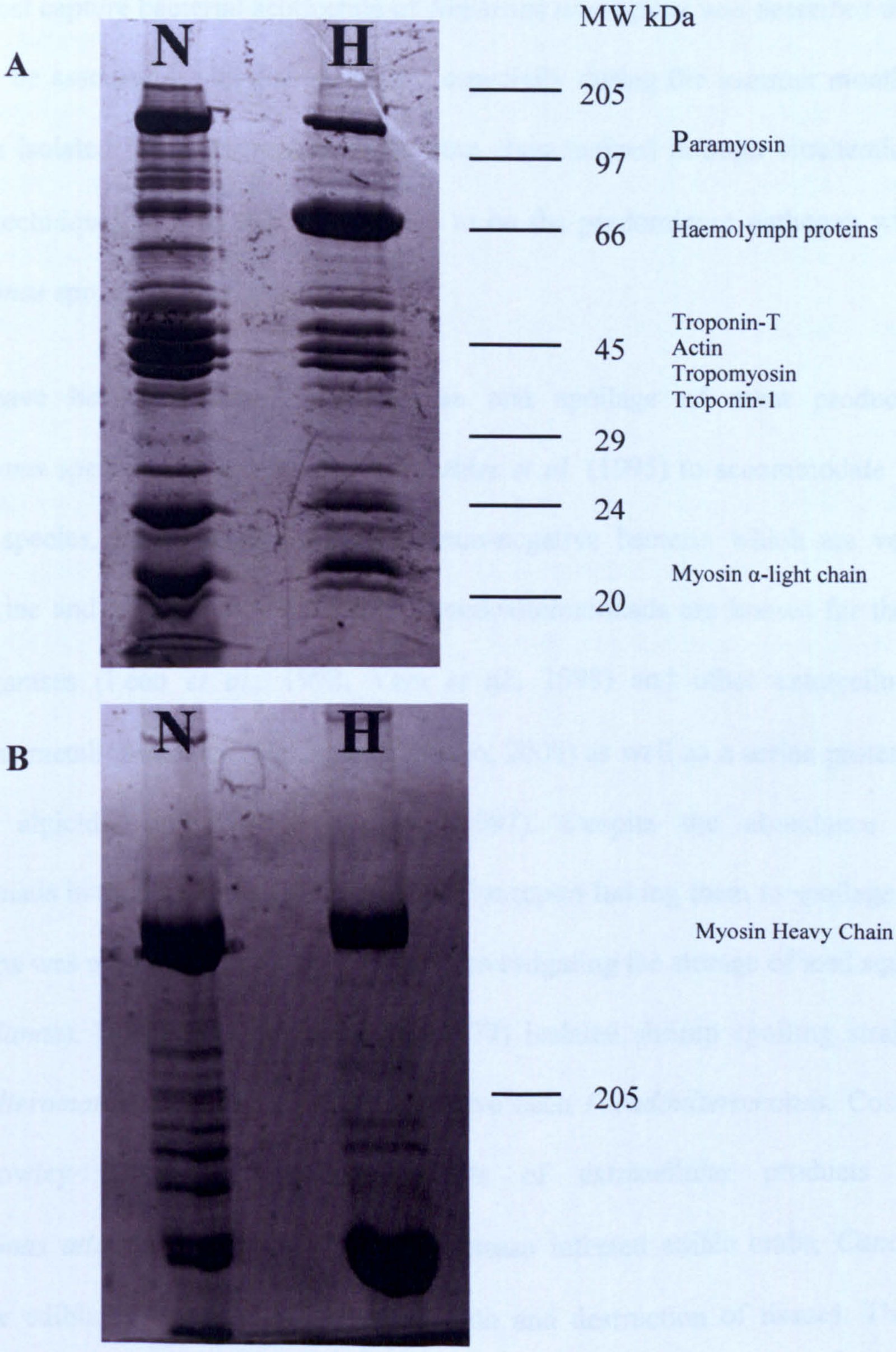


Figure 7.10: 12.5 % (A) and 5% (B) SDS-PAGE gels illustrating the degradation of abdominal muscle tissue in *N. norvegicus* experiencing high levels of bacteraemia (N), the lane on the right is muscle tissue from an apparently healthy *N. norvegicus* (H). The molecular mass (MW) standards are indicated on the right by kilodaltons (kDa) in addition to the names of the protein bands.



Chapter 8

Extracellular proteases and possible virulence factors of two marine bacteria believed to be implicated in a vibriosis infection of *Nephrops norvegicus.*

8.1 INTRODUCTION

In chapter 7 a post capture bacterial septicemia of *Nephrops norvegicus* was described that was believed to be associated with fishery losses, especially during the summer months. Bacterial strains isolated from diseased animals were characterized through biochemical and molecular techniques. *Vibrio* spp. were found to be the predominant pathogen with *Pseudoalteromonas* spp. also being abundant.

Both genera have been associated with disease and spoilage of meat products. *Pseudoalteromonas* species, first described by Gauthier *et al.* (1995) to accommodate 12 "*Alteromonas*" species, are naturally occurring gram-negative bacteria which are very common in marine and estuarine environments. Pseudoalteromonads are known for their extracellular agarases (Leon *et al.,* 1992; Vera *et al.,* 1998) and other extracellular enzymes, such as metalloproteases (Hoffman & Decho, 2000) as well as a serine protease that possesses algicidal activity (Lee *et al.,* 1997). Despite the abundance of Pseudoalteromonads in the marine environment, the first report linking them to spoilage of marine organisms was not until Paarup *et al.* (2002), investigating the storage of iced squid (*Todaropsis eblanae*). However, van Spreekens (1977) isolated shrimp spoiling strains with typical "*Alteromonas*" reactions, which may have been *Pseudoalteromonas.* Costa-Ramos & Rowley (2003) studied the effects of extracellular products of *Pseudoalteromonas atlantica* (isolated from shell-disease infected edible crabs, *Cancer pagurus*) on the edible crab and reported rapid death and destruction of tissues. They

concluded that it was premature to comment on the importance of *P. atlantica* in shell disease syndrome until other factors, such as the synergistic role of bacteria in lesion development, had been considered.

Vibrio species have been implicated in numerous outbreaks of disease especially wherever crustaceans are held in artificial or stressful situations (Sindermann, 1977).

Vibrio spp. are important in the initial stages of soft tissue breakdown and resultant bacterial proliferation (Duncan, 1993) and are pathogenic bacteria capable of overcoming the host immune system and then multiplying in the body fluids and internal organs. Once a virulent strain of bacterium has gained access into the host tissue, disease and death are normally imminent. Virulance factors, such as cytolytic and haemolytic toxins, have been described for *V. damsela* (Kreger, 1984; Kreger *et al.*, 1987; Toranzo *et al.*, 1993).

The pathogenicity of fish pathogenic *Vibrio* species have been extensively studied (Egidius, 1987; Austin & Austin, 1993) and extracellular virulence factors such as haemolysin, proteases and cytotoxins have been suggested to play a significant role in diseases (Montero, 1998).

Microorganisms growing on muscle secrete a wide variety of hydrolytic enzymes, particularly proteases (Jackson *et al.*, 1997). In contrast to microbial intracellular enzymes, which are regulated within the cell, extracellular enzymes are free to degrade substrate, thereby causing extensive damage to food constituents (Venugopal, 1990). Many of the clinical symptoms of diseases, such as tissue destruction and necrotic lesions, are believed to be the result of proteases secreted by pathogens. Extracellular products (ECP) degrade the host extracellular matrix proteins allowing the parasite to spread through the host tissue (Schulte & Scholze, 1989).

Virulence factors assist pathogens in the invasion and colonization of host tissues through the evasion of host defences and the spread of infection (Moss *et al.*, 1995). In *Vibrio* species pathogenic to fish and shellfish, enzymatic properties and the virulence of proteases produced have been well studied (Inamura *et al.*, 1985; Deane *et al.*, 1987; Nottage & Birkbeck, 1987; Lee *et al.*, 1997), although there have been few studies on ECP of *Pseudoaltermonas* species.

The aim of this study was to investigate the extracellular proteases secreted by two strains of gram-negative bacteria isolated from *N. norvegicus* with vibriosis, and to further understand the role of these strains in the spoilage of host muscle tissue. Protease activity was assessed spectrophotometrically through the degradation of azocasein under a range of temperature and pH assay conditions. Through SDS-PAGE analysis proteases of the isolates were observed and the affect on *N. norvegicus* muscle proteins demonstrated. Identification of possible virulence factors and measurement of enzyme activities in the extracellular proteases was performed using the API ZYM system.

8.2 MATERIALS AND METHODS

8.2.1 Effect of temperature on bacterial growth.

During the study of the bacterial septicaemia of *Nephrops norvegicus* described in chapter 3, 10 bacterial isolates were removed from necrotic animals and stored at -80°C using the Protect System (Technical Services Consultants, Lancaster, UK). The extracellular products (ECP) and virulence factors of two of these isolates, N8 and N10, were studied. Through a range of biochemical and molecular tests these isolates were identified as a *Vibrio* spp. and *Pseudoalteromonas* spp. respectively. Following 16s rRNA sequencing,

N8 demonstrated 99% homology with *Vibrio* spp. (AY262019) and N10 demonstrated 99% homology with *Pseudoalteromonas* spp. (AY040229).

The effect of temperature on the growth of isolate N8 was measured using an odometer. The required isolate, N8, which had been stored at -80°C, was grown up 2-3 times on marine agar plates to provide isolated colonies. 50 ml of Tryptone Soya Broth (TSB) (made up according to the manufacturer's instructions) was then inoculated with a single colony and grown overnight at 20°C. The following day 0.5 ml of the suspension was added to 50 ml of TSB in conical culture flasks and grown for 12 h at 10, 15 and 25°C. The bacterial growth rate at each temperature was assayed in triplicate. The optical density of the suspension was recorded, at $OD_{600,}$ at the start and every 2 h thereafter for 12 h.

8.2.2 Preparation of extracellular products

To obtain cell cultures along the bacterial growth rate curve, isolates N8 and N10 were cultured as described in section 8.2.1. The procedure was identical, apart from incubating the samples at 20°C and sampling every 3 h for 12 h. At the start, and at each sampling point thereafter, 3 ml of suspension was removed and centrifuged at 4000 rpm for 10 minutes. The supernatant was removed and passed through a 0.2 µl acrodisc filter and frozen at –80°C.

8.2.3 Spectrophotometric assay for protease activity

Protease activities of the cell free suspension were assayed by the method of Sarath *et al.* (1989). Azocasein substrate (3 % w/v) was prepared by dissolving azocasein (Sigma) in 0.1 M Tris-HCl, pH 8.0 to, and centrifuged at 10,000 rpm for 10 min. The supernatant was removed and stored on ice ready for use. 75 µl of the cell free suspension was incubated with 125 µl of the azocasein substrate at 37°C for 12 h. The addition of 600 µl of ice cold

10 % (w/v) trichloroacetic acid (TCA) terminated the reaction. The mixtures were held at 4°C for 30 min and centrifuged at 9,000 rpm for 5 min to pellet the precipitated proteins (including undigested azocasein). 0.6 ml of supernatant was removed and mixed with 0.7 ml 1.0 M NaOH and the absorbance read at 440nm on a spectrophotometer. One unit of protease activity was defined as the enzyme activity resulting in an absorbance of 1.0 in a 1 cm cuvette. Samples of TSB without the addition of bacteria were run as controls and assays were carried out in triplicate.

8.2.4 Effect of temperature and pH on protease activity

The effect of temperature on protease activity of the bacteria-cell-free culture medium samples was examined spectrophotometrically as described in section 8.2.3. However in this case substrate-sample mixtures were incubated at 3, 5, 10, 13, 17, and 23°C. At each temperature samples were assayed in triplicate.

The effect of pH on protease activity of the bacteria-cell-free culture medium was examined spectrophotometrically and by substrate-impregnated SDS-PAGE. For the spectrophotometric assay the procedure was carried out as described in section 8.2.3 with the following minor modifications. Azocasein substrate (3% w/v) was dissolved in 0.1 M Tris-HCl, pH 7.0 and 8.0. 75 µl of the bacteria-cell-free culture medium was incubated with 125 µl of the azocasein substrates at the different pH values at 17°C for 12 h and protease activity measured as detailed in section 8.2.3.

8.2.5 Effect of proteases on host muscle proteins

The method of Small (2004) was used, with minor modifications, to investigate the effect of the proteases on the muscle proteins of *N. norvegicus*. 100 mg of abdominal flexor muscle was homogenised in 500 µl 0.1 M Tris-HCL, pH 8, and centrifuged at 3000 rpm

for 10 min. Muscle protein supernatant was removed and placed on ice prior to use. Samples of protein supernatant (20 µl), mixed with 20 µl samples of bacteria-cell-free supernatant (from 10 h on the curve obtained in section 8.2.2) and 20 µl 0.1 Tris-HCl, ph 8.0 were incubated at 10°C for 12 h. Controls included the incubation of 0.1 M Tris-HCl, pH 8.0, cell-free supernatant, and fresh culture medium under the same conditions. Protein degradation was evaluated by standard SDS-PAGE analysis, using 7.5 % and 12.5 % acrylamide resolving gels, with 4 % stacking gels.

8.2.6 Substrate-impregnated SDS-PAGE

The non-denaturing gel electrophoresis methods of La Peyre *et al.* (1995) and Small (2004) were used with minor modifications to separate the proteases in the bacteria-cell-free culture medium. Abdominal muscle tissue from *Nephrops norvegicus*, homogenised in 0.1 M Tris-HCl, was added to the 12.5 % (w/v) acrylamide resolving gel to obtain a final concentration of 0.2 % (w/v) muscle. 5 µl of bacteria-cell-free culture samples were mixed with 15 µl of electrophoresis sample buffer (62.5 mM Tris HCl, pH 6.8, containing SDS (2 % w/v), ß-mercaptoethanol (5% w/v), glycerol (10% w/v) and 0.004 % pyronin Y) before being loaded onto the *N. norvegicus* muscle tissue impregnated gels. The gels were run at 30mA constant current for 90 minutes using a BioRad mini-Protean system, containing Tris-glycine buffer. Following electrophoresis gels were incubated for 30 min at 4°C in Triton X-100 (2.5% v/v in ddH20) to remove SDS and reactivate the resolved proteins. Proteolytic activity was detected by incubating the gels in 0.1 M Tris-HCl, pH 8 for 18 h at 15°C. The gels were fixed and stained with Coomassie blue concentrate and destained until proteolytic bands became evident. Apparent molecular weights of the proteases were determined from their mobility relative to known protein standards. This procedure was repeated with the exception that gels were incubated in 0.1 M Tris-HCl, pH 7.

8.2.7 API ZYM enzyme analysis

The enzymatic activities of isolates N8 and N10 were determined utilising the API ZYM system according to the manufacturer's guidelines (Biomérieux). Briefly, isolated colonies were cultured overnight in TSB, centrifuged at 4000 rpm and re-suspended in a sterile 3 % NaCl solution to obtain a turbidity of 5-6 McFarland (1.5-1.8 x 10^9 bacteria ml^{-1}). 65 µl of this suspension was added to each cupule and the test strips were incubated for 4 h at 20°C. Following incubation, 1 drop of ZYM A (API; tris-hydroxymethyl-aminomethane, hydrochloric acid, sodium laurel sulphate, H_2O) and ZYM B (API; fast blue BB, 2-methoxyethanol) was added to each cupule and the colour allowed to develop for 5 min. The test strips were then read and the results scored using the following activity classification; 0, negative reaction; 1-2 weak activity; 3, 4 or 5, strong activity. Each isolate was analysed in triplicate.

8.3 RESULTS

8.3.1 Effect of temperature on bacterial growth

The results indicate there was a clear effect of temperature on the growth rate of isolate N8 (Fig. 8.1). At 25°C the culture progressed through the growth phases within 12 h, reaching the stationary phase of the growth curve after only 8 h. The growth at 15°C was slower, with the bacterial culture beginning to enter the logarithmic phase of growth after 12 h. After 12 h at 10°C the culture was beginning to enter the acceleration phase.

8.3.2 Spectrophotometric assay for protease activity

No proteolytic activity could be detected from isolate N8. Attempts to modify the conditions of the assay to encourage proteolytic activity were unsuccessful. Isolate N10

demonstrated logarithmic growth between the 3 and 9 h sampling times, and then entered the deceleration phase (Fig. 8.2). Proteolytic activity increased with the cell density of the culture, although not with the same pattern of increase. Thus no proteolytic activity of the isolate N10 culture was detected until 6 h post inoculation, when minimal activity was detected (0.11 ± 0.12 U ml^{-1}). Thereafter, between 6 and 9 h post inoculation it increased greatly (approximately 35 fold) to 3.98 ± 0.50 U ml^{-1}, and increased a further 2 fold by 12 h (6.75 ± 0.38 U ml^{-1}). No proteolytic activity was detected in un-inoculated culture medium.

8.3.3 Effect of temperature and pH on protease activity

Proteolytic activity of the bacteria-cell-free culture medium of isolate N10 increased with temperature (Fig. 8.3). The largest increases in proteolytic activity were between 3 and 5°C, and between 13 and 23°C, with the proteolytic activity at 23°C (13.84 ± 0.11 U ml^{-1}) being approximately 8-fold that of 3°C (1.64 ± 0.07 U ml^{-1}).

At 17°C the pH of the assay was reduced from 8.0 down to 7.0. This did not have a significant impact on the proteolytic activity (t-test, 2.98; p value, 0.097) of the bacteria-cell-free culture medium.

8.3.4 Effect of proteases on host muscle proteins

SDS-PAGE analysis of the effect of ECP from isolates N8 and N10 on the abdominal muscle structure revealed that the proteases had a selective impact on the muscle proteins. Figures 8.4 and 8.5 (12.5% and 7.5% resolving gels respectively) demonstrate that the ECP of isolate N10 completely degraded the myosin heavy chain, troponin-T, troponin-I, and Paramyosin. There was also complete degradation of a number of unidentified muscle proteins with an approximate size of 110 kDa, and tropomyosin appeared to be

significantly reduced in quantity, as is especially evident in Figure 8.5. However it was also evident that a number of proteins, namely actin, the myosin α-light chain, and a number of other unidentified proteins at 50 and 80 kDa, remained unaffected by proteolytic activity.

Proteases contained in the bacteria-cell-free culture medium by isolate N8 had no effect on the muscle proteins of *Nephrops norvegicus.*

8.3.5 Substrate-impregnated SDS-PAGE

SDS-PAGE gels impregnated with deep abdominal flexor (DAF) muscle proteins from *N. norvegicus* revealed multiple proteases in the bacteria-cell-free culture medium of isolate N10. The gel was incubated in 0.1M Tris-HCl, pH 8.0 and 6 zones of proteolysis are evident (Fig. 8.6) with estimated molecular masses between 100 and 30 kDa. This was repeated with the pH of the buffer reduced to 7.0, which resulted in the same number, sizes and intensity of proteolytic areas (results not presented).

8.3.6 API ZYM enzyme analysis

The API ZYM profiles of the 2 isolates (N8 and N10) are presented in Table 8.1.

Isolate N8 demonstrated strong alkaline phosphatase, leucine arylamidase, β-galactosidase, α-galactosidase, naphthol-AS-BI-phosphohydrolase, trypsin, and acid phosphatase activities. Weak positive reactions were observed for esterase (C_4), lipase (C_{14}), valine arylamidase, α-chymotrypisn, α-glucosidase, β-glucosidase and α-mannosidase. Negative reactions were observed for all other enzymes tested.

Isolate N10 demonstrated strong alkaline phosphatase, esterase (C_4), esterase lipase (C_8), leucine arylamidase, valine arylamidase, trypsin, and naphthol-AS-BI-phosphohydrolase

activities. Weak activities were observed for cystine arylamidase, α-Chymotrypisn, α-mannosidase, N-Acetyl-β-glucosaminidase, α-glucosidase and negative reactions were observed for all other enzymes tested.

8.4 DISCUSSION

Although aerobic spoilage bacteria are able to produce proteolytic enzymes, the production of such enzymes is believed to be delayed until the late logarithmic phase of growth (Jackson *et al.*, 1997). It has been well established that proteolysis only occurs at populations greater than 10^8 cfu.cm^2, when spoilage is well advanced and bacteria are approaching their maximum cell density (Dainty *et al.*, 1975; Finne, 1982). A similar pattern was observed in the present study where considerable proteolysis was only observed late in the logarithmic phase.

This chapter has demonstrated that proteolytic activity was observed isolate N10, but not with isolate N8. It is unclear why no proteolytic activity was detected in isolate N8, the *Vibrio* spp. An extracellular protease purified from *Vibrio pelagius* by Farto *et al.* (2002) demonstrated proteolytic activity. The proteolytic assay was repeated at different pH values, with different media (casein and *N. norvegicus* muscle tissue impregnated SD-PAGE) and at different ambient temperatures but no activity could be detected. It may be mentioned that in earlier trial experiments (not documented in the thesis), isolate N8 incubated in casein-impregnated marine agar plates did demonstrate some proteolytic activity. The reasons for this are unclear, but further trials under different conditions (eg. with alternative buffers to the Tris-HCl used here) are required.

Temperature is the most important factor influencing the growth of mesophilic bacteria such as *Vibrio* sp. (Jackson *et al.*, 1997). Since most pathogenic bacteria are mesophiles,

chilling shellfish prevents their growth. In the present study, growth was observed at all temperatures but was strongly temperature dependent.

Increasing the ambient temperature of the spectrophometric assay also resulted in increased proteolytic activity. Venugopal (1990) reviewed the extracellular proteases of contaminant bacteria in fish spoilage and reported that the proteolytic potential of psychotrophic organisms has not been well documented. Decreasing the temperature from 30 to 0°C increased the proteolytic activity of pseudomonads (Peterson & Gunderson, 1960). In the present study isolate N10, *Pseudoalteromonas* spp., demonstrated the opposite.

In bacterial septicaemias of marine crustaceans, extensive tissue damage is often observed, indicating the significant role of proteases in the degradation processes (Edgerton *et al.*, 1995; Jiravanichpaisal *et al.*, 1995; Camus *et al.*, 1998). The present study has demonstrated that proteases present in the culture medium of isolate N10 selectively degraded the protein structure of *N. norvegicus* abdominal muscle. The proteases completely degraded the myosin heavy chain, troponin-T, troponin-I, paramyosin and a number of unidentified muscle proteins approximately 110 kDa in size with tropomyosin also significantly reduced in quantity. However it was also evident that a number of proteins remained unaffected by proteolytic activity, namely actin, the myosin α-light chain, and a number of other unidentified proteins at 50 and 80 kDa. A similar pattern of degradation was observed by Small (2004) who suggested that through the selective degradation of key structural proteins, such as the myosin heavy chain, tissue structure is lost facilitating the tissue penetration and degradation by the pathogen.

Substrate-PAGE analysis of ECP from the isolates failed to reveal any evidence of proteolytic bands from isolate N8, but indicated the presence of eleven bands in the ECP of isolate N10. These bands demonstrated profile similarities to ECP of *P. atlantica* (Costa-

Ramos & Rowley, 2003), and other *Vibrio* ECP (Nottage & Birkbeck, 1987). Seven Bands were observed, ranging from 120 KDa to 25 KDa, although a number of lower molecular weight bands may represent breakdown products of the higher molecular weight proteases (Montero, 1998).

Although production of proteinases as virulence factors has been demonstrated in experimental *Vibrio* infections of *Ostrea edulis* larvae (Nottage & Birkbeck, 1987), there have been reports of virulence factors other than proteases in the ECP of related bacteria. Thus Lee & Ellis (1990) demonstrated that a glycerophospholipid, cholesterol acyltransferase complex with lipopolysaccharide (LPS) are a major lethal toxin of *Aeromonas salmonicida.*

In chapter 2 it was reported that total haemocyte counts reduced as internal bacteria concentrations of the haemolymph increased. It has been reported in a number of cases that extracellular proteins can modify the immune system of a host. In the Atlantic salmon (*Salmo salar*) extracellular proteases secreted by the bacteria *Aeromonas salmonicida* resulted in leucopenia facilitating the spread of the bacteria (Ellis *et al.,* 1981; Hussain *et al.,* 2000). Montero (1998) reported reduced clotting ability and hemocytopenia resulting from the experimental infection of *N. norvegicus* with *Vibrio harveyi.* Hemocytopenia resulting from bacterial infections have also been observed in the blue crab, *Callinectes sapidus,* and the American lobster, *Homarus americanus* (Stewart *et al.,* 1969; Johnson, 1976).

The use of the API ZYM system ensures a degree of standardisation rarely found when individual species are studied by different investigators (Poh & Loh, 1988). The advantage of the API ZYM system is that it enables the determination of enzymatic profiles after 4 h of incubation, whereas other methods are more protracted. The technique is rapid, simple

to achieve and inexpensive compared to molecular techniques (García-Martos *et al.*, 2000); however, the specificity of the technique is uncertain (the enzymatic profile of bacterial species may not be very specific, since such patterns may be shared by more than one species). Despite this, the results obtained here indicate that it may constitute a good marker for epidemiological purposes.

In the present study, different enzymatic profiles were established for the two isolates, N8 (*Vibrio* spp.) and N10 (*Pseudoalteromonas* spp). This will allow rapid identification of isolates in the future, as well as suggesting a number of possible virulence factors. Isolate N8, a *Vibrio* spp., demonstrated strong acid phosphatase (AP) activity. Remaley *et al.* (1984) suggested that parasite-derived acid phosphatases inhibit the production of host-derived superoxide ions. Further studies are required to confirm whether acid phosphatase is a virulence factor of isolate N8, allowing it to invade and cause pathological damage to *N. norvegicus*.

Reactive oxygen species (ROS) are produced by stimulated haemocytes during a respiratory burst, which forms an integral part of the crustacean immune system (Thornqvist & Söderhall, 1997). Small (2004) hypothesised that AP secreted by cells of the dinoflagellate, *Hematodinium*, may cause suppression of ROS production by *N. norvegicus* haemocytes by dephosphorylating the enzymes involved in the manufacture of ROS, such as protein kinase C. AP has been demonstrated to inhibit superoxide ion production in human neutrophils (Remaley *et al.*, 1984) as well as in bivalve haemocytes by *Perkinsus marinus* (Volety & Chu, 1995) and *Bonamia ostreae* (Hervio *et al.*, 1988).

Small (2004) warns that AP may be involved in the sequence of events that lead to cell division and is therefore not a virulence factor. Phosphatases are involved in a number of cellular processes such as DNA synthesis (Brautigan, 1992), cell cycle regulation

(Freeman & Donoghue, 1991) and signal transduction (Walton & Dixon, 1993). However if AP was only involved in these processes and functioning internally it should not be detectable in the culture medium as was found in the present study.

Both isolates demonstrated strong leucine arylamidase activity. Extracellular N-terminal proteolytic activity by leucine arylamidase has been observed in a number of pathogens (Grehn *et al.*, 1991; Dettori *et al.*, 1995; Farto *et al.*, 1998), and has been implicated as a virulence factor in a *Vibrio* strain infecting turbot (Farto *et al.*, 1998).

The chapter suggests that isolate N10 is able to cause the pathological damage observed in *N. norvegicus* with bacterial septicaemia (chapter 7), but it is likely that there is a complex pathway to the final condition. Virulence factors, such as leucine arylamidase or acid phosphatase, released from other bacterial strains may allow the spoilage bacteria to invade and destroy muscle structure. To substantiate a specific bacterium as the cause of disease, Koch's postulates have to be observed: the researcher must find the same pathogen in each diseased individual, isolate the pathogen from a diseased animal and grow the bacteria in culture, induce the disease through challenge studies by transferring the pathogen from the culture and isolate the same pathogen from the experimental animals after the disease develops (Campbell, 1996). Future studies should therefore concentrate on challenge studies of *N. norvegicus* with bacterial strains isolated from necrotic individuals. It is likely that a number of species may be able to induce the necrotic condition because immuno-suppression triggered through the stress of the fishery process rather than the presence of a particular pathogen may be the key factor in the onset of the condition.

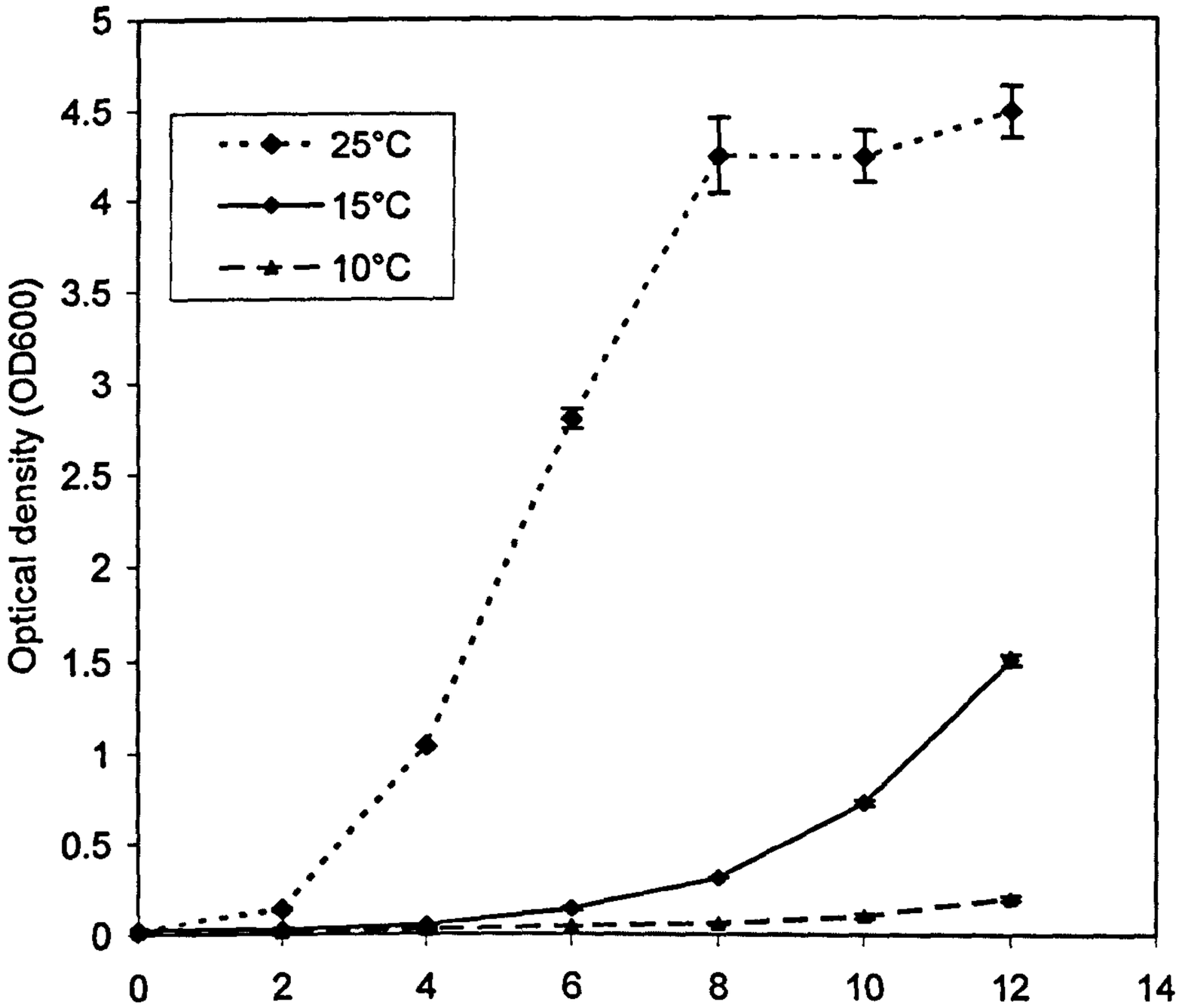
A number of possible virulence factors of the two isolates studied have been suggested and future work should concentrate on the cytotoxity of these ECP to *N. norvegicus.*

Table 8.1: Enzyme activities of isolates N8 and N10 with the API ZYM system.

Cupule No.	Enzyme assayed for	Isolate	
		N8	**N10**
1	Control	Negative	Negative
2	Alkaline phosphatase	Strong	Strong
3	Esterase (C_4)	Weak	Strong
4	Esterase lipase (C_8)	Negative	Strong
5	Lipase (C_{14})	Weak	Negative
6	Leucine arylamidase	Strong	Strong
7	Valine arylamidase	Weak	Strong
8	Cystine arylamidase	Negative	Weak
9	Trypsin	Strong	Strong
10	α-Chymotrypisn	Weak	Weak
11	Acid Phosphatase	Strong	Negative
12	Naphthol-AS-BI-phosphohydrolase	Strong	Strong
13	α-galactosidase	Strong	Negative
14	β-galactosidase	Strong	Negative
15	β-glucoronidase	Negative	Negative
16	α-glucosidase	Weak	Weak
17	β-glucosidase	Weak	Negative
18	N-Acetyl-β-glucosaminidase	Negative	Weak
19	α-mannosidase	Weak	Weak
20	α-fucosidase	Negative	Negative

Figure 8.1: The effect of the temperature on the mean growth rate, measured as optical density of the suspension, of isolate N8.

Figure 8.2: The effect of bacterial cell density on the protease activity of the bacteria-cell-free culture medium of isolate N10 over a 12 h period. Mean ± SD, N = 3.


25°C
15°C
10°C
Optical density (OD600)
Time (h)

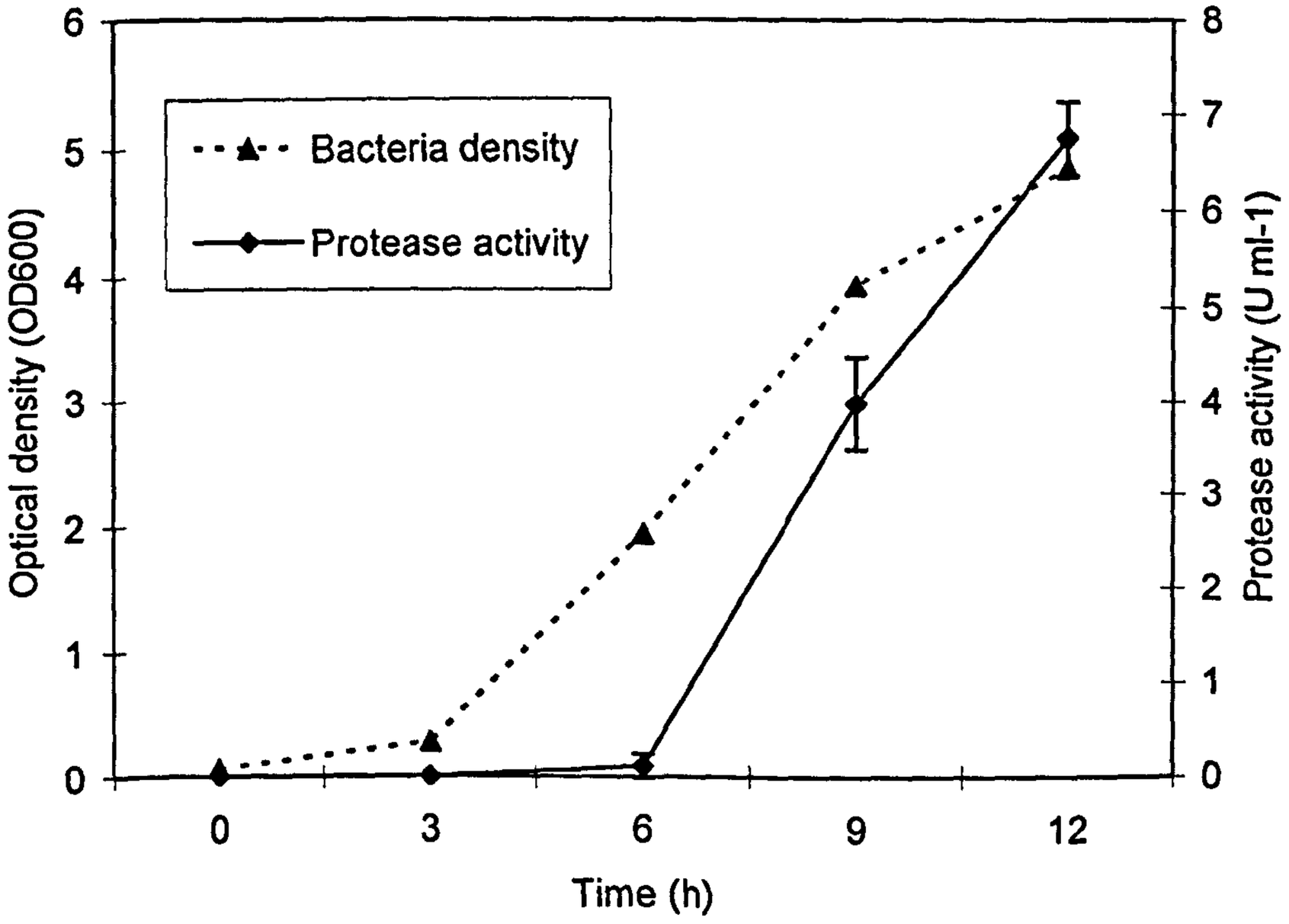
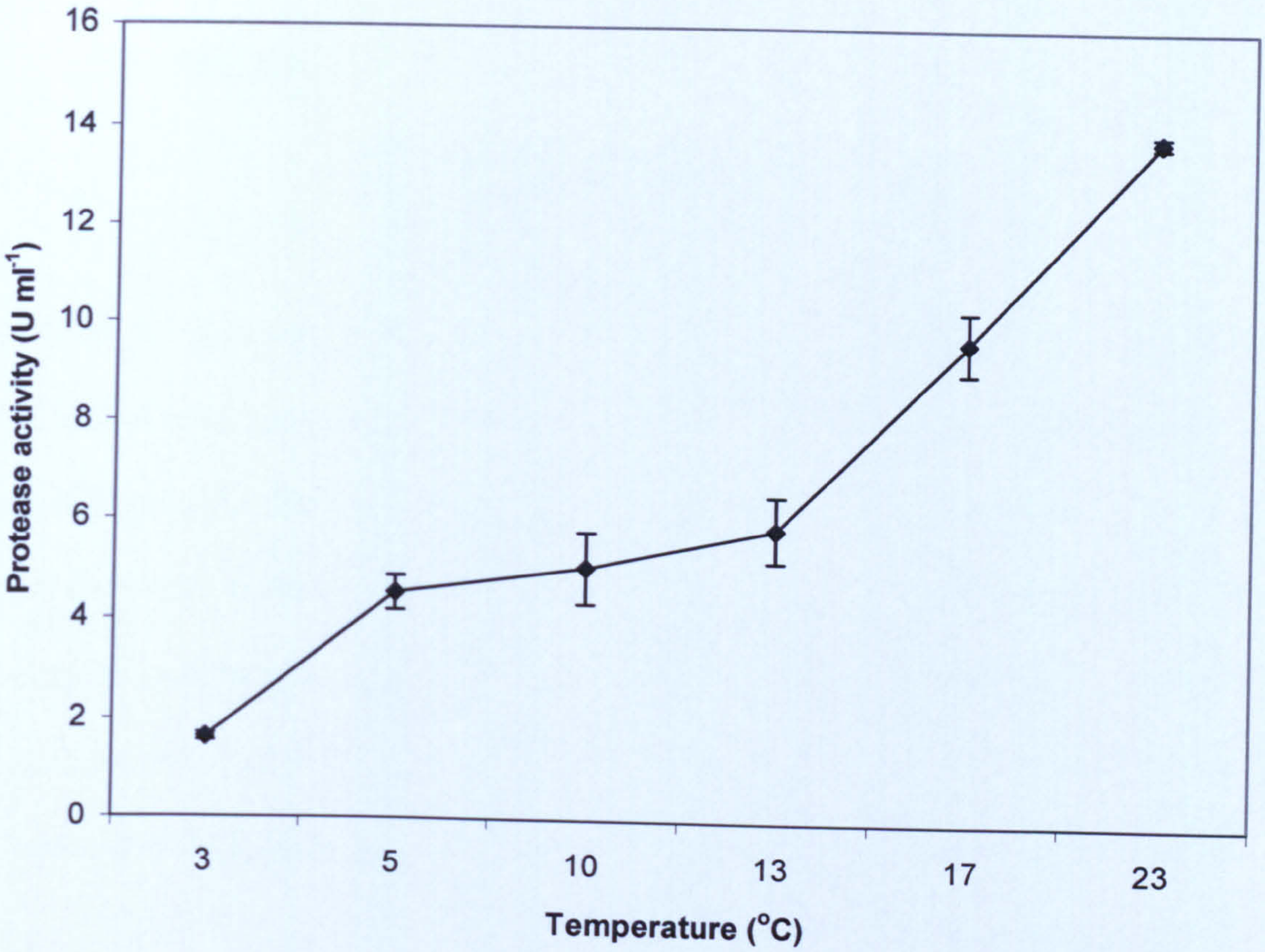
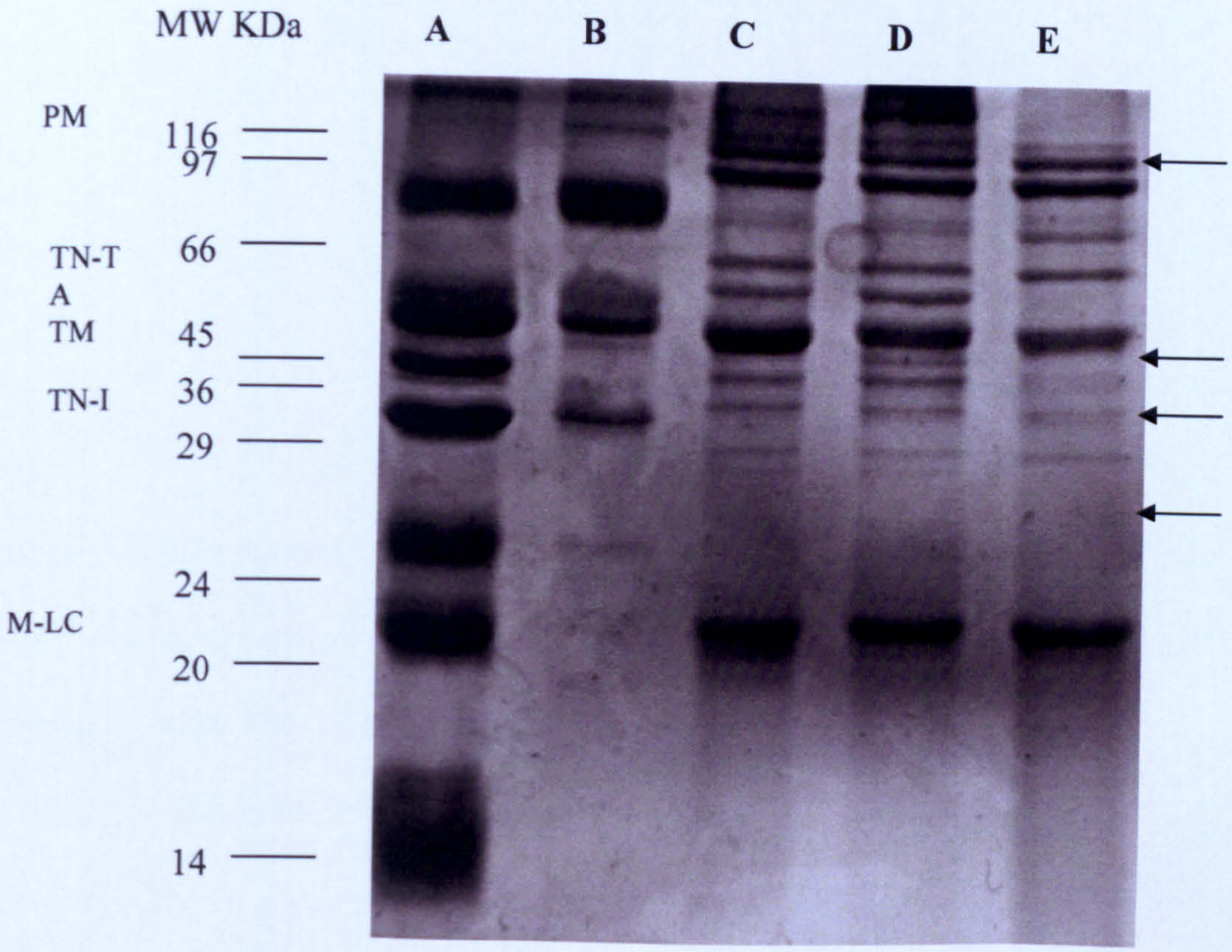
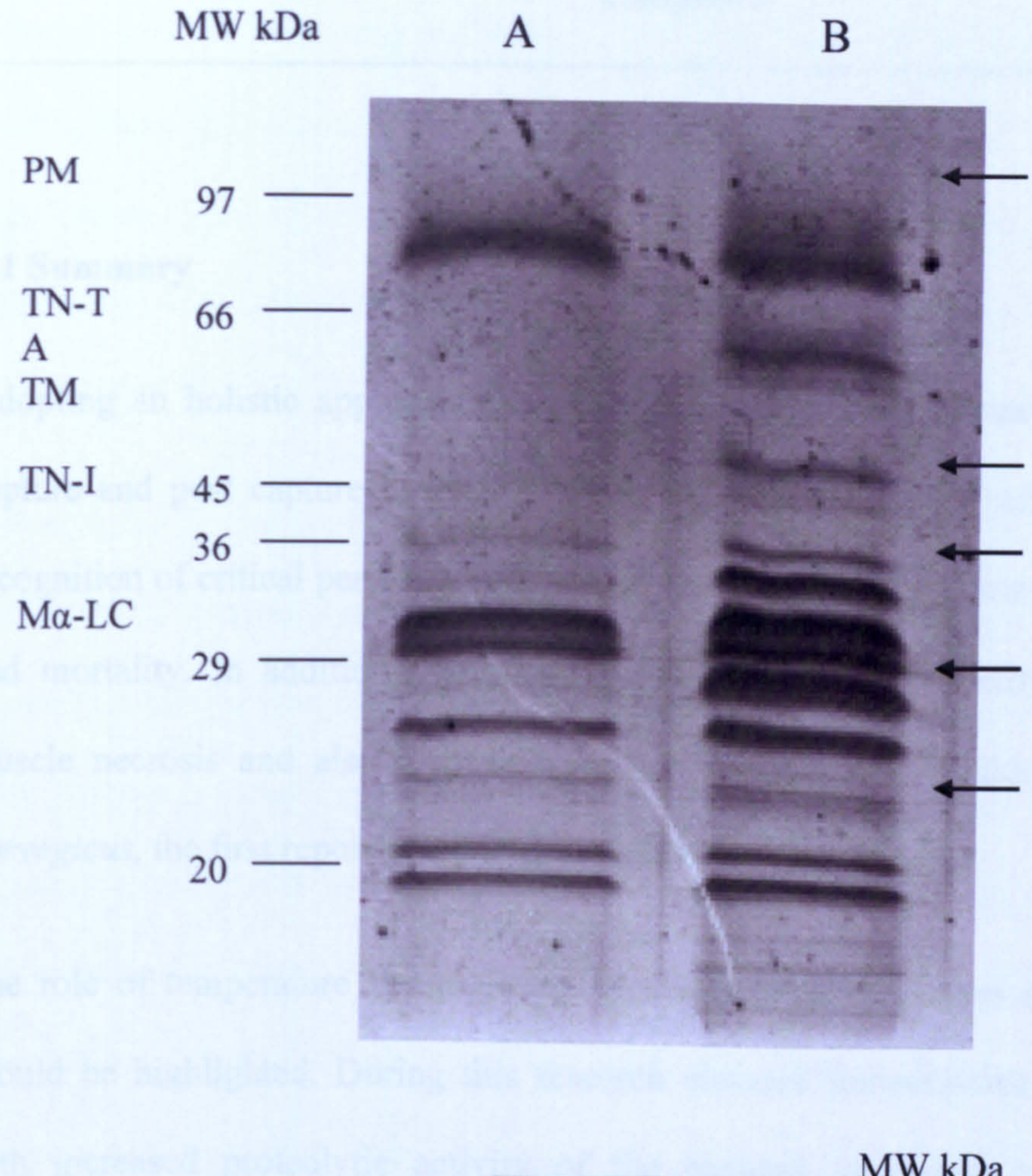

Bacteria density
Protease activity
Optical density (OD600)
Protease activity (U ml-1)
Time (h)

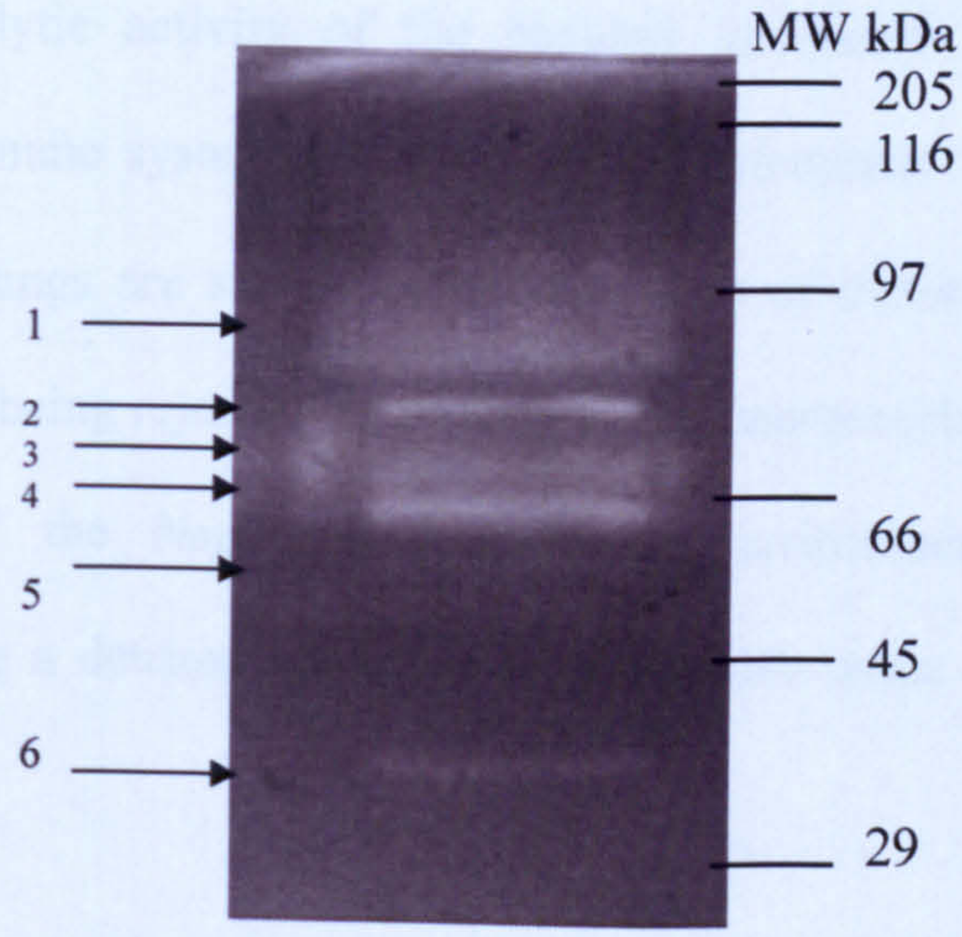
Figure 8.3: Effect of temperature on the protease activity of the bacteria-cell-free culture medium of isolate N10. Mean ± SD, N = 3.

Figure 8.4: Digestion of deep abdominal muscle (DAF) proteins of *Nephrops norvegicus* by proteases present in the bacteria-cell-free culture media of isolates N8 and N10. Lane A, low molecular weight markers; lane B, high molecular weight markers (sizes are indicated on the left in kilodaltons (kDa)); lane C, DAF protein sample incubated in the absence of proteases; Lane D, DAF protein sample incubated in the presence of proteases from isolate N8 and Lane E, DAF protein sample incubated in the presence of proteases from isolate N10. Arrows indicate degradation of protein bands. Names of the protein bands are given on the right. 12.5 % (w/v) acrylamide resolving gel. Key: PM, Paramyosin; TN-T, troponin-T; A, actin; TM, tropomyosin; TN-I, troponin-I; M α-LC, myosin α-light chain.


Protease activity (U ml-1)
16
14
12
10
8
6
4
2
0
3
5
10
13
17
23
Temperature (°C)


MW KDa
A
B
C
D
E
PM
116
97
66
TN-T
A
TM
45
36
TN-I
29
24
M-LC
20
14


MW kDa
A
B
PM
97
TN-T
66
A
TM
TN-I
45
36
Mα-LC
29
20


MW kDa
205
116
97
66
45
29
1
2
3
4
5
6

Chapter 9

General discussion

9.1 Summary

Adopting an holistic approach this thesis has detailed the stressors associated with the capture and post capture processes of the *Nephrops* fishery. Such data will help in the recognition of critical periods in the post capture period that promote poor stock condition and mortality. In addition, the research has furthered our understanding of idiopathic muscle necrosis and also identified another pathological condition affecting *Nephrops norvegicus,* the first report of a bacterial infection in the species.

The role of temperature in the pathologies and stress responses observed in this study should be highlighted. During this research elevated temperatures have been associated with increased proteolytic activity of the bacteria, increased bacterial growth rates, suppression of the immune system and a significant disruption to the physiology of *N. norvegicus*. These findings are supported by the effect of climatic temperatures on the proportion of the catch being rejected for the live export market (chapter 5). At all levels of the holistic study of the *Nephrops* fishery has environmental temperature been demonstrated as having a detrimental affect on the health status and meat quality of *N. norvegicus.*

Section 1.4 reported that stress is one of the most important factors in lobster health, and detailed the sequence of events that occurs in a stress response (Fig. 1.2, Evans, 1999). If the stressor is mild, only temporary disturbances occur; however, if the stressor is more severe, detrimental long-term effects can occur. A number of researchers have described

these detrimental outcomes such as reduced growth, reduced resistance to disease and reduced survival (Pickering & Pottinger, 1989; Lee & Wilkins, 1992; Iwama *et al.*, 1997). During the capture and post capture periods of the *N. norvegicus* fishery, it has been demonstrated that lobsters are exposed to both mild and extreme stressors with severe detrimental long term affects occurring, such as the two post capture conditions described in chapters 6 and 7.

Research by Seafish (UK fish industry authority) demonstrated the profit advantages for vessels which landed higher quality whitefish at Peterhead market (Curtis & Martin, 2003). Recently a further study illustrated that increased revenues for whole *N. norvegicus* results from adopting better on-board handling practices (Curtis *et al.*, 2005). The report "Economics of quality at sea for *Nephrops*" (Curtis *et al.*, 2005) reported that the control of temperature, use of ice and not over filling boxes resulted in a much greater packout rate (the volume of whole *N. norvegicus* actually packed for sale at processing plants). The report concluded that increasing the packout rate from 80% to 90% resulted in a £20,000 (12%) increase in annual revenue for a fishing vessel.

Through research described in this thesis, a Code of Practice for the capture, handling and transport of commercially exploited decapod crustaceans will be suggested. It is hoped that by improving the treatment of decapod crustaceans, both during the capture and post capture periods, increased post capture survival will result. Better post capture survival will reduce the amount of *N. norvegicus* being rejected at processing plants as well as the high mortality of discarded animals returned to the sea after trawl capture (Ulmestrand *et al.*, 1998). As Stentiford & Neil (2000) suggested, IMN and the opportunistic bacterial infection may be responsible for the high mortality observed in discarded *N. norvegicus*.

Better handling and treatment of the *N. norvegicus* portion of the catch will also lead to greater meat quality. Aerobic bacteria present in *N. norvegicus* experiencing bacterial septicaemia exceed that allowed in cooked whole crustaceans by EC community legislation (Decision 93/51/EEC) (Anon, 2002). Further research should investigate the negative impact of elevated bacteria counts and alterations in the carbohydrate dynamics on the taste profile of *N. norvegicus* abdominal muscle. Unlike *Hematodinium*, the two conditions described here do not appear to affect natural populations of *N. norvegicus* so a management plan is not required. However routine microbiological surveys should be undertaken to ensure that human disease causing pathogens, such as *Vibrio parahaemolyticus*, are not present and human health concerns can be allayed resulting in consumer confidence in the meat quality of *N. norvegicus*.

9.2 Treatment of post capture conditions

The literature has described that regression of IMN is possible if the crustacean is removed from a stressor immediately following the initiation of the condition. Effective prevention and treatment of bacterial infections in crustaceans has been demonstrated through the use vaccines (Stewart & Zwicker, 1974; Itami *et al.*, 1991; Keith *et al.*, 1992), immune stimulants (Raa, 2000), probiotics (Rengpipat *et al.*, 1998) and antibiotics (Stewart & Cornick, 1967; Diggles *et al.*, 2000). However, it is the view of the author that the most effective treatment of the conditions affecting *N. norvegicus* is to reduce the stress experienced during the fishery processes, which results in the manifestation of diseases. Other measures are likely not to be economically viable (Beard & McGregor, 1991). Thus it makes economic sense to prevent rather than cure.

9.3 Recommended code of practice for the *Nephrops* fishery

The formation of this code of practice was made possible by research contained in this thesis as well as viewing other codes of practice for other crustacean and fish species (Beard & McGregor, 1991; Anon, 2000a; Anon, 2000b; Estrella, 2002).

Capture

Once the catch is in the net there is little control over the conditions experienced, however, our research has allowed us to make a number of recommendations for the trawl capture of *N. norvegicus*.

- For the capture of *N. norvegicus* destined for the live transport or to be sold as whole animals it is suggested that the trawl duration be 1 h. Increasing the duration of the trawl results in a higher mortality rate and greater number of animals rejected at the processing plant. Although physiological and immunological indicators of stress demonstrated only small variations with prolonged trawl duration, there were significant increases in the amount of physical damage caused.
- During the spring (April/May), when many *N. norvegicus* are soft, or on hot days (above 25°C) it may be economically viable to place the entire catch on ice immediately on landing. Where animals are held in warm conditions bacteria levels in the muscle elevate, thereby risking rejection from market, including diversion to other product types, for example being tailed as "scampi".
- It has been demonstrated that the time of day that trawling is conducted affects the degree of stress experienced by *N. norvegicus*. Animals caught in the morning were more stressed (in terms of elevated haemolymph L-lactate concentrations), although this was overshadowed by the longer recovery period these animals experienced

before docking. Following trawl capture and subsequent exposure and handling, *N. norvegicus* should be placed in running seawater undisturbed for at least 8 h, before further handling and sorting.

Handling

Physical damage provides routes of entry for possible bacterial spoilers such as pseudomonads. The following guidelines will ensure that physical damage is kept to a minimum and lobsters are held in the appropriate conditions.

- Keep the deck wet and as cool as possible while fishing. This prevents temperature shock when the catch is landed on deck.
- The landing of the catch onto the deck should be done with care to minimise physical damage to fish and exposure to the warming and drying effects of sun and wind. Sort the catch and remove *N. norvegicus* as quickly as possible so as to minimise the duration of air exposure.
- During trawl capture, oxygen deficient mud can be swept into the cod end. This material may not only directly interfere with the gill function (clogging the gills and damaging fine gill filaments) but also compete for available oxygen. To mitigate this, lobsters should be washed in seawater before being placed in holding facilities.
- Minimise exposure to sunlight and wind, these quickly dry out lobsters and strong sunlight is also likely to damage the eye stalks. Keep crustaceans out of bright lights as much as possible. Bright light has been shown to be a stressor and may reduce survival rates.
- Do not throw crustaceans. Cracked carapaces allow entry of spoilage bacteria, present on the shells, into the animal. When sorting the catch, lobsters should be handled and

transferred carefully to a nearby box to minimise the distance fish fall and subsequent damage.

- Handling of crustaceans should be carried out as quickly and gently as possible. Avoid picking up animals by their claws or limbs to prevent shedding.
- Equipment should be designed to prevent physical damage to the animals.
- On hot days it is advised that lobsters packaged within 30 minutes of landing on deck should be kept alive, the remainder of the catch should be placed on ice.

Holding conditions

The conditions in which crustaceans are held and transported must not cause further stress. This will keep the animals healthy and improve product quality. During holding it is essential that the oxygen concentration of the water is kept as high as possible as the rate of recovery from stress is dependant on the oxygen concentration of the water. A period of recovery before transportation, following landing, is essential if high packout rates are to be observed.

- *N. norvegicus* should be left in circulated seawater for **at least 8 h** following trawl capture before further handling and transportation. This recovery period has a large affect on the mortalities observed in the following 48 h period.
- Seawater in holding tanks/containers should be circulating at a rate that ensures that the water is saturated with oxygen; stressed lobsters will rapidly deplete oxygen concentrations in standing water.
- Tanks should be tested for "dead spots" where the seawater is not fully circulated, food dyes can be used to observe possible "dead spots". In these areas lobsters will rapidly deplete oxygen concentrations.

- Do not store *N. norvegicus* if they are badly damaged, weak (limp legs and tail) or recently moulted (soft shells).
- Do not attempt to put more stock into a holding tank than it is designed to hold. Overstocking of the systems must be avoided as this increases disease risks and increases stress on the animals due to the inability of the system to cope with oxygen demand and metabolic wastes.
- Avoid undue disturbance to the animals during holding periods.
- Minimise the number of times animals are removed from the water, and work as quickly as possible when transporting.
- Keep water temperatures within the range 5-10°C to maximise oxygen saturation of the water and decrease the metabolic rate of the animals. Ensuring dissolved oxygen concentrations are maintained will help the recovery of animals from handling.
- Crear & Allan (2002) provide guidelines increasing the flow rate with elevated air temperatures.
- Avoid using water from estuaries or harbours as pollution or variations in salinity may make it unsuitable for the holding of crustaceans.
- It is essential to carry out regular monitoring of the animals to check for body colour, missing appendages, fouling of the shell or gills, and muscle condition. Weak and dead animals should be removed daily; if not water quality will be affected.
- Remove legs or claws which have been dropped as these rapidly result in increased ammonia levels in the water and can result in mass mortalities.
- Monitor use of ice for stock destined for live transport. Shipping crates may contain or be covered in ice, however live *N. norvegicus* should not be allowed direct contact with ice, this not only avoids temperature shock by also prevents contact with fresh water from melting ice which can be fatal.

Transport

- Minimising transport times from the quayside to the processing facility reduces initial stress on the animals and improves welfare.
- Do not unload *N. norvegicus* catches during the hottest part of the day.
- Transport water should be disposed of into the sewage water system and not into a storm water drain. This minimises the risk of disease transfer to local waters (this is especially a concern when transferring lobsters from NW Scotland to coastal sites in continental Europe, such as Barcelona in the Mediterranean, two very different ecosystems.

Acclimation

- On arrival at the processing plants, water temperature changes, if necessary, should be gradual with a maximum instantaneous change of less than 5°C. Large variations in salinity have been associated with IMN (Venkataramaiah, 1971) and turgid lobster syndrome (Diggles, 1999).

Feeding

- Do not feed crustaceans in holding systems at processing plants. Feeding results in increased organic matter in the water, an increased amount of excretion of waste products and during the digestion of food lobsters use up more oxygen from the water.

The data obtained in recent studies (Curtis *et al.*,, 2005; Chapter 5) indicated that there is a large difference in how skippers treat their catch. The above guidelines aim to improve meat quality and minimise mortalities of *N. norvegicus* during trawl capture. It is hoped

that by maximising the return of the fishery, less pressure will be placed on *N. norvegicus* stocks and a sustainable future for the fishery can be achieved.

9.4 Further work

In the discussions for each results chapter a range of further studies are suggested. However, a few suggestions should be highlighted. The impact of trawl duration has been demonstrated, further studies should concentrate on the impact of trawling at different depths on the stress experienced by *N. norvegicus*. Pressure differences during hauling will be considerable when bringing the catch up from 100m depth. Further studies into the causative pathogen of the bacteraemia observed in chapter 7 are encouraged, during the project preliminary challenge studies were undertaken without success, Koch's postulates must be fulfilled if the pathogen is to be identified.

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Appendix 1a: Development of IMN in *Nephrops norvegicus* following the December trawl, scored out of 12. Each line represents a single *Nephrops norvegicus.*

Appendix 1b: Development of IMN in *Nephrops norvegicus* following the January trawl, scored out of 12. Each line represents a single *Nephrops norvegicus.*

Appendix 1c: Development of IMN in *Nephrops norvegicus* following the February trawl, scored out of 12. Each line represents a single *Nephrops norvegicus.*