EFFECTS OF *HEMATODINIUM* INFECTION ON THE NORWAY LOBSTER, *NEPHROPS NORVEGICUS* (L.)

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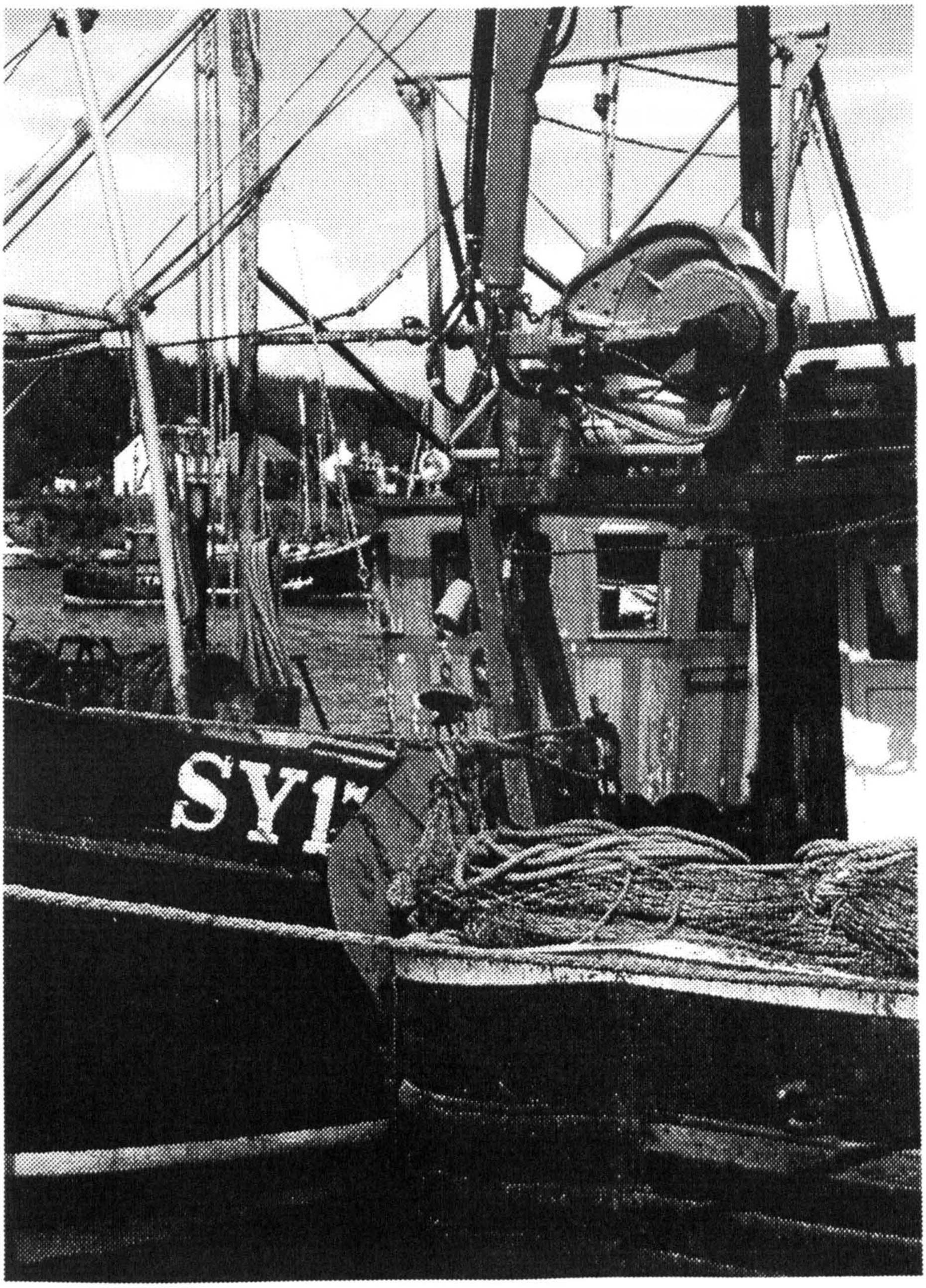

Nephrops trawlers at Tarbert, Loch Fyne (G.D. Stentiford)

Candidate's Declaration

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

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Summary

1. The Norway lobster (*Nephrops norvegicus*) is one of the most valuable shellfish resources in the north east Atlantic ocean and considerable knowledge exists with regard to its biology and life history. During the late 1980s, populations *of N. norvegicus* were shown to harbour an infection by a parasite of the genus *Hematodinium* (Dinoflagellata: Syndinidae). Although studies on the pathology, progression and prevalence of infection have since been carried out, considerable gaps exist in our knowledge of the effects of parasitism on host life history and cycling of the parasite in the field. This study aims to develop techniques for monitoring *Hematodinium* infection in natural populations of *N. norvegicus* and to study the interactions between parasite-induced pathological changes and the life history of host animals.

2. The two major field methods (body colour and pleopod index) for the diagnosis of infection in *N. norvegicus* by *Hematodinium* were compared. The pleopod index was then used to collect infection prevalence data in the Clyde Sea area over a period of 31 months from December 1998 to August 2000. Peak infection prevalence occurred during the spring and was highest in small lobsters and in females. Mean infection severity increased during the main infection season (Jan - May), highlighting the progressive nature of patent infection. Infection prevalence was synchronous between the sexes in some years but not in others. The proportion of recently moulted, infected males in the late season extended the duration of peak infection prevalence in certain years. Sites with a smaller mean animal size showed higher infection prevalence. The methods available for monitoring *Hematodinium* infection in *N. norvegicus* are evaluated. The relationships between fishing pressure, population structure of *N. norvegicus* and seasonal *Hematodinium* infections are also discussed.

3. While useful for studying advanced (patent) infections of the haemolymph, the pleopod diagnostic method failed to identify low-level haemolymph (sub-patent) and tissue-based (latent) infections. The development and application of an immunoassay for the detection of antigens from *Hematodinium* in *N. norvegicus* is described. Infected tissue and haemolymph samples were detected as multiple-band reactions to a polyclonal antibody (anti-*Hematodinium*). The sensitivity limit of the method was 204 parasites.mm^3 - which is approximately ten times more sensitive than the pleopod diagnosis method. Use of the immunoassay on tissue samples taken from catches

showed that the pleopod method considerably under-diagnosed infection prevalence in the early part of the season, though this under-diagnosis decreased as infected lobsters in the field progressed from latent and sub-patent to patent infections. The immunoassay failed to detect any infected lobsters during the summer months, suggesting that infection may not be carried over from one season to the next. Data are discussed in relation to the epidemiology of latent and sub-patent *Hematodinium* infections, to the transmission of parasites to other hosts and to the use of this immunoassay for the routine assessment of *Hematodinium* infection prevalence in *N. norvegicus* populations.

4. The haemolymph free amino acid (FAA) composition of *N. norvegicus* at different stages of infection by *Hematodinium* sp. was determined by reverse phase high performance liquid chromatography (HPLC). Uninfected animals had a total haemolymph FAA concentration of 3.79 $\mu mol.ml^{-1}$. The concentrations of several FAAs, notably serine, were reduced in early infections (Stage 1), while at later Stages (2-4) several FAAs were increased in concentration. The most significant change was in taurine, which increased by 13-fold (from 0.22 to 2.56 $\mu mol\ ml^{-1}$) in Stage 4, when its relative contribution to the total FAA was 41.6%. Possible causes of these changes in the lobster haemolymph FAAs are the breakdown of host tissues, haemocyte lysis, a host stress response and the release of FAAs from parasite cells. These results have implications for the biology of *N. norvegicus*, and indicate that the taurine:serine ratio in the haemolymph provides a sensitive diagnostic measure of patent *Hematodinium* infections.
5. Changes in the biochemistry and ultrastructure of the deep abdominal flexor (DAF) muscles were studied in *N. norvegicus* at different stages of infection by *Hematodinium*. Muscles from infected lobsters showed slight, but significant increases in total water content, greatly depleted glycogen reserves and an altered free amino acid profile. However, protein concentration and composition remained unchanged. Parasitic infection also caused an alteration in sarcolemmal structure and localized disruption of myofibrillar bundles around the fibre periphery. The implications of these changes for normal escape swimming are evaluated. The altered carbohydrate titre could reflect the parasites acting as a carbohydrate sink in the haemolymph, a disruption of normal tissue glycogenesis, or some alteration in the host's hormonal regulation. The changes described could also adversely affect the taste, texture and marketability of infected meat.

6. The effects of *Hematodinium* on carbohydrate metabolism were examined in *N. norvegicus*. Five stages of infection were observed, including uninfected, sub-patently infected, lightly infected (Stage 1), and moderately and heavily infected (Stages 2 and 3-4, respectively). Lobsters that were in Stages 1-4 of infection had significantly lower levels of hemolymph glucose than uninfected or sub-patently infected animals. These results were accompanied by significantly lower levels of hepatopancreatic glycogen in Stages 2-4 compared to Stages 0-1. Due to the disruption of the normal feedback loops that control the release of crustacean hyperglycaemic hormone (CHH) from the sinus gland, plasma concentrations increase with infection severity. The increase in CHH concentrations occurred concomitantly with reduced concentrations of plasma glucose and tissue glycogen. Data are discussed from the perspective that the parasite places a heavy metabolic load on the host lobster.
7. A post-capture muscle necrosis of rapid onset has been identified in *N. norvegicus*. Economic losses, due to mortality of these animals in transport, were encountered by Scottish wholesalers during the summer and autumn of 1999. The pathology causes a loss of the normal function of the abdomen, thus preventing the normal 'tail flip' swimming. Electron microscopy of affected tissue failed to reveal any obvious causative agent but showed a disruption of sarcomeric organization, a loss of Z-line material, a condensation of myofibrils and an infiltration of necrotic regions by granulocytes. SDS-PAGE of affected muscle tissue revealed a great reduction of most of the major contractile proteins. The condition most closely resembles idiopathic muscle necrosis, a pathology previously reported from both wild and cultured crustaceans. Damage to the integument in conjunction with exposure to various stressors during and immediately following capture is the most likely cause of the pathology. The rapid onset of the pathology has implications for the post-capture handling procedure for *N. norvegicus* and their subsequent vivier transport to market, and may also be partially responsible for the high mortality rate of undersized *N. norvegicus* returned to the sea after capture and aerial emersion.
8. Various components of swimming performance were measured in uninfected *N. norvegicus* and compared to animals at different stages of infection by *Hematodinium*. Animals showed a progressive decline in overall swimming performance as infection severity increased, with reductions in the number of tail-flips performed, the number of swimming bouts and the total distance travelled by swimming. The velocity of the first (giant-fibre mediated) tail flip and average velocity over the swimming bout were also

significantly reduced in infected lobsters. Possible reasons for this decreased swimming performance are suggested, and the implications of this for predator avoidance of infected lobsters in the benthic habitat, and for capture of *Nephrops* by trawl rigs, are discussed.

9. The pattern of tail flipping in uninfected and *Hematodinium*-infected *N. norvegicus* over a complete swimming sequence, and the subsequent performance following 4 h of recovery are described. Swimming in uninfected *N. norvegicus* was divided into two phases, the first comprising 80 to 100 high power tail flips, and the second comprising a variable number of less powerful flips which precede cessation of swimming. Lightly infected *N. norvegicus* showed a similar pattern, though the number of flips comprising the second phase was much less. Heavily infected *N. norvegicus* produced a smaller number of weak flips before cessation of swimming. Following recovery, uninfected *N. norvegicus* produced 84.3 % of the flips produced in the initial trial, this reduction being due to a smaller number of Phase 2 tail flips. Infected lobsters showed a lower recovery rate than uninfected animals, this being proportional to the severity of infection. The metabolic basis of this swimming pattern and the reasons for less Phase 2 tail flips being produced during *Hematodinium* infection are discussed in relation to the catchability of infected *N. norvegicus* by trawlers and predators.

10. Time-lapse video recording was used to study the burrow-related behaviour of uninfected and *Hematodinium*-infected *N. norvegicus* in aquarium conditions. Uninfected lobsters were more active during the hours of darkness, while infected lobsters were equally as active in the light and dark periods. The absolute number of departures performed by infected lobsters (70 day^{-1}) was more than double that of uninfected lobsters (30.1 day^{-1}), while the duration of burrow departures performed by infected lobsters (258.5 $s.departure^{-1}$) was more than six times greater than in uninfected lobsters (38.7 $s.departure^{-1}$). This led to a more than ten times increase (from 1.7 to 19.4 %) in the percentage of the day spent out of the burrow by infected lobsters. The altered burrow-related behaviour could be due to the increased nutritional or oxygen requirements of infected lobsters, both of which may cause the host to emerge from the burrow onto the sediment surface. Increased time spent out of the burrow has implications for the catchability of infected lobsters by trawlers and by predators, and for the estimation of infection prevalence from trawl-caught samples.

11. In Conclusion, this study has greatly increased our understanding of the complex relationship between *Hematodinium* parasites and their hosts. By linking biochemical

and physiological data to effects observed in the field, it has been shown that *Hematodinium* infection may provide an excellent model system for studying stress responses in aquatic invertebrate hosts. The study has described in much greater detail than before the effect of parasitism on host lobsters, and has related these effects to the moult cycle and to host condition. Furthermore, it has shown how changes in host behaviour and locomotion during infection may be directly or indirectly linked to biochemical and physiological changes brought about by infection. The implications of these changes in the life history of *N. norvegicus* for the availability of infected lobsters to be captured by trawlers and predators is discussed.

Contents

Chapter 1

General Introduction.

The Norway lobster (Nephrops norvegicus).

The Norway lobster, *Nephrops norvegicus* (L.) is a burrowing decapod crustacean which is an important member of the marine benthic community on soft sediments, and over the past 50 years it has been the subject of an important fishery in the northeast Atlantic. Due to its availability in large numbers throughout the year, its adaptability to aquarium conditions and its convenience for laboratory studies on whole animals and isolated tissues, it has proved to be a model species for research on various aspects of crustacean biology and ecology. Laboratory studies and field observations, addressing aspects of its general biology; including feeding ecology (Loo *et al.*, 1993; Cristo, 1998), reproduction (Farmer, 1974a) and moult cycle (González-Gurriarán, 1998) and of its behaviour (Rice & Chapman, 1971; Farmer, 1974b,c; Aréchiga & Atkinson, 1975; Atkinson & Naylor, 1976; Newland & Chapman, 1989) have led to considerable advances in our understanding of the life history of *N. norvegicus* in the field. Although other studies have been concerned with *N. norvegicus* as a fisheries target (Tuck *et al.*, 1997a,b; Merella *et al.*, 1998; Sardá, 1998), there are still considerable gaps in our knowledge of the population dynamics and fisheries biology of this important species (Briggs, 1997).

Up until the 1950s, these lobsters were usually discarded by fishermen due to their troublesome blockage of trawl nets, thereby impeding fin-fish capture (Briggs, 1997). However, more recently they have become one of the most important shellfish species captured in the northwest Atlantic, with annual landings of around 60,000 t (Tuck *et al.*, 1997a,b). Large fisheries for *N. norvegicus* exist around the south and west coasts of

Iceland, the North sea, the west coast of France, the Irish sea, the Kattegat, the Skagerrak, the Bay of Biscay, the Adriatic and the Tyrrhenian sea (Dow, 1980). It also supports a major fishery in the United Kingdom, with the Scottish fishery contributing over 76 % of this (22,000 tonnes, £57 million in 1999). The bulk of Scottish landings (c. 80 %) are from trawler capture, with lobsters usually being 'tailed' at sea and landed as 'scampi'. Creeling on the other hand is generally used for the capture of larger animals in sheltered waters or where trawling is not feasible. Creel-caught animals are usually landed live, after which they are transported via specially designed 'vivier' vehicles, often to supply the lucrative continental European markets (Anon, 1999).

The natural history of *N. norvegicus* impinges in many ways upon its availability to the fishery. As lobsters can only be captured when present on the surface of the sediment, both trawl and creel capture depend upon lobsters leaving their burrows (Fig. 1), and out-of-burrow activity provides the most likely explanation for the short term variations seen in catches (Farmer, 1974c). Burrow excursions are principally for the acquisition of food, and periods of maximal activity may coincide with the presence of principal prey items (Atkinson & Naylor, 1976). Female lobsters, which spend much of the winter within their burrows incubating eggs, are largely unavailable to the fishery during these times, causing a strong predominance of males in the catches (Farmer, 1974b). The feeding ecology of the female lobster during incubation is not well understood, though suspension feeding may play a significant role in nutrient supplementation (Loo *et al.*, 1993). Following spawning, females emerge from the burrow to feed, moult and be mated by hard-shelled male lobsters. During this period, the sex ratio within the catch can return to approximately 1:1; the different sex ratios at different times of the year represent the significant seasonal changes in the burrow-oriented behaviour of female lobsters (Farmer, 1974a).

Once on the surface of the sediment, capture by trawlers is further affected by the ability of lobsters to perform escape swimming. Following disturbance by the ground rope of the

trawl (Fig. 2); lobsters undergo a series of rapid abdominal flexions and extensions (tail flips), which propel the animal backwards and away from the source of threat (Newland *et al.*, 1992). Both the speed and endurance of tail flip swimming have implications for capture by trawl nets. In a study of the reaction of *N. norvegicus* to trawl nets, two capture zones have been observed: the 'catching zone' immediately in front of the net mouth, and the 'peripheral herding zone', where tail flipping lobsters are apparently herded (into the catching zone) by the otter boards of the trawl. Lobsters which are in the catching zone or herded in to this zone by the otter boards are usually overtaken and captured, while those tail flipping away from the catching zone evade capture (Newland & Chapman, 1989). Once within the net, the retention of captive lobsters is dependent upon the size of the lobster, the mesh size and the crowding of the net with other species.

In addition to the possibility of physical damage from the trawling process itself (Symonds & Simpson, 1971), after landing on the deck of a fishing vessel, *N. norvegicus* are exposed to a significant period of air-emersion during sorting of the catch. This period of emersion may be expected to lead to considerable physiological stress caused by extremes of temperature, light, desiccation and hypoxia (Santos & Keller, 1993; Jussila *et al.*, 1997; Paterson & Spanoghe, 1997; Chapman *et al.*, 2000). Stress responses occur when regulated physiological systems are pushed beyond their normal limits of operation by external stressors acting upon them (Paterson & Spanoghe, 1997). Failure of all or part of these regulatory responses will lead to increasing physiological disturbance and ultimately death (Morris & Airriess, 1998) and such stresses have been blamed for considerable post-harvest losses in commercially important crustacean fisheries (Cawthorn, 1997; Paterson & Spanoghe, 1997). The cumulative effect of these stresses has also been implicated in the low survivorship of undersized discarded *N. norvegicus* (Ulmestrand *et al.*, 1998). Creel-capture is thought to impart considerably less stress upon *N. norvegicus* than trawl-capture (Wileman *et al.*, 1999). However, in addition to the aggressive conflicts and starvation

which may occur within creels, lobsters will be exposed to an array of stressors during emersion, handling, pounding and transportation to the market place (Whyman *et al.*, 1985; Spicer *et al.*, 1990; Paterson & Spanoghe, 1997; Schmitt & Uglow, 1997). As such, any lobster in sub-optimal condition at the point of capture (e.g. recently moulted, diseased, damaged etc.) may be less likely to survive post-capture handling, storage and transportation (see Zhou & Shirley, 1995). Additionally, loss of physiological condition in the post-harvest period has been implicated in the deterioration in the flavour and texture of crustacean meat (Boyd & Sumner, 1973).

Disease as an additional stressor.

Infectious and non-infectious diseases affect both wild and cultured crustacean species. An understanding of crustacean pathogens and the natural reactions of host animals to disease is necessary for the efficient husbandry of cultured species and for the management of wild stocks. Non-infectious pathologies, such as those brought about by heat, cold, detergents, pesticides and heavy metals, may be important factors in the regulation of host populations (Pauley, 1975a). They may also cause significant economic losses in aquaculture situations, with disease events normally coinciding with overcrowding, handling, hypo- or hyperthermia, hyperactivity, changes in salinity or hypoxia (see Akiyama *et al.*, 1982; Nash *et al.*, 1987).

However, the majority of infections in crustaceans are caused by pathogenic microorganisms. The most important pathogens include representatives of the viruses, bacteria, fungi and protozoa. Over 30 species of virus have been described infecting crustaceans (Brock & Lightner, 1990), some of which, such as the one causing Taura syndrome in the shrimp *Penaeus vannamei*, cause significant economic losses under aquaculture conditions (Tu *et al.*, 1999). Bacteria have been reported as residual inhabitants of the normal crustacean haemolymph (Colwell *et al.*, 1975). The Family Vibrionaceae

(especially *Vibrio* spp., *Aeromonas* spp.) most commonly cause bacterial infections (Lightner, 1977), and these are often associated with host stress reactions to sub-optimal environmental conditions (Lightner & Lewis, 1975; Johnson, 1976; Stewart, 1980). External pathologies of the carapace, such as 'rust disease' and 'black spot' have been attributed to chitinivorous species of *Vibrio* and *Pseudomonas* (Baross *et al.*, 1978). Bacterial colonization may also occur as a secondary effect of infection by other parasites. In these cases, impairment of the normal immune response of the host caused by the primary pathogen may allow infestation by secondary opportunists (Meyers *et al.*, 1987; Field *et al.*, 1992).

Diseases caused by fungi may also have significant impact on crustacean populations. Shell diseases, commonly in conjunction with bacterially-induced lesions, have been attributed to various fungal species. One such disease in European species of crayfish is Burned Spot disease. In this, fungal hyphae growing within the cuticle cause dark lesions and breaches to appear, which allow secondary invasions by bacteria to occur. A similar disease, Black Mat syndrome (caused by the ascomycete *Trichomaris invadens*) has been described in Tanner crabs (*Chionoecetes bairdi* and *C. opilio*) from Alaska. In this infection, the connective tissue surrounding the organs may be largely replaced by fungal hyphae, with the highest prevalence of infection being found in mature female crabs (Brock & Lightner, 1990). The most notable example of a fungal disease of wild crustacean populations is 'Crayfish Plague' caused by the Phycomycete *Aphanomyces astaci*. First reported in Italy in the mid-1800s, the disease spread rapidly through mainland Europe, destroying many native populations of *Astacus astacus*, *A. leptodactylus* and *Austropotamobius pallipes* by the early 1900's (Johnson, 1983). North American species of crayfish, which show resistance to the parasite, can however act as carriers. It has thus been suggested that Crayfish Plague may have been introduced to Europe with imported animals and subsequently formed epizootic infections in native populations (Johnson, 1983).

Infections caused by yeasts have also been described in *Daphnia*, *Artemia*, amphipods and freshwater crayfish (Brock & Lightner, 1990).

It is likely that all decapod crustaceans harbour protistan parasites, although relatively few species are known due to the small number of decapod species that have been examined (for review see Sprague & Couch, 1971). The most important protozoan parasites of crustaceans are the microsporidians, with over 140 species described (Brock & Lightner, 1990). The microsporidians are intracellular parasites with great reproductive capacity. Transmission is normally by ingestion of spores by the host organism. Spores hatch and sporoplasms gain entry to the primary site of infection (usually the gut wall) via the spores' polar filaments. From here, the infection can spread to target tissues and organs. Once inside the target cell, a period of rapid multiplication (binary fission of schizogony) is followed by sporogony, whereby sporonts divide into sporoblasts and then to spores. These spores can then infect other host cells, often completely replacing the cell contents with parasite material (Canning, 1977). Microsporidian parasites are commonly found infecting the skeletal muscles (Findley *et al.*, 1981; Olson & Laman, 1984; Dennis & Munday, 1994) the hepatopancreas (Anderson *et al.*, 1989; Kabre, 1992), the gut wall (Kelly, 1979) and the reproductive tissues (Baticados & Enriquez, 1982) of crustaceans. In addition to the economic threat of microsporidiosis to the survival of cultured species, the presence of infections in wild crustacean populations may impact upon reproductive output and stock recruitment (by causing parasitic castration – Breed & Olson, 1977) and may also affect the marketability of infected animals (Olson & Laman, 1984).

Protozoan parasites of the subphylum Sarcomastogophora are generally thought to play a minor role in crustacean diseases (Sprague & Couch, 1971). However, one species of the family Paramoebidae, *Paramoeba perniciosa*, the causative agent of Grey Crab disease in the blue crab (*Callinectes sapidus*), causes seasonal infections along the eastern coast of the USA (Johnson, 1977). Moribund specimens have a translucent, grey appearance, with

internal symptoms that include lysis of muscle and blood cells and a reduction in carbohydrate and protein reserves (Pauley *et al.*, 1975). The parasite is responsible for commercially significant mortalities in wild populations and epizootics in holding tanks (Sawyer *et al.*, 1970; Johnson, 1988).

Ciliate diseases are also relatively uncommon in crustacean hosts. The early descriptions of ciliate infections of crabs were attributed to the genus *Anophrys,* but more recently these forms have been transferred to the genus *Paranophrys* (Sparks *et al.*, 1982). They are generally described as being problematic under hatchery and holding conditions (Bang *et al.*, 1972) but have also been reported in wild populations (Poisson, 1930; Armstrong *et al.*, 1981). Recently, studies on the infection by the scuticociliate *Anophryoides haemophila* have suggested this to be one of the three most important diseases in the American lobster (*Homarus americanus*), contributing significantly to post-capture economic losses (Cawthorn, 1997). Though initially described as being 'blood parasites', ciliate infections are now known to be systemic pathogens, capable of invading all major tissues (Sparks *et al.*, 1982). Death of the host is probably due to a major disruption of normal haemolymph and tissue function caused by the massive proliferation of parasites which accompanies late infection (Armstrong *et al.*, 1981).

In addition to their importance in the phytoplankton, the dinoflagellates are found as symbionts in a phyletically wide range of marine invertebrates (Trench, 1987). The first well-documented parasitic life style amongst the dinoflagellates was by Pouchet in 1885. He described *Gymnodinium pulvisculus* (later named *Oodinium pauchetti*) as an ectoparasite on *Oikopleura* (Pouchet, 1885 in Coats, 1999). However, knowledge of parasitic dinoflagellates was poor until the contributions of Chatton were collected in a volume of parasitic dinoflagellates in the 1920s and reviewed in the 1930's (Chatton & Poisson, 1931 in Taylor, 1987). The work of Chatton was considerably expanded by Jean and Monique Cachon in the mid 1900s, who described the cytology and life history of

numerous parasitic dinoflagellate forms (for review see Cachon & Cachon, 1987). It is now known that approximately 35 genera, representing four orders of dinoflagellates (the Phytodiniales, Gymonodiniales, Blastodiniales, and Syndiniales) contain species which are known to be parasitic. The majority of these are parasites of marine and estuarine organisms (Coats, 1999). In addition to the crustaceans, parasitic dinoflagellates are known to infect algae, protozoa, annelids, molluscs, salps, tunicates, rotifers and fishes (with over 140 species out of the 2000 described being parasitic) (Shields, 1994). Concerns over the importance of dinoflagellates as agents of disease in marine fisheries have led to a large expansion in the body of literature on this subject. In particular, much interest is now focussed on aspects of parasite-host ecology and on the molecular identification of parasitized hosts and the different parasite taxa responsible (Coats, 1999).

A number of parasitic dinoflagellate 'tribes' have been distinguished: the Blastodinida, Duboscquodinida, Syndinida and the Dinococcidia (which differ in morphology of the vegetative stage, in nuclear development and in their structural and metabolic relationship with the host) (Cachon & Cachon, 1987). The Blastodinida, Duboscquodinida and the Syndinida have life cycles consisting of two phases: the vegetative phase (trophont) and the reproductive phase (sporont). The reproductive phase leads to the formation of two dissimilar forms of bi-flagellate dinospore ('swarmers') which arise from different parent individuals and ensure dispersal and new infection (Cachon & Cachon, 1987).

The Syndinidae are generally considered as parasites of the haemocoel of metazoan hosts. They occur mainly as plasmodial forms which continue to grow and multiply until production of the motile spore stage. There are no chloroplasts and osmotrophy is the rule during the trophic phase (flagella not present), where lipid and polysaccharide inclusions suggest active feeding at the expense of the host. Sporogenesis is simple, with multiplication of the nuclei, plasmodia and cytoplasmic divisions up until the last generation of sporocysts, following which the bi-flagellate spores are produced and

liberated (Cachon & Cachon, 1987). Four genera of syndinids are known to be parasitic of crustaceans; *Actinodinium*, *Trypanodinium*, *Syndinium* and *Hematodinium*. Of these, *Actinodinium* (in copepods) and *Trypanodinium* (on copepod eggs) have not been well documented, while *Syndinium* and especially *Hematodinium* have been studied by a number of workers (Shields, 1994). Infection by *Syndinium turbo* and *S. gammarai* have been described in copepods and inoculation presumably occurs by ingestion of dinospores by the host. Pathology includes degeneration of the host musculature, castration and eventually death. It is assumed that such infections may play a significant role in the regulation of host populations (Shields, 1994).

Infections caused by Hematodinium spp.

Members of the genus *Hematodinium* are primarily parasites of decapods. Questions relating to host specificity are complicated by limited species descriptions and the lack of recognized morphological traits for sorting taxa of some parasitic dinoflagellates (Coats, 1999). Until recently, there was only one described species in the genus *Hematodinium*. This type species, *Hematodinium perezi*, was first described from the portunid crabs *Carcinus maenas* and *Liocarcinus depurator* in European waters (Chatton & Poisson, 1931). More recently, description of a second species, *H. australis* (Hudson & Shields. 1994) and molecular studies showing differences in *Hematodinium* isolates from other crustacean hosts (Hudson & Adlard, 1996), indicate a higher species diversity and host specificity than first thought (Coats, 1999).

Hematodinium infection has now been reported from a number of crab hosts from around the world, including *Callinectes sapidus* (Newman & Johnson, 1975; Messick, 1994), *Cancer pagurus* (Latrouite *et al.*, 1988), *Cancer irroratus*, *Cancer borealis* (Maclean, 1978); *Chionoecetes bairdi* (Meyers *et al.*, 1987; Eaton *et al.*, 1991; Love *et al.*, 1993), *Chionoecetes opilio* (Taylor & Khan, 1995), *Necora* (= *Liocarcinus*) *puber* (Wilhelm &

Boulo, 1988; Wilhelm & Mialhe, 1996), *Ovalipes ocellatus* (Maclean, 1978) *Portunus pelagicus* (Hudson & Shields, 1994), *Scylla serrata* (Hudson & Lester, 1994), *Trapezia areolata* and *T. coerulea* (Hudson *et al.*, 1993). *Hematodinium*-like dinoflagellates have also been described from several species of pandalid shrimp (Bower *et al.*, 1993, Meyers *et al.*, 1994) and in benthic amphipods (Johnson, 1986).

During the early 1980s, a low incidence of *N. norvegicus* in a moribund state, with dull orange colouration and a milky-white haemolymph, were captured by fishermen in the Firth of Clyde, western Scotland. Due to its coincidence each spring with the main moulting period, the condition was designated 'post-moult trauma'. By the late 1980s, the increasing incidence of lobsters in this poor condition began to evoke comment from fishermen and processors and led to a regular sampling program to define its seasonality and geographic incidence (Field, 1992). Further studies on these affected lobsters led to the discovery that the condition was in fact caused by a parasitic dinoflagellate, similar to the *Hematodinium* species described from other species of decapod. This was the first description of *Hematodinium* infection in a lobster (Field *et al.*, 1992).

The *N. norvegicus* isolate of *Hematodinium* has now been serially cultured at 8°C for a number of years and a putative life cycle for the parasite has been described. The parasite appears to undergo a series of developmental changes which eventually lead to the production of the motile dinospore stages. However, successful transmission of parasites to uninfected hosts has not been demonstrated to date, suggesting that the life-cycle described from *in vitro* cultures may not fully explain events in the field (Appleton & Vickerman, 1998). The presence of *Hematodinium* infection in the hermit crab *Pagurus bernhardus* in the Clyde fishery (Appleton & Vickerman, 1998) and the description of *Hematodinium* infection in several species of benthic amphipod (Johnson, 1986) means that the involvement of an intermediate/alternative host in *Hematodinium* transmission to *N. norvegicus* cannot be ruled out. The *Hematodinium* isolate from *C. sapidus* has also been

serially cultured (Shields & Messick, 1997), though here, transmission to uninfected hosts has been demonstrated by serial passage of infected haemolymph (Shields & Squyars, 2000).

To date, the major field diagnostic method for the detection of *Hematodinium* infection in *N. norvegicus* has been the pleopod method of Field & Appleton (1995), in which a pleopod is assessed for the presence of agglutinated parasite and hemocyte material under low-power microscopy. In addition to its rapidity and adaptability to field conditions, a further advantage of this method is that it allows infection severity to be assessed on a four point scale, which allows the progression of infection to be charted. This has been used to show that patent *Hematodinium* infection (present in the haemolymph) progresses from low haemolymph burdens during light infections to the production of swarming spore masses at the termination of infection, after which the host lobster dies (Field & Appleton, 1995; Appleton & Vickerman, 1998). However, the pleopod method fails to diagnose low-level (sub-patent) haemolymph infections and tissue-based (latent) *Hematodinium* infections (Field & Appleton, 1996). For this reason, a polyclonal antibody, was raised against the cultured *Hematodinium* sp. originally isolated from *N. norvegicus* and used in an indirect fluorescent antibody technique (IFAT) study to show that apparently uninfected lobsters (using the pleopod method) can harbour low level infections outside of the main season (Field & Appleton, 1996). Observations made in this IFAT study suggested that the epidemiology of latent and sub-patent infections can only be investigated by using diagnostic methods which are considerably more sensitive than the pleopod method. However, the pleopod method has been used to show that epizootics of *Hematodinium* infection, similar to those already described in other commercially important crustacean hosts from southeast Alaska, France and the eastern United States (Newman & Johnson, 1975; Latrouite *et al.*, 1988; Wilhelm & Boulo, 1988; Meyers *et al.*, 1987, 1990; Wilhelm & Mialhe, 1996) occur in *N. norvegicus* populations at various sites on the west coast of

Scotland (Field *et al.*, 1992, 1998). For this reason, the pleopod method remains a powerful field tool for the diagnosis of patent infections.

Rationale for the current study.

The large fishery for *N. norvegicus* necessitates careful stock assessment and effort control (Tuck *et al.*, 1997b). While an important feature of such assessment is the estimation of natural (*M*) and fishing (*F*) mortalities, little information is available on the former (Chapman, 1980). However, a major contributor to natural mortality in any population is disease, with infectious organisms also affecting growth, reproduction and egg survival in their hosts. As such, assessment of natural mortality in fisheries models should make use of data on disease prevalence where available (Kuris & Lafferty, 1992). The prevalence of *Hematodinium* infection in *N. norvegicus*, especially during epizootic seasons is now being considered as an important natural mortality factor in *N. norvegicus* stock assessment models (Anon, 1997) and as such, estimation of the proportion of natural mortality (*M*) attributable to *Hematodinium* infection requires accurate assessment of prevalence in natural populations. The diagnostic sensitivity and reliability of the pleopod method therefore requires investigation, while further development of the IFAT method of Field & Appleton (1996) into a rapid, sensitive and non-subjective immunoassay will allow for infection prevalence data from different fisheries, at different European locations, to be compared with greater confidence.

Any feature which alters the burrow emergence pattern or the escape swimming ability of *N. norvegicus* may be expected to impact upon their capture by trawlers. Due to pathological (Field & Appleton, 1995) and physiological (Taylor *et al.*, 1996) deterioration which occurs *in N. norvegicus* with patent *Hematodinium* infections, it has been suggested infected lobsters may spend longer periods out of the burrow and may be less able to escape from predators and trawlers than their uninfected counterparts (Field *et al.*, 1992,

Field & Appleton, 1995). As such, estimations of *Hematodinium* infection prevalence in lobsters from trawl-caught samples may give a false estimation of true levels of infection prevalence in the natural populations. In order to assess the accuracy of infection prevalence estimates in trawler-caught samples, an investigation into the burrow-oriented behaviour and the escape swimming ability of *Hematodinium*-infected *N. norvegicus* is required.

Studies on *Hematodinium* infections of other commercially-important crustacean hosts species have described major pathological disruptions to the muscle and haemolymph (Meyers *et al.*, 1987; Taylor & Khan, 1995; Wilhelm & Mialhe, 1996). Similar gross observations on pathological progression of infection (such as milky-white haemolymph, infiltration of tissue and organs by parasite cells and general morbidity) have also been made for *Hematodinium*-infected *N. norvegicus* (Field *et al.*, 1992; Field & Appleton, 1995), though to date, only one study has provided data on the physiological changes which occur in haemolymph and tissues during infection (Taylor *et al.*, 1996).

Changes in the biochemical and physiological characteristics of *Hematodinium*-infected *N. norvegicus* tissues may have implications at several different levels. At market level, biochemical alterations to the meat of heavily-infected animals may render it unmarketable, a problem already encountered in *Hematodinium*-infected tanner crabs (*C. bairdi* and *C. opilio*) (Meyers *et al.*, 1987; Taylor & Khan, 1995). At the fisheries level, ultrastructural and biochemical disruptions to the abdominal muscles may impinge upon the escape-swimming capability of infected lobsters, while the utilization of host resources by the parasites may lead to physiological starvation, an effect which may manifest itself as an increase in out-of-burrow foraging – both of which may lead to increased catchability of infected lobsters relative to their uninfected counterparts. Alternately, parasite-mediated biochemical and physiological alterations in the host could be used to map the pathological

progression of infection, thereby leading to a richer understanding of the ecological relationship between parasite and host.

The aims of this thesis are based on the above considerations, and are:

1. To use existing field diagnostic methods for studying the epidemiology of patent *Hematodinium* infection prevalence in natural populations of *N. norvegicus* and to investigate prevalence in relation to the size, sex, moult status and population structure of host animals (Chapter 2).
2. To develop the sensitive IFAT method into a multi-sample, reproducible diagnostic method for the detection of sub-patent and latent *Hematodinium* infections in field-caught samples of *N. norvegicus* (Chapter 3).
3. To investigate the effects of *Hematodinium* infection on the biochemistry and physiology of the haemolymph and tissues of *N. norvegicus* (Chapters 4, 5 and 6).
4. To investigate whether *Hematodinium* infection renders *N. norvegicus* more available to the fishery and to predators by causing changes in the locomotory ability (Chapters 8 and 9) and burrow-related behaviour (Chapter 10) of infected lobsters.
5. To integrate laboratory-based and field data to provide a detailed picture of the overall ecological effect of *Hematodinium* infection on *N. norvegicus* (Chapter 11 – Conclusions and Prospects).

While not being an initial aim of this thesis, chapter 7 provides the first description of a seasonal, post-capture necrotic muscle pathology in *N. norvegicus* from the west coast fishery. This chapter reports on a disease which is distinct in both its seasonality and its pathology from *Hematodinium* infection, and thus provides a useful reference for the prevention of misdiagnosis in the field.

Each of the data chapters in this thesis has been written as independent pieces of research and Chapters 2-10 inclusive have been submitted separately as scientific papers. As such, the chapters can be read independently without the need for cross referencing to other chapters, though all chapters do refer to each other by cited references, which are presented at the end of each chapter. Details on the status of the manuscript, co-authors, publication title, journal name and publication date are given on the title page of Chapters 2-10.

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Fig. 1. The Norway lobster, *Nephrops norvegicus* in typical 'guarding' position at the entrance to its burrow (from Erwin & Picton, 1995).

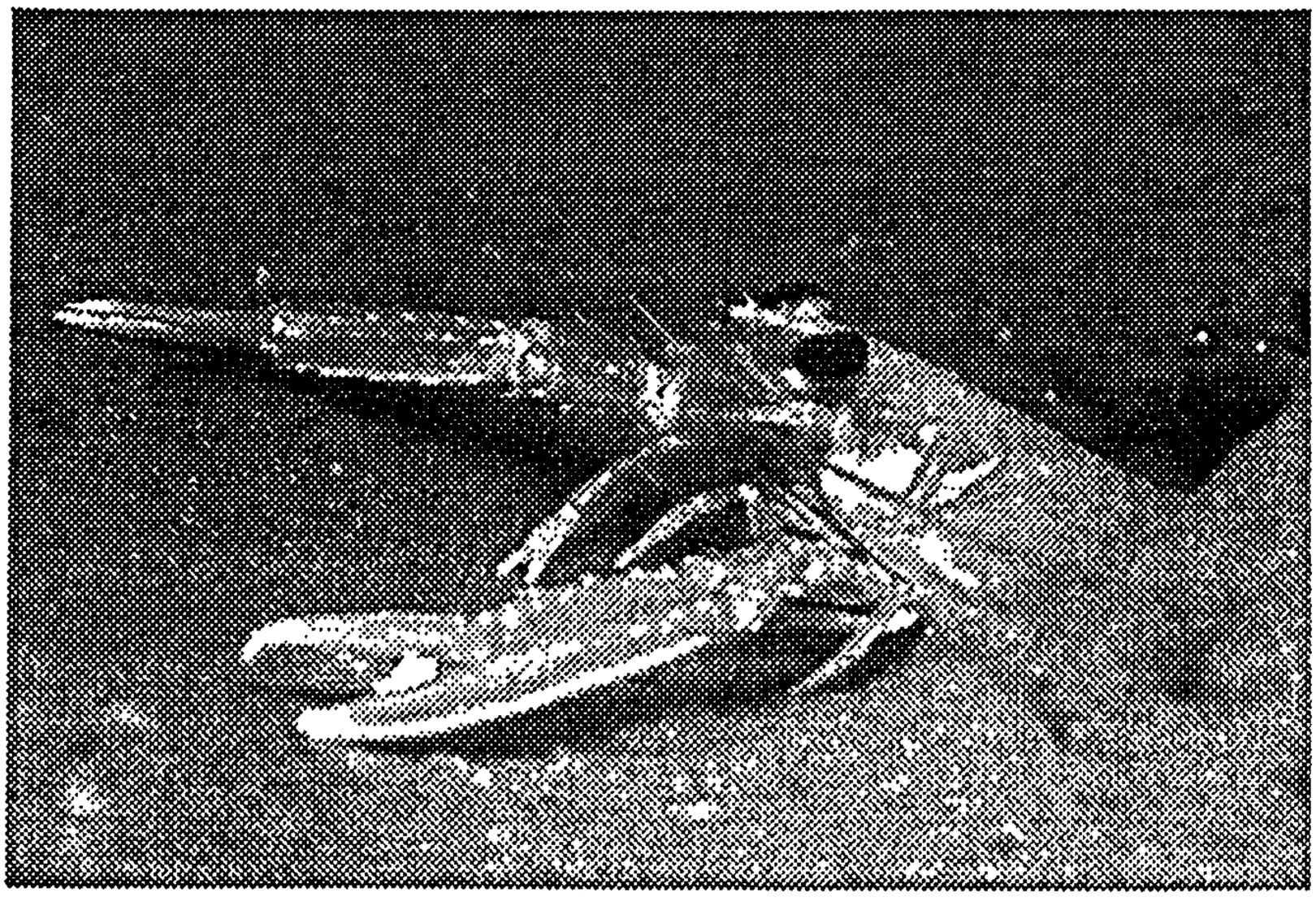
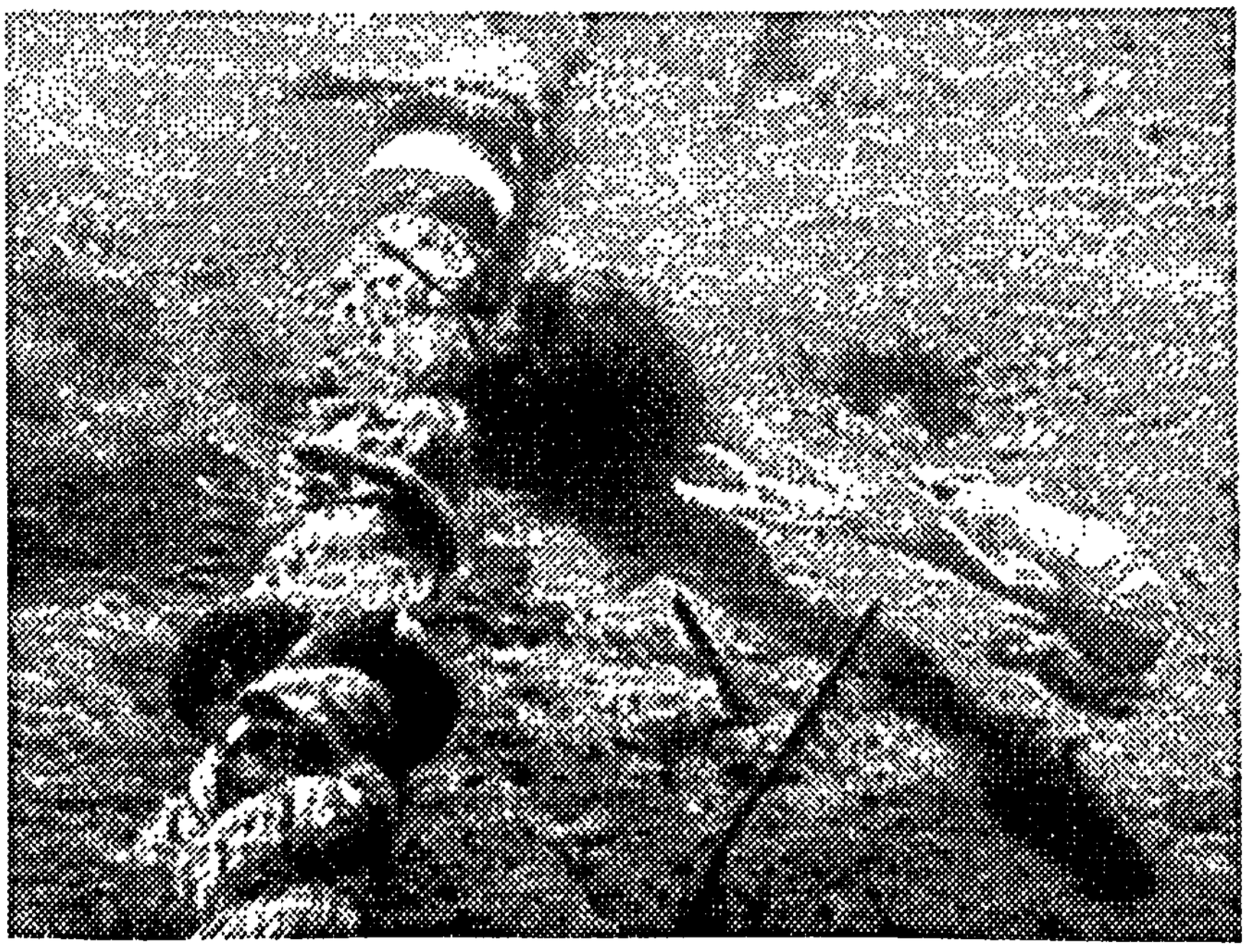


Fig. 2. The Norway lobster, *Nephrops norvegicus* undergoing active escape-swimming following disturbance by the ground rope of a trawl net (from Newland, 1985).



Chapter 2

The relationship of *Hematodinium* infection prevalence in a Scottish *Nephrops norvegicus* population to seasonality, moulting and sex.

Abstract.

The two major field methods (body colour and pleopod index) for the diagnosis of infection in the Norway lobster, *Nephrops norvegicus* (L.) by the dinoflagellate parasite *Hematodinium* have been compared. Using the more reliable method, the pleopod index, which scores infection severity on a four point scale, infection prevalence data have been collected from a single fishing ground in the Clyde Sea area, western Scotland over a continuous period of 31 months. Peak infection prevalence occurs during the spring and is highest in small *N. norvegicus* (mean carapace length 28.1 mm ± 0.67 in females and 30.9 mm ± 0.50 in males) and in females. Mean infection severity increased from 1.05 ± 0.03 during the low season (July - December) to 2.59 ± 0.19 by the end of the main infection season (May), highlighting the progressive nature of patent infection over time. Data collected separately for male and female lobsters show that infection prevalence is synchronous between the sexes in some seasons but not in others. Additionally, the proportion of recently moulted, infected males in the late season extends the duration of peak infection prevalence in certain years. Data from two adjacent fishing grounds in the Clyde Sea area has also shown that infection prevalence depends upon the population structure at a given site – the site with smaller animals showing the highest prevalence. The methods for monitoring *Hematodinium* infection for the assessment of natural mortality attributable to infection are evaluated. The relationships between fishing pressure, population structure of *N. norvegicus* and seasonal *Hematodinium* infections are also discussed.

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Introduction.

Populations of the Norway lobster (*Nephrops norvegicus*) provide one of the most valuable shellfish resources in the north east Atlantic, with annual landings in excess of 60,000 tonnes (Tuck *et al.*, 1997). A major fishery for *N. norvegicus* exists in waters surrounding the United Kingdom, with the Scottish fishery contributing over 76% of this (£57 million in 1999 – FRS, 2000, unpublished). The majority of the landings are from trawler capture, with lobsters being 'tailed' for sale as 'scampi'. Larger animals, captured by baited creels are often exported live to continental Europe by specially-designed 'vivier' vehicles. The large fishery for *N. norvegicus* necessitates careful stock assessment and effort control (Tuck *et al.*, 1997) and an important feature of such assessment is the estimation of natural (M) and fishing (F) mortalities. While fishing mortality is directly related to effort (E), little information is available on the rate of natural mortality in *N. norvegicus* populations (Chapman, 1980). A major contributor to natural mortality in any population is disease, and infectious organisms may also affect growth, reproduction and egg survival in their hosts. As such, assessments of natural mortality in fisheries models should make use of data on disease prevalence where available (Kuris & Lafferty, 1992).

Infections by parasitic dinoflagellates of the genus *Hematodinium* have been reported in a number of commercially important crustacean hosts (Newman & Johnson, 1975; Maclean, 1978; Messick, 1994; Meyers *et al.*, 1987; Latrouite *et al.*, 1988; Wilhelm & Boulo, 1988; Hudson & Shields, 1994; Hudson & Lester, 1994; Taylor & Khan, 1995; Wilhelm & Mialhe, 1996). Stocks of *N. norvegicus* on the west coast of Scotland have been also been shown to harbour infections by *Hematodinium* (Field *et al.*, 1992). Since the initial descriptions of disease etiology, diagnosis and pathology (Field *et al.*, 1992; Field & Appleton, 1995, 1996), further studies have described the *in vitro* life-cycle of the parasite (Appleton & Vickerman, 1998) and have revealed that significant alterations in host physiology (Taylor *et al.*, 1996), biochemistry (Stentiford *et al.*, 1999 – Chapter 4,

Stentiford *et al.*, 2000a – Chapter 5), locomotory performance (Stentiford *et al.*, 2000b – Chapter 8) and behaviour (Stentiford *et al.* 2001a; in manuscript – Chapter 10) are associated with parasitism.

A number of methods are available for the detection of *Hematodinium* infection in *N. norvegicus*. The simplest involves external assessment of the altered carapace coloration and opacity that accompanies patent infection. This assessment has the advantage that it can be performed rapidly in the field, but its value as a diagnostic tool has not been established, since no systematic study has been made of the relationship between parasite burden and colour change. The method that has been used most routinely is the pleopod infection staging technique of Field & Appleton (1995), in which a pleopod is assessed for the presence of parasite and hemocyte material under low-power microscopy. This method assigns infection severity on a four point scale, which allows the progression of infection to be charted. The current study has compared the sensitivity and reliability of the body colour and pleopod methods for the detection of patent infections. Observations on the prevalence of *Hematodinium* infection in crabs have suggested that there is a highly seasonal epidemiology, with peak infection occurring over a relatively narrow time period, followed by a longer period of undetectable or low level infection prevalence (see Shields, 1994). Studies on *Hematodinium* infection in Scottish *N. norvegicus* populations have shown similar features of seasonal epidemiology, along with evidence that prevalence is higher in small lobsters than in large lobsters, and in female lobsters than in male lobsters (Field *et al.*, 1992, 1998; Field & Appleton,1995). Meyers *et al.* (1990) have reported that *Hematodinium* infection prevalence is highest in post-moult *C. bairdi*. Moulting has also been suggested as the major predisposing factor for *Hematodinium* infection of *N. norvegicus*, though the relationship remains unclear (Field *et al.*, 1992, 1998).

From a general survey of *Hematodinium* infection in *N. norvegicus* captured from the west coast of Scotland, it has been shown that the Clyde sea area generally has the highest

prevalence levels (Field *et al.*, 1998). Here we report the results of a continuous long-term study of infection prevalence, whereby the pleopod diagnostic technique was applied to a major *N. norvegicus* fishing ground in the Clyde Sea area. Data are discussed in relation to the importance of long-term *Hematodinium* infection monitoring in commercially important crustacean populations, and the requirement for accurate infection prevalence data for use in stock assessment models for the *N. norvegicus* fishery.

Methods.

Capture of lobsters.

Norway lobsters (*Nephrops norvegicus*) were caught from a depth of approximately 80 m, using 90 minute tows of a standard otter-trawl (70 mm mesh size). The start of the towing period was always between 0900 h and 1000 h to minimise any effect of differential burrow emergence behaviour on catch composition (Atkinson & Naylor, 1976) and the effect of capture time on infection prevalence (Field *et al.*, 1998). The main capture site was at a location immediately south of Little Cumbrae (LC) in the Clyde sea area (55.41°N, 4.56°W) or in one instance, from the adjacent Bute-Cumbrae channel (BC) (55.46°N, 4.59°W) in the Clyde Sea area, Scotland, UK. Both sites are fished commercially. Trawls were made each month over the period from February 1998 to August 2000 inclusive (n = 31).

Following capture, all non-target species were separated from the catch and a random 5 kg sub-sample of *N. norvegicus* was removed for assessment of sex, carapace length, approximate moult stage and infection stage. Carapace length was measured from the rim of the eye socket to the posterior mid-point of the carapace. Moult stage was assigned as either intermoult (IM - no setal withdrawal in the pleopod and a rigid carapace) or recently moulted (RM – no setal withdrawal in the pleopod and a flexible 'paper' carapace) (see Aiken, 1980). *Hematodinium* infection diagnosis was carried out either in the laboratories

of the University Marine Biological Station Millport (UMBSM) or at the Division of Environmental & Evolutionary Biology, University of Glasgow, Scotland, UK. All sub-samples were stored in a cool, damp environment following capture and were generally alive for *Hematodinium* infection diagnosis. Pleopods were viewed under low-power light microscopy (x 40 magnification). The accumulation of agglutinated parasite and haemocyte material was used to assign the appropriate stage, whereby Stage 0 is apparently uninfected and Stages 1 to 4 are patently infected. The accuracy of the body colour and pleopod diagnosis methods was assessed by comparing the results from two different scorers (one naïve and one experienced). Both scorers assessed each lobster in a standard sub-sample for *Hematodinium* infection using the body colour method (loss of shell translucency, vivid coloration) and the pleopod method (as above). Results from the two scorers were compared at the end of the trial to assess the inter-operator sensitivity and reliability of the two methods.

Analysis of data.

Comparisons of the mean size of infected male and female lobsters and the mean size of uninfected and infected lobsters from the LC and BC sites were performed either by one-way analysis of variance (ANOVA) for normally distributed data, or by a Mann-Whitney test for non-normal distributions. Tests were considered significant if $p < 0.05$.

Results.

Body colour vs pleopod diagnosis.

By comparing the scores obtained by the experienced and naïve operators, it was possible to assess the sensitivity and reliability of the two major field diagnosis methods. In relation to the pleopod method, the body colour assessment underestimated infection prevalence by approximately 50 %, even when used by the experienced operator (Fig. 1a). Of those

infected lobsters thus misdiagnosed by the body colour method, the majority were of Stage 1 infection for both the naïve and experienced scorer. However, no heavily infected lobsters were missed by the colour diagnosis method by either operator (Fig. 1b). The pleopod scores show that the naïve operator estimated infection prevalence to be approximately 9 % higher than the experienced operator (Fig. 1a). Of these over-estimates, again the majority were lightly-infected (Stage 1) animals.

Hematodinium prevalence at the LC site.

Infection prevalence data obtained using the pleopod method at the LC site over three consecutive fishing seasons are shown in Figure 2a. During the summer and autumn (July to November), the prevalence of patent infection was minimal in both male and female lobsters (< 5%), with the initial increase occurring during December in each of the seasons studied. Prevalence of patent infection showed a marked increase during the period between January and March, and peaked in April or May. Overall infection prevalence reached a maximum of 20 - 25% in each of the three seasons studied, though prevalence in female lobsters was as high as 35% during the 1998 and 2000 seasons. However, the elevated level of infection prevalence in female lobsters had a lesser effect on the overall infection prevalence, due to the reduced proportion of female lobsters in the catch during the late winter and early spring periods (Fig. 2b). Following the peak (May to July), a sharp reduction in the prevalence of patently infected lobsters occurred.

Severity of patent Hematodinium infection.

As well as allowing *Hematodinium* infection prevalence to be assessed at the population level, the pleopod staging method also allows the severity of the infection in lobsters (on a scale of Stage 1 to Stage 4) to be assessed The mean infection severity measured in lobsters displaying patent infection, captured at the LC site over the three consecutive seasons is

shown in Fig. 2c. Over this period, all *Hematodinium* infected lobsters captured during the 'Low Season', i.e. outwith the main infection season of July - December, had very low parasite burdens, with a mean infection stage of 1.05 ± 0.03 (n=12). At the beginning of the main infection periods (February), the mean severity of infection in infected lobsters increased significantly to 2.04 ± 0.02 (n=3) ($p < 0.05$), and then to 2.59 ± 0.19 (n=3) ($p = 0.05$) (heavy *Hematodinium* parasite burden) by the end of the main infection season (May). These data indicate a progressive nature of patent infection over the main infection period.

Size of infected lobsters.

The monthly data for mean carapace size of lobsters captured at the LC site are given in Fig. 3. In addition to a reduction in mean carapace lengths which occurred during the autumn (September to October) of each season studied, the mean carapace length of male lobsters was significantly smaller in the 1999 season (29.2 ± 0.4 mm) than in the1998 season (32.3 ± 1.15 mm) ($p < 0.05$). However, no significant reduction in carapace length was seen in females over the same period (30.7 ± 1.4 mm in 1998; 27.8 ± 0.7 mm in 1999) ($p = 0.081$). In the 2000 season, the mean sizes of male and female lobsters (30.7 ± 0.5 and 28.2 ± 0.6 mm respectively) were not significantly different to those in the 1998 season ($p > 0.05$).

The mean carapace length of *Hematodinium*-infected female lobsters (28.1 mm ± 0.67) was significantly smaller than that of infected male lobsters (30.9 mm ± 0.50) captured from the LC site ($p < 0.001$). In order to test whether the mean carapace length of the *N. norvegicus* population at a particular site affects the prevalence of *Hematodinium* infection in that population, two geographically-close sites (LC and BC) were sampled on the same day in February 1999. Overall infection prevalence at the BC site was 15.5%, while at the

LC site, prevalence was 21.6%. The mean carapace length of uninfected lobsters captured at the BC site (37.5 ± 0.47 mm) was significantly greater than for lobsters at the LC site (28.6 ± 0.26 mm) ($p < 0.001$). Similarly, the mean carapace length of *Hematodinium*-infected lobsters captured at the BC site (36.2 ± 0.52 mm) was significantly greater than those from the LC site (28.1 ± 0.42mm) ($p < 0.001$). When the mean carapace lengths of uninfected and infected lobsters from the LC site were compared, it was found that the difference was not significant ($p = 0.239$). At the BC site, the mean carapace length of infected lobsters was also not significantly different from that of uninfected lobsters ($p = 0.066$). This suggests that the size distribution of infected lobsters captured at each site was not significantly different from the size distribution of uninfected lobsters captured at the same site (Fig. 4 a,b).

Hematodinium infection and the moulting period.

The relative proportion of recently moulted (RM) and intermoult (IM) *N. norvegicus* in the catch gives an indication of the main moulting period and allows an assessment to be made of the synchrony of moulting amongst individuals in the population. Moult data from the LC site are shown in Figure 5a. Large numbers of RM lobsters appeared in the catch during the spring and summer of each year, but the relative proportion of RM lobsters appeared to vary between seasons (with a particularly high proportion of RM females during the summer of 1998). The reduction in the proportion of RM lobsters in the catch during late summer seems to be due to the progression of moulted lobsters into the IM state as the season advances. The fact that there is a higher proportion of RM female lobsters than RM male lobsters each spring and summer is suggestive of more synchronous moulting period in females than males, even though RM lobsters of both sexes are found in the catch throughout the year.

By combining data for moult status and infection prevalence and comparing the prevalence level in RM and IM lobsters, it is possible to investigate the relationship between moulting the onset of disease patency in each sex. Figure 5b shows infection prevalence data for RM and IM female lobsters at the LC site. In the 1998 season, prevalence was initially high in IM female lobsters, and increased in RM female lobsters as the season progressed. In the 1999 season, IM prevalence also rose initially, though the prevalence level in RM females did not increase to the level seen in the 1998 season. Finally, in the 2000 season, the prevalence level in IM and RM female lobsters was similar for the whole season. In contrast, male lobsters displayed a similar pattern of IM and RM infection prevalence for each of the seasons studied (Fig. 5c), with RM infection prevalence being consistently higher than IM infection in the late season (April-May).

In some seasons, data for overall infection prevalence at the LC site (Fig. 2a) show an apparent 'plateau' of peak infection prevalence between February and April-May (e.g. the 1999 season). When data from the 1999 season for RM and IM female lobsters are compared to those of RM and IM male lobsters, it can be seen that infection prevalence peaks later in males than in females and that the plateau of infection prevalence described above is caused by an increase in infection prevalence in RM males (Fig. 2a and 5c). During other seasons (e.g. 2000), when male and female infection prevalence peaks occur at the same time, a sharper peak of infection prevalence occurs (see dashed lines between Fig. 5b and 5c).

Discussion.

The body colour vs pleopod diagnostic methods.

The body colour diagnostic method uses external features of infection (vivid colouration, opaque carapace) to estimate prevalence. It is rapid and can be carried out aboard research and fishing vessels. However, the current study has shown that it may underestimate

infection prevalence relative to that determined by the pleopod method, by approximately 50 %. No improvement in diagnostic accuracy occurred with experience, with both the naïve and experienced operators failing to diagnose large numbers of early-stage (Stage 1) infections. This suggests that while this method may be useful for the selection of heavily infected lobsters from the catch for pathological studies, the colour changes in lobsters with light infections are too minor to be used for accurate diagnosis. As such, the body colour method probably has little use in generating accurate data for modelling the proportion of natural mortality (*M*) attributable to *Hematodinium* infection (Field *et al.*, 1992).

The pleopod diagnosis method detected considerably more infected lobsters than the body colour method, but the higher prevalence estimate made by the naïve operator suggests that some subjectivity exists in this method, especially for the diagnosis of light infections. As the pleopod method determines the presence of *Hematodinium* parasites in the haemolymph, any other changes to the haemolymph which occur following capture are likely to affect diagnostic accuracy. In the hours following capture, colonization of the haemolymph by bacteria was often observed, making visual assessment of light infections more difficult. Sample freshness is therefore important, especially for the diagnosis of light infections. However, overall, the pleopod diagnostic method provides a reliable, rapid and relatively transferable tool for infection assessment and is therefore useful for preliminary studies in *N. norvegicus* fisheries where the presence of *Hematodinium* infection is unconfirmed.

Hematodinium infection epidemiology.

By use of the pleopod diagnostic method, the current study has highlighted the seasonal, epidemic nature of *Hematodinium* infection in a population of *N. norvegicus* contributing to the important commercial fishery in the Clyde Sea area and reinforces earlier reports of high *Hematodinium* infection prevalence at other sites on the west coast of Scotland (Field

et al., 1992, 1998). During the study period, three discrete seasonal episodes of *Hematodinium* infection occurred (in each case, during the spring), in which overall prevalence reached 20-25 %. Previous data from the Clyde Sea area have shown that peak infection prevalence can be as high as 70 % in certain years (Field *et al.*, 1992), highlighting the potential for considerable variation in the absolute level of infection prevalence between years.

In the present study, infection prevalence was highest in female lobsters (especially during the 1998 and 2000 infection seasons), and this may be understood in terms of the life history of the female. In *N. norvegicus*, the egg-rearing period may last for up to 8 months of the year, during which time the female lobster remains for the most part, within the burrow (Farmer, 1974a). Following spawning, the female lobster emerges from the burrow (reflected by the increase in the proportion of female lobsters in the catch during late spring in this study) to feed, moult and be mated by a hard-shelled male lobster (Farmer, 1974b). It has been proposed that mechanical disruption to the soft cuticle of the female lobster during copulation may lead to infection by motile spores of the *Hematodinium* parasite entering at the trauma site (Field *et al.*, 1992). However, although feasible, this would not explain transmission to male lobsters and such a route of transmission has not been demonstrated experimentally.

During periods of food scarcity (e.g. in the extended time period spent within the burrow during incubation), it has been suggested that lobsters may resort to suspension feeding in order to supplement their normal nutritional requirements (Loo *et al.*, 1993). In this way, it is possible that lobsters may ingest motile or encysted (see John & Reid, 1983) forms of the *Hematodinium* parasite which may exist in the sediment or in the burrow water. Similarly, the hermit crab (*Pagurus bernhardus*), which also harbours infections of *Hematodinium* on the Scottish *N. norvegicus* fishing grounds is known to resort to filter feeding when other food sources are scarce (Gerlach *et al.*, 1976). Ingested parasite forms may penetrate the

gut wall and lie dormant in tissues such as the hepatopancreas until development of patent infection at some point in the future (Appleton & Vickerman, 1998). It seems likely that ingestion of spores via suspension or filter feeding, or of vegetative forms and/or spores by predation (of hermit crabs) or cannibalism are the most likely routes of transmission to and from *N. norvegicus*. Predation on amphipods which are also known to harbour *Hematodinium* infections (Johnson, 1986) is also a possible route of transmission to *N. norvegicus* and *P. bernhardus*, though studies of *Hematodinium* infection in Scottish amphipod species have not been made.

The pleopod diagnosis method allows some assessment to be made of the absolute *Hematodinium* parasite burden in infected *N. norvegicus*. Field *et al.* (1992) showed that the majority of infected lobsters captured by trawling were of Stage 1 or 2 infection. However, the current study has shown that while mean infection severity in infected *N. norvegicus* is low during the summer months (mean approximately Stage 1), this increases as the infection season progresses (up to a mean of Stage 3). Progression of infection through the main season is suggestive of a slow incubation of the parasite in the haemolymph, and reinforces previous data which showed that the *Hematodinium* parasite burden increased by 100-fold over a period of 100 days in the haemolymph of aquarium-held infected lobsters (Appleton *et al.*, 1997). The slow incubation of patent *Hematodinium* infection in the field may increase the chance of parasite transmission via cannibalism or predation, and occurs concomitantly with the progressive utilization of host tissue and haemolymph storage products (Stentiford *et al.*, 1999 – Chapter 4; 2000a – Chapter 5). The final sporolation phase of the parasite, whereby parasite forms become motile and emerge from the host lobster (Appleton & Vickerman, 1998) probably occurs when all available host resources have been depleted (Stentiford *et al.* 2001b. in manuscript – Chapter 6) and may explain why sporolation of the parasite can occur in lobsters displaying only Stage 1 or 2 infection (Appleton & Vickerman, 1998).

Hematodinium infection and the moult cycle.

An association between patent *Hematodinium* infection and moulting has been described by a number of workers, though due to the inherent complexities of the moulting process, the details of this relationship remain unclear (Meyers *et al.*, 1987; Field *et al.*, 1992, 1998; Messick, 1994). By assessment of shell condition, the current study has shown that in some years an extended infection season is caused by a high infection prevalence in RM male lobsters during the late season (April-May). In other years, where infection prevalence rises and falls more abruptly, prevalence in RM and IM males and RM and IM females coincides, creating a sharper infection peak. It is probable that in seasons of very high peak infection prevalence (> 70%), the infection prevalences in male and female lobsters peak at the same time. Therefore, in terms of absolute natural mortality (*M*) attributable to *Hematodinium* infection, the length of the infection season may be as important as the absolute peak prevalence level

Parasitism in relation to host condition.

The general condition of the host may help to establish the degree to which parasitic infections are successful, with hosts in the best physiological condition harbouring the largest parasite burdens (Schmidt and Platzer, 1980). Female crustaceans have relatively larger amounts of hepatopancreatic tissue than males as a means of supplying some of the nutritional requirements for egg rearing and spawning (Farmer, 1974b). The association of moulting with patent *Hematodinium* infection in *N. norvegicus* may thus be directly related to the nutritional status of the host and the nutritional requirements of the parasite. In decapods, the highest concentration of hepatopancreatic storage products (e.g. lipids and glycogen) are found in the pre-moult period (Johnson, 1980). During the final stages of the pre-moult and in the post-moult periods, storage material is mobilized (from tissue to haemolymph) to sustain the animal over the non-feeding stages of the life-cycle (Icely &

Nott, 1992). Recently, it has been shown that the plasma concentration of the crustacean hyperglycaemic hormone (CHH), a hormone involved in carbohydrate homeostasis in the tissues and haemolymph of crustaceans (Santos & Keller, 1993), is increased by up to 100-fold in the hours leading up to moulting, with levels returning to normal following ecdysis (Chung *et al.*, 1999). In relation to this, a study of the carbohydrate and CHH dynamics of *Hematodinium*-infected lobsters has shown that the concentration of CHH is elevated significantly during sub-patent infection and that the increased concentration of CHH in the plasma at this time may create haemolymph conditions that are suitable for rapid growth of the parasite population (Stentiford *et al.* 2001b, in manuscript – Chapter 6). This may explain the coincidence of peak infection prevalence with the main moulting period for *N. norvegicus*. Additionally, due to larger volumes of these storage tissues, the higher incidence of *Hematodinium* infection in female lobsters may reflect some relative advantage of the female host to the parasite. This benefit may be enhanced in smaller lobsters, which contain relatively larger amounts of hepatopancreatic material per unit size, store larger amounts of reserve material for moulting (Heath & Barnes, 1970), and in the case of males, moult more frequently than larger lobsters (Sarda, 1995, González-Gurriarán *et al.*, 1998). All of these features are likely to make them a better resource for *Hematodinium* parasite growth and transmission. The consistently higher peak in RM infection prevalence in male lobsters also suggests that, in males at least, infection may be carried through the moult. As such, lobsters with latent and low-level haemolymph infections may survive the moult, with severe infections developing after the moult, while those entering the moult with severe infections may perish during ecdysis. Further research on this complex issue is required to determine the link between *Hematodinium* infection and the moult.

Hematodinium infection and the fishery for N. norvegicus.

A reduction in the mean carapace length of the *N. norvegicus* population on a particular fishing ground is one symptom of stock over-exploitation (Sarda, 1998). The data presented in this study suggest that the mean carapace length of lobsters has reduced at the LC site in the Clyde Sea area over the past 3 years. This reduction may be due to high recruitment at the same site over the same period (Marrs *et al.*, 2000). The significant difference in mean carapace length between the BC and LC sites also suggests that the population structure of *N. norvegicus* may differ between geographically-adjacent sites. While Field *et al.*, (1998) showed that the mean carapace lengths of *Hematodinium* infected *N. norvegicus* may differ between sites, they did not compare the mean size of the whole catch to the mean size of infected animals. The current study has shown that for two grounds with infected animals of different mean sizes, the mean size of infected lobsters was not significantly different to that of the whole sub-sample at either the LC or BC sites. However, the overall prevalence at the two sites was different, suggesting that population structure may play a part in the overall *Hematodinium* infection prevalence. Large populations of small, size-matched, rapidly growing individuals may lead to strong moulting synchrony amongst individual lobsters. As described above, it may be the populations with such characteristics that experience the highest *Hematodinium* infection prevalence each season. Further investigations on the effect of carapace size distributions on infection prevalence should be carried out to determine the importance of fishing pressure in the generation of population structures suitable for *Hematodinium* epidemics to occur.

Due to the severe pathological effects associated with advanced *Hematodinium* infections, the survival of infected lobsters under aquarium conditions (Field *et al.*, 1992, Field & Appleton, 1995) and the sporolation response which leads to the death of the host lobster (Appleton & Vickerman, 1998), it is unlikely that recovery from patent infections is

possible. As such, seasons of high infection prevalence have been associated with reductions in landing per unit effort (LPUE) and burrow density (Field *et al.*, 1998). Accurate prevalence estimates should allow a *Hematodinium* infection mortality factor (M_H) to be incorporated into natural mortality (M), with this factor being greater during seasons in which infection prevalence is highest. Previous attempts to incorporate the high observed prevalence of *Hematodinium* infection into analytical stock assessments of the Clyde Sea area modelled the effect as an additional loading on the natural mortality rate M (since animals showing symptoms of patent infection usually die) (Anon, 1997). However, the exercise was not entirely successful, leading, as it did, to unrealistic estimates of recruits entering the fishery.

Following this, the accuracy of infection prevalence estimates from trawl caught *N. norvegicus* samples has been questioned, due to the severely reduced escape swimming capacity (Stentiford *et al.*, 2000b – Chapter 8) and the increased out-of-burrow activity (Stentiford *et al.* 2001a, in manuscript – Chapter 10) of infected lobsters. Increased catchability of infected lobsters relative to their uninfected counterparts may lead to considerable overestimation of true prevalence on a particular fishing ground. Furthermore, if infected animals are likely to be more susceptible to predation through a reduced ability to escape, then the infection may not necessarily add to overall natural mortality, but rather replace a proportion of it. Taken together, the altered catchability of patently infected lobsters and the subjectivity in detecting light infections raise doubts about the accuracy of the pleopod method for estimating patent *Hematodinium* infection prevalence in natural populations, and about the usefulness of these data for estimating natural mortality due to *Hematodinium* infection. Development of sensitive molecular methods for the non-subjective detection of latent and sub-patently infected lobsters, which are less likely to show behavioural changes due to parasitism, should allow these issues to be resolved.

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Fig. 1A and B. (A) Percentage *Hematodinium* infection prevalence detected using the body colour and pleopod diagnostic methods by the naïve and experienced operators. **(B)** Number of lobsters diagnosed as infected by the pleopod method but not by the body colour method as a function of pleopod infection stage.

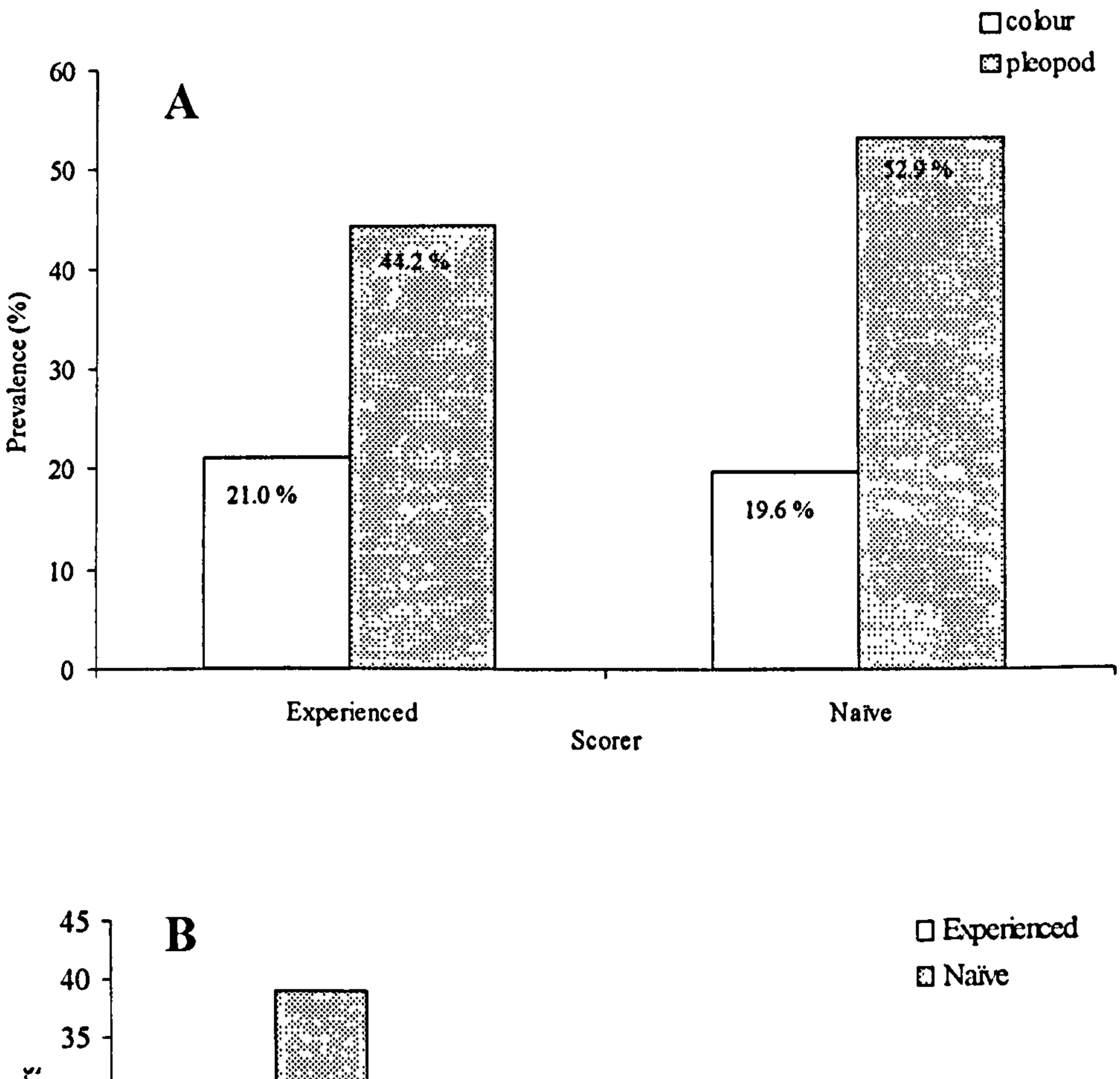
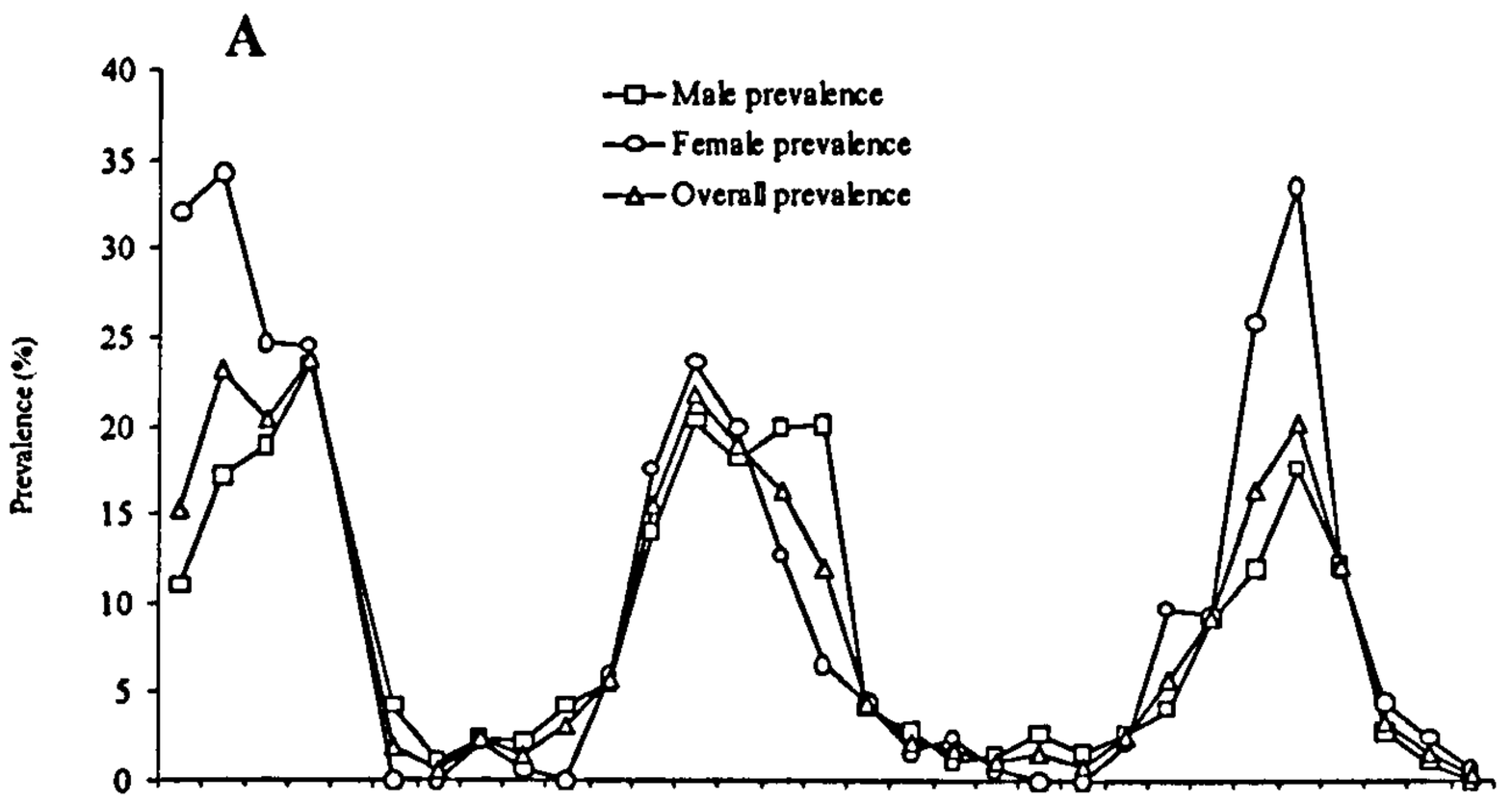
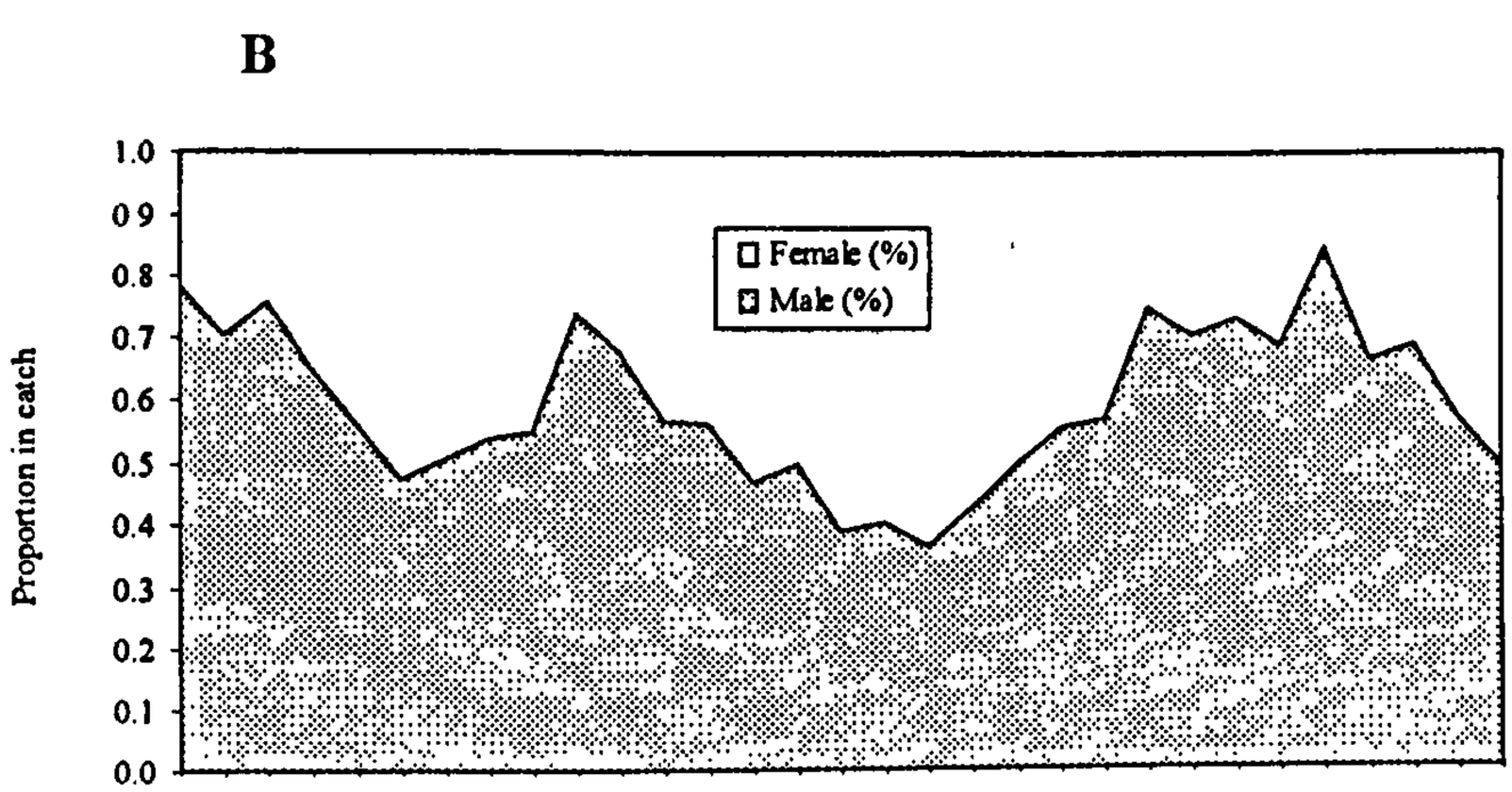


Fig. 2A, B and C. (A) Pleopod-derived *Hematodinium* infection prevalence, **(B)** proportion of males and females and (C) average pleopod-derived infection stage, in monthly samples of *N. norvegicus* captured from the Little Cumbrae (LC) site between February 1998 and September 2000.





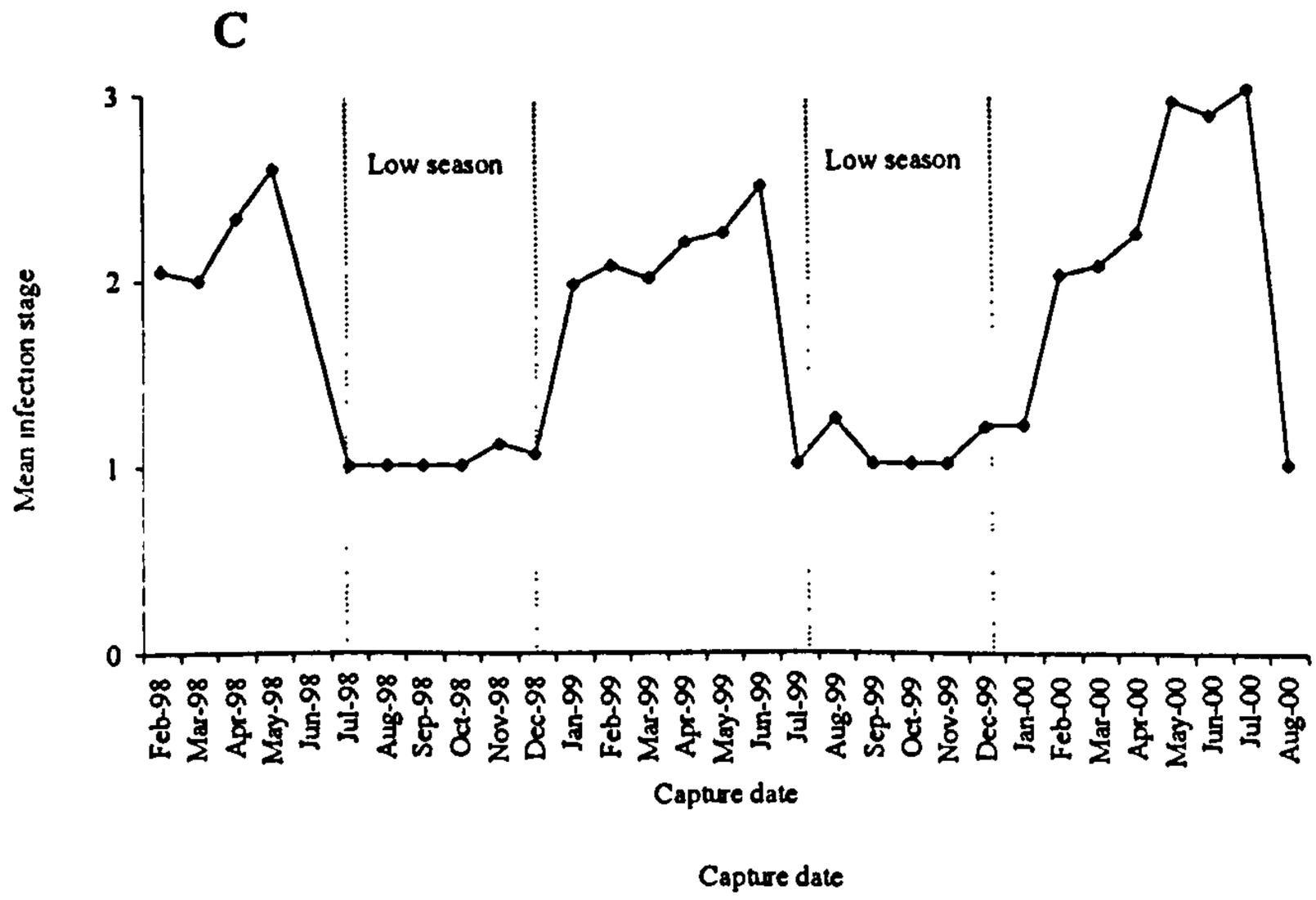


Fig. 3. Mean carapace length of monthly samples of *N. norvegicus* captured from the Little Cumbrae (LC) site between February 1998 and September 2000.

Fig. 4A and B. Carapace length frequency distributions of *N. norvegicus* from the Little Cumbrae **(A)** and Bute-Cumbrae channel **(B)** sites. Uninfected lobsters are shown as clear bars and infected lobsters as black bars.

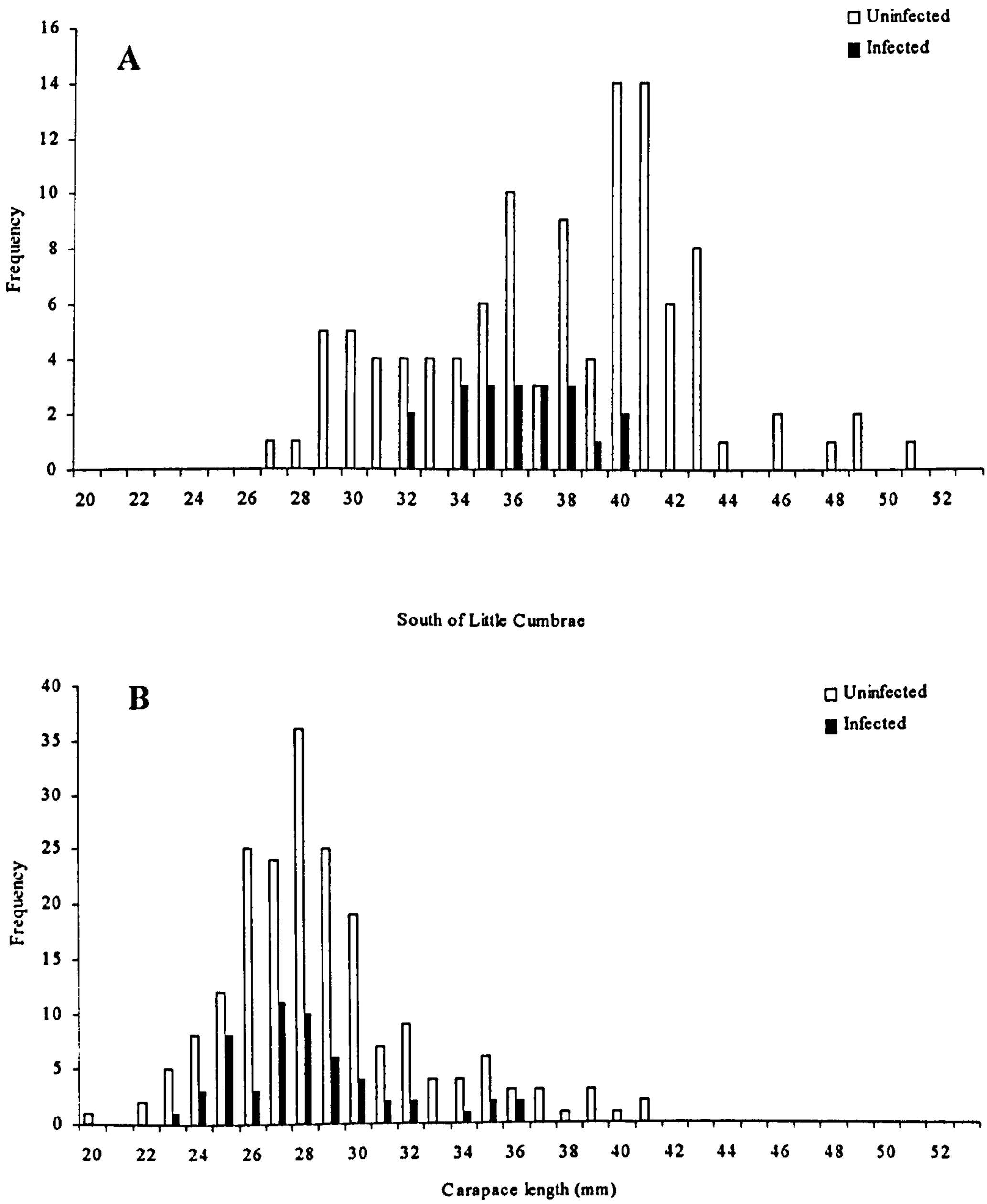
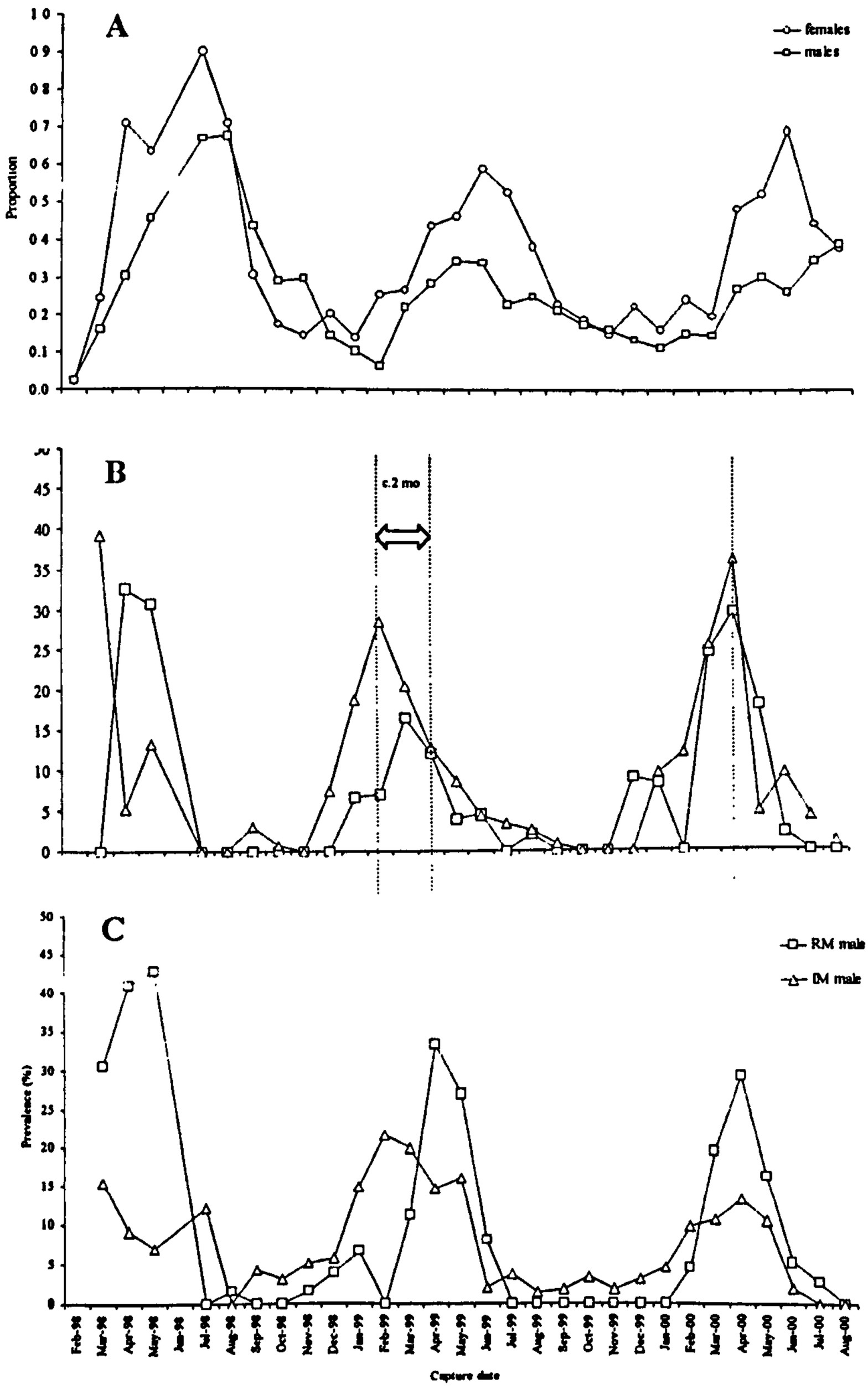


Fig. 5A, B and C. **(A)** Proportions of recently moulted (RM) and intermoult (IM) male and female *N. norvegicus*. (B) Infection prevalence in RM and IM female and (C) male lobsters at the Little Cumbrae site between March 1998 and September 2000. Dashed lines on figures B and C compare RM and IM infection prevalence seasons in female and male lobsters (see main text).



Chapter 3

Development and application of an immunoassay diagnostic technique for studying *Hematodinium* infections in *Nephrops norvegicus* populations.

Abstract

Patent *Hematodinium* infections of the Norway lobster, *Nephrops norvegicus* can be detected with a morphological method (the pleopod diagnosis), but this fails to identify low-level haemolymph (sub-patent) and tissue-based (latent) infections. The current study describes the development and application of an immunoassay for the detection of antigens of the parasite *Hematodinium* in the Norway lobster, *Nephrops norvegicus*. Infected tissue and haemolymph samples were detected as multiple-band reactions to a polyclonal antibody (anti- *Hematodinium*). By comparing haemolymph and tissues samples from the same lobsters, it was possible to distinguish latent from sub-patent infections. The sensitivity limit of the method was 204 parasites.mm^3 - approximately ten times more sensitive than the pleopod diagnosis method. Use of the immunoassay on tissue samples taken from catches taken in the Clyde Sea area, Scotland, U.K. showed that the pleopod method considerably under-diagnosed infection prevalence in the early part of the season, though this under-diagnosis decreased as infected lobsters in the field progressed from latent and sub-patent to patent infections. However, the immunoassay failed to detect any infected lobsters during the summer months, suggesting that infection may not be carried over from one season to the next. Data are discussed in relation to the epidemiology of latent and sub-patent *Hematodinium* infections, to the transmission of parasites to other hosts and to the use of this immunoassay for the routine assessment of *Hematodinium* infection prevalence in *N. norvegicus* populations.

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Introduction

The Norway lobster (*Nephrops norvegicus*) is the subject of an important fishery in the northeast Atlantic, with annual landings in excess of 60 000 t per annum making it one of the most valuable lobster resources in the world (Tuck *et al.*, 1997). Populations of *N. norvegicus* range from Iceland and northern Norway to the Mediterranean sea and the Moroccan coast (Figueiredo & Thomas, 1967). An infection of *N. norvegicus* by the parasitic dinoflagellate *Hematodinium* was first described in Scottish populations by Field *et al.* (1992), and since this initial description *Hematodinium* infection has also been reported from populations in the Irish sea (McAliskey & Briggs, 1997), the German bight (Dr F. Redant, personal communication) and the Swedish Skaggerak and Kattegat (Mr M. Ulmestrand, personal communication). However, it remains to be determined whether this infection extends to *N. norvegicus* populations at more southerly locations along the coastline of mainland Europe and in the Mediterranean sea.

External manifestations of *Hematodinium* infection of *N. norvegicus* include an opaque, vividly coloured carapace, general morbidity (Field & Appleton, 1995), a reduction in swimming performance (Stentiford *et al.* 2000a – Chapter 8) and altered burrow-related behaviour patterns (Stentiford *et al.* 2001a, in preparation – Chapter 10), while internal pathological effects include marked changes in host physiology (Taylor *et al.*, 1996) and biochemistry (Stentiford *et al.*, 1999 – Chapter 4, 2000b – Chapter 5). These pathological effects are similar to those caused by *Hematodinium* infections of other crustacean host species (Meyers *et al*, 1987; Hudson & Shields, 1994; Messick, 1994; Hudson, 1995; Love *et al.*, 1996; Shields & Squyars, 2000). A tentative life-cycle of the *N. norvegicus* isolate of *Hematodinium* has been described from *in vitro* cultivation and observations on aquarium-held lobsters (Appleton & Vickerman, 1998), although transmission to uninfected hosts has not been demonstrated to date. In addition, the seasonal nature of *Hematodinium* infection in *N. norvegicus* has been described in a number of studies (Field *et al.*, 1992, 1998) and

has recently been examined in detail for a single fishing ground in the Clyde sea area, Scotland, UK (Stentiford *et al.* 2001b, in preparation – Chapter 2).

A number of methods are available for the detection of *Hematodinium* infection in *N. norvegicus*. The simplest involves external assessment of the altered carapace colouration and opacity, which can be performed rapidly in the field. However, the insensitivity of this method, especially for detecting early infections, has recently been demonstrated (Stentiford *et al.*, 2001b, in preparation – Chapter 2). The method which has been used most routinely is the pleopod staging technique of Field & Appleton (1995), whereby a pleopod is removed and assessed for the presence of parasites and hemocytes using low-power microscopy. This method assigns an infection severity score based on a four point scale, which allows the progression of infection to be followed over its seasonal cycle (Field *et al.*, 1992, 1998; Stentiford *et al.*, 2001b, in preparation – Chapter 2) and has greatly facilitated laboratory studies on the pathological progression of infection (Stentiford *et al.*, 1999 – Chapter 4, 2000b – Chapter 5). It seemed probable, however, that the pleopod diagnosis method, which scores only patent infection, would fail to identify latent (tissue-based) and sub-patent (low level haemolymph parasitaemia) infections and so underestimate prevalence.

Modern diagnostic methods based on molecular technology have increased the sensitivity of detection for many infective agents. Immunoassays, which detect parasite antigens, utilize specific antibodies produced against parasite antigens. These can be applied in assays based on Western blotting or the immunofluorescence antibody technique (IFAT) and in enzyme-linked-immunosorbant-assays (ELISA). Such methods have facilitated the sensitive diagnosis of important pathogens of aquatic hosts. These include the detection of Taura syndrome virus (TSV) in penaeid shrimps (Poulos *et al.*, 1999), *Penaeus monodon*-type baculovirus (MBV) in *Penaeus monodon* (Hsu *et al.*, 2000), *Renibacterium salmoninarum* in Atlantic salmon (*Salmo salar*) (Griffiths *et al.*, 1996), *Sphaerospora*

dicentrarchi in the European sea bass (*Dicentrarchus labrax*) (Muñoz *et al.*, 2000) and *Cryptocaryon irritans* in barrimundi (*Lates calcarifer*) (Bryant *et al.*, 1999).

An IFAT, developed using antibodies raised against *in vitro* cultured *Hematodinium* parasites originally isolated from *N. norvegicus*, has revealed that some apparently uninfected *N. norvegicus* in fact harbour latent or sub-patent infections outside of the main infection season (Field & Appleton, 1996). However, the epidemiology of these low-level infections, and their progression towards patent infections (detectable using the pleopod method), were not undertaken. The current study aimed to develop the IFAT technique of Field & Appleton (1996) into an immunoblotting assay, and apply the assay developed to analyze infection in a major Scottish *N. norvegicus* fishery over a 12 month period. Hepatopancreatic tissue was chosen as the study tissue as this has been suggested as a likely location of latent *Hematodinium* infection in *N. norvegicus* (Field & Appleton, 1996) and is well perfused with haemolymph, and so allows detection of sub-patent as well as latent infections. The data obtained are discussed in relation to *Hematodinium* infection epidemiology in natural populations, the non-subjective monitoring of *Hematodinium* infection in other European populations, and the provision of accurate data on infection-associated mortality for use in stock assessment models.

Methods.

Capture of lobsters.

Norway lobsters (*Nephrops norvegicus*) were caught using 90 min tows of a standard otter-trawl (70 mm mesh size) from a depth of approximately 80 m, at a location immediately south of Little Cumbrae (LC) in the Clyde sea area (55.41°N, 4.56°W). The start of the towing period was always between 0900 h and 1000 h to minimise any effect of differential burrow emergence behaviour on catch composition (Atkinson & Naylor, 1976) and the effect of capture time on infection prevalence (Field *et al.*, 1998). Monthly samples

were taken between October 1999 and September 2000 (inclusive) for the assessment of *Hematodinium* infection prevalence using the pleopod diagnosis method of Field & Appleton (1995). Bi-monthly samples of lobster tissue were also taken from sub-samples of the catch over the same period and tested for *Hematodinium* infection using an immunoassay based on Western blotting for parasite antigens as detailed below. The animals used were stored alive in a cool, damp environment (usually on ice) following capture for transfer to the laboratory for analysis. Pleopods were viewed using low power light microscopy (x 40 magnification). The accumulation of agglutinated parasite and haemocyte material was used to assign the infection stage according to Field & Appleton (1995). Stage 0 is apparently uninfected and Stages 1 to 4 are stages of increasingly severe patent infection.

Tissue preparation and immunoassay conditions.

Tissue samples from a standard subset of 60 lobsters (30 each of male and female), diagnosed as Stage 0 by the pleopod method, were prepared for immunoassay diagnosis. Small blocks (c. 2 mg) of hepatopancreatic tissue were excised, placed into 200 µl SDS-sample buffer (62.5 mM Tris-Hcl pH 6.8, 12.5 % glycerol, 1.25 % β-mercaptoethanol) and immediately heated in a water bath at 95-100°C for 4 min. The denatured samples were then frozen at –20°C until analysis (usually within 1 week).

Samples were defrosted immediately before analysis and 10 µl aliquots were subjected to discontinuous SDS-PAGE, performed according to the method of Laemmli (1970) with a 12.5 % acrylamide separating gel and a 4 % acrylamide stacking gel. Electrophoresis running conditions were as described by Neil *et al.* (1993). Following separation of proteins, gels were transferred to a nitrocellulose membrane (Amersham Life Science) in a buffer of 20 mM Tris, 150 mM glycine and 20 % (v/v) methanol at 100 V for 45 min

(BioRad mini-transblotter). Membranes were blocked overnight at 4°C in 5 % (w/v) milk powder in Tris-buffered saline (TBS) (20 mM Tris base pH 7.6, 137 mM sodium chloride, 3.8 % (v/v) HCl with 0.1 % Tween 20 and 0.1 % gelatin). After blocking, membranes were rinsed in TBS (pH 7.6) with 0.1 % Tween 20 and 0.1 % gelatin, following which the primary rabbit antibody (anti-*Hematodinium* lysate – for production protocol see Field & Appleton, 1996) was applied for 4 h at a dilution of 1/1000 in TBS (as above). Blots were then washed at room temperature five times for 10 min each, with vigorous shaking. The secondary antibody (anti-rabbit-HRP conjugate) was then applied at a concentration of 1/1000 in TBS (as above) for 1 h at room temperature and developed using chemi-luminescence reagents (Pierce Co.) for 5 min and standard developer and fixer (Kodak Co.) onto ECL film (Amersham Life Science). Known *Hematodinium*-positive and uninfected haemolymph samples were used as controls. *Hematodinium*-positive samples were recognised by the presence of multiple-bands or smears due to reaction of numerous parasite proteins with the polyclonal primary antibody. The immunoassay-derived estimate of sub-patent/latent (SPL) infection was used to re-calculate the pleopod-derived prevalence estimate for *Hematodinium* infection in *N. norvegicus*, of both sexes, in the original sub-samples.

To test the detection sensitivity of the western blotting method, *Hematodinium* parasite cell density in the haemolymph of an infected lobster was determined microscopically. Haemolymph was withdrawn from the base of the fifth pereiopod and then transferred into an Improved Neubauer counting chamber viewed with a digital video camera (JVC TK-C1381) through a standard light microscope. Digital images were captured using Q-Video ImageLab™ software and a count of parasite cells was made later according to the method of Baker *et al.* (1966). Samples of the same haemolymph were diluted to 10 % in SDS-sample buffer, serially two-fold diluted, and subjected to SDS-PAGE and Western blotting

as described above. The sensitivity of the method was defined as the minimal number of parasites that could be detected by the Western blotting technique.

To determine whether the Western blotting method could be used to distinguish between latent (parasites only in tissues) and sub-patent (low level haemolymph parasitaemia) *Hematodinium* infections, haemolymph and hepatopancreas samples from a small number of animals scored as Stage 0 by the pleopod method were run alongside one another. Detection of antigens in the hepatopancreas material only was taken to indicate a latent infection, whereas detection of antigens in the hepatopancreas and in the haemolymph was taken to indicate a sub-patent infection.

Results.

Severity of infection through the year.

As well as allowing *Hematodinium* infection prevalence to be assessed at the population level, the pleopod staging method allows assessment of infection severity within individual lobsters (Stages of infection 1 to 4). The data presented in Figure 1 show the percentage of infected lobsters at the different stages of infection over the whole study period. In the early season (October and November), all infected lobsters captured displayed only very light infections (Stage 1). However, as the season progressed an increasing proportion of the infected lobsters captured were more severely infected (Stages 2 and 3-4) so that by June and July all infected lobsters captured were severely infected. These data suggest that *Hematodinium* infection severity is progressive in individual lobsters. Peak infection prevalence occurred in April.

Sensitivity of the Western blot method.

The use of serial dilutions of infected haemolymph, containing a known density of parasites, allowed the sensitivity of the immunoassay to be determined. The sensitivity

limit was 204 parasites.mm^3 of haemolymph (lane D, Fig. 2). When a greater number of parasites were present, numerous bands were detected with the polyclonal anti-*Hematodinium* antibody (lanes B and C), but with fewer parasites no bands were detected. There was also reaction to the 66 kDa protein (bovine serum albumin) and two other proteins (molecular masses of > 200 kDa and c. 97 kDa) in the molecular weight (M_r) marker protein mixture (lane A).

By applying the same method to excised hepatopancreas samples from field caught lobsters, previously diagnosed as Stage 0 by the pleopod method, sub-patent and/or latent infections were detected. Here, infected samples appeared as heavy, multiple-band reactions (see lobster sample numbers. 29, 33, 36, 43 and 44 in Fig. 3), while samples of apparently uninfected lobsters showed no reaction to the anti-*Hematodinium* antibody.

Incorporation of immunoassay data into pleopod prevalence analysis.

Immunoassay-derived infection prevalence data for SPL infections were used to re-calculate pleopod-derived infection prevalence estimates for the corresponding month. The immunoassay allowed for much earlier detection of infection in female lobsters (October) than was possible with the pleopod method (first detected in December) (Fig. 4A). By December, the immunoassay-derived estimates showed that infection prevalence was dramatically greater (over 25 %) than the pleopod analysis had suggested (less than 5 %). A similar situation was revealed with the February samples. By April ('peak season' from pleopod diagnosis - > 30 % infection), there were fewer SPL infections. By June, pleopod prevalence had reduced to approximately 5 %, and there were no SPL infections. This suggests that following the peak of patent infection in April, there were either no new *Hematodinium* infections or newly-infected animals had parasite burdens below the limit of detection with the immunoassay method (Fig. 4A).

A similar pattern in the immunoassay and pleopod-derived infection prevalence estimates was also seen for male lobsters (Fig. 4B), though patent infections appeared earlier (October) and overall prevalence at the peak of infection (April) was approximately half that of females.

When the data for immunoassay and pleopod-derived infection prevalence were plotted separately, it was possible to examine the epidemiological nature of SPL and patent *Hematodinium* infection in *N. norvegicus*. In females, the SPL prevalence rose to a peak in February and then began to fall (suggesting that all animals showing patent infection within a given season were infected by February). Following this peak, SPL infections advanced to a patent state, with this peaking in April. As shown in Figures 4A and 4B, when patent infection prevalence was at its highest (April) the prevalence of SPL infection had reduced to very low levels. The decline in SPL infection to zero in June suggests that infections may not have been carried over from one season to the next, but were instead acquired at some point after late summer (shown by the low level of sub-patent infection in October) (Fig. 5A). SPL and patent infection in male lobsters showed a similar epidemiological pattern, though here, the overall prevalence was considerably lower than in female lobsters (Fig. 5B). The time period between a given level of SPL infection prevalence and the same level of patent infection prevalence (shown by the 'phase-shifted' nature of the prevalence peaks), may indicate the approximate incubation time of the *Hematodinium* parasite from the SPL to patent state. Data presented here suggest that this time period may be between 2 and 4 months. It is important to note that the prevalence of SPL infection was approximately equal to the prevalence of patent infection that followed. This suggests that lobsters carrying a sub-patent or latent infection will display the symptoms of patent infection within the same season (Fig. 5a and 5b).

Discriminating between latent and sub-patent infections.

Detection of parasite antigens in the tissue and not in the haemolymph is suggestive of a latent *Hematodinium* infection, while presence of antigens within the tissues and haemolymph of Stage 0 animals (by the pleopod method) is suggestive of a sub-patent infection. By assaying the haemolymph and hepatopancreas of Stage 0 animals, it was shown that the Western blot method was able to discriminate between latently (lanes D^1 and D^2, Fig. 6) and sub-patently (lanes B^1 and B^2, and C^1 and C^2, Fig. 6) infected lobsters. It was found that there were differences in the degree of antigen reactivity in the haemolymph of sub-patently infected lobsters (compare lanes B^2 and C^2, Fig. 6). This is consistent with there being progressive colonization of the haemolymph by the parasites subsequent to initial invasion from the tissues.

Discussion.

In the current study, further development of the IFAT described by Field & Appleton (1996) into a multi-sample immunoblotting assay has allowed for the detection of SPL *Hematodinium* infections in field-caught samples of *N. norvegicus*. The Western blotting assay detected parasite densities of 204 parasites.mm^3 and above. This method is therefore approximately ten times more sensitive than the pleopod diagnostic method, in which Stage 1 infected animals have burdens of over 2000 parasites.mm^3 (see Field *et al.*, 1992: Field & Appleton, 1995). The technique worked well for both diluted haemolymph and for tissue samples, with there being no reaction of the polyclonal antibody to *N. norvegicus* material. Reactions between the anti-*Hematodinium* antibody and the proteins in the M_r marker probably reflect the presence of antibody against components of the parasite's culture medium (which included fetal calf serum) which would have been present on the surface of injected *Hematodinium* parasites during the initial production of the antibody (see Field & Appleton, 1996).

Latent *Hematodinium* infections of *N. norvegicus* are thought to involve parasites residing in tissues such as the hepatopancreas and muscle (see Appleton & Vickerman, 1998), while in low-level sub-patent infections parasites are also present in the haemolymph (Field & Appleton, 1996). As the main purpose of the immunoassay in the current studies was to detect all infections (latent and sub-patent) not detectable by the pleopod staging method, hepatopancreas samples were used. Immunoblotting of these did not distinguish between latent and sub-patent infections. Hence no distinction was made between sub-patent and latent infection states for immunoassay-derived prevalence calculations.

The data showed that the pleopod diagnostic method considerably under-estimates the prevalence of *Hematodinium* infection during the early season, but also that the degree of under-estimation is reduced as the main infection season (February to April) progresses. This reduction is likely to be due to the development of latent and sub-patent and latent infections into pleopod-detectable patent infections. The similarity in the amplitude of the SPL (immunoassay-derived) and patent (pleopod-derived) infection peaks suggests that all animals with a SPL infection within a given season will also express a patent infection within the same season. In addition, data for average patent infection severity (derived from monthly pleopod diagnosis) showed that a steady increase in infection severity occurred as the season progressed. As such, most infected animals captured at the end of the season had considerably larger parasite burdens than those captured at the start of the season. Data for infection severity progression is suggestive of a relatively slow incubation of the parasite in the haemolymph, and reinforces previous data which showed that the *Hematodinium* parasite burden increased by 100-fold over a period of 100 days in the haemolymph of aquarium-held infected lobsters (Appleton *et al.*, 1997). The slow incubation of patent *Hematodinium* infection in the field may increase the chance of parasite transmission via cannibalism or predation. The increase in parasite number

correlates well with the progressive utilization of host tissue and haemolymph storage products (Stentiford *et al.*, 1999 – Chapter 4, 2000b – Chapter 5).

Most studies on *Hematodinium* infections of decapod crustaceans have proposed a long, asymptomatic latency, followed by a shorter period of parasite proliferation and spore release (Hudson & Shields, 1994). Colonization of a new host may either occur shortly after ecdysis or parasites may remain in the host through the moult (Shields, 1992). If infection is acquired within one season and remains in a latent state until the following season, it should be possible to detect the same level of latent infection prevalence out of season as patent infection in the following season. In the current study, the increasing SPL prevalence up to February, suggests that either acquisition of infection occurs within the same season that full patency develops, or that the immunoblotting technique failed to diagnose very low burdens of parasites outside of the main infection season. Similarly, the reduction in the prevalence of sub-patent infections to zero in June suggests that either Stage 0 animals at this time are completely free from infection, or that very low-level latent infections are undetectable using this assay. The comparison of tissue and haemolymph samples from the same lobster showed that latent infections could be distinguished from sub-patent infections using the immunoblotting technique. However, it may be that the parasite burden within the tissues of latently infected lobsters also differs significantly between individuals and over time. Lobsters infected with very low tissue-based parasite burdens (below 204 parasites.mm^3) would not be detectable with the current immunoassay. Alternatively, upon initial infection, the *Hematodinium* parasite may reside in different tissues or organs to those tested (see Field & Appleton, 1995, 1996).

If acquisition of infection does occur within the same season as patent infection is expressed, transmission is unlikely to be occurring via lesions in the cuticle of newly moulted lobsters as the main moulting season for *N. norvegicus* is during the spring and early summer (Farmer, 1974). In this case, acquisition of parasites is more likely to be

occurring by the direct ingestion of vegetative stages or spores of the *Hematodinium* parasite (Appleton & Vickerman, 1998; Stentiford et al., 2001b, in manuscript – Chapter 2). Sensitive diagnosis of *Hematodinium* infection by the use of the polymerase chain reaction (PCR) has been demonstrated (Hudson & Adlard, 1994, 1996). However, no follow-up studies over longer periods have been carried out. Utilization of a sensitive PCR method for the detection of very low parasite burdens is required if issues relating to the latency and transmission of the *Hematodinium* parasite in *N. norvegicus* are to be resolved.

In order to make reliable comparisons of *Hematodinium* infection prevalence between different European *N. norvegicus* fisheries, the diagnostic method must be standardized and reproducible in the hands of different operators. While the pleopod diagnostic method has some merit for estimating *Hematodinium*-associated mortality in *N. norvegicus*. the insensitivity of the method, in addition to the subjectivity of assessment between different scorers (Stentiford *et al.*, 2001b, in preparation – Chapter 2), suggests that the pre-season measurement of SPL infections (by immunoassay) may provide an alternative means of estimating *Hematodinium* infection prevalence in the field. Additionally, due to the phase-shifted nature of the immunoassay-derived prevalence peak from the pleopod-derived prevalence peak (by a period of 2-4 months), it is likely that immunoassay-derived prevalence data could be used as an early warning signal for the level of patent infection level that will appear in the fishery. As such, the immunoblotting technique described here has furthered our knowledge on the epidemiology of *Hematodinium* infection in *N. norvegicus* and the development of this into a rapid, non-subjective enzyme-linked-immunosorbant-assay (ELISA) is being pursued.

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Fig. 1. Percentages of *Hematodinium*-infected *N. norvegicus* at each infection stage, as judged by the pleopod staging method, in monthly samples from the Little Cumbrae site between October 1999 and September 2000. Overall monthly infection prevalence (%) in the catch is given above each bar.

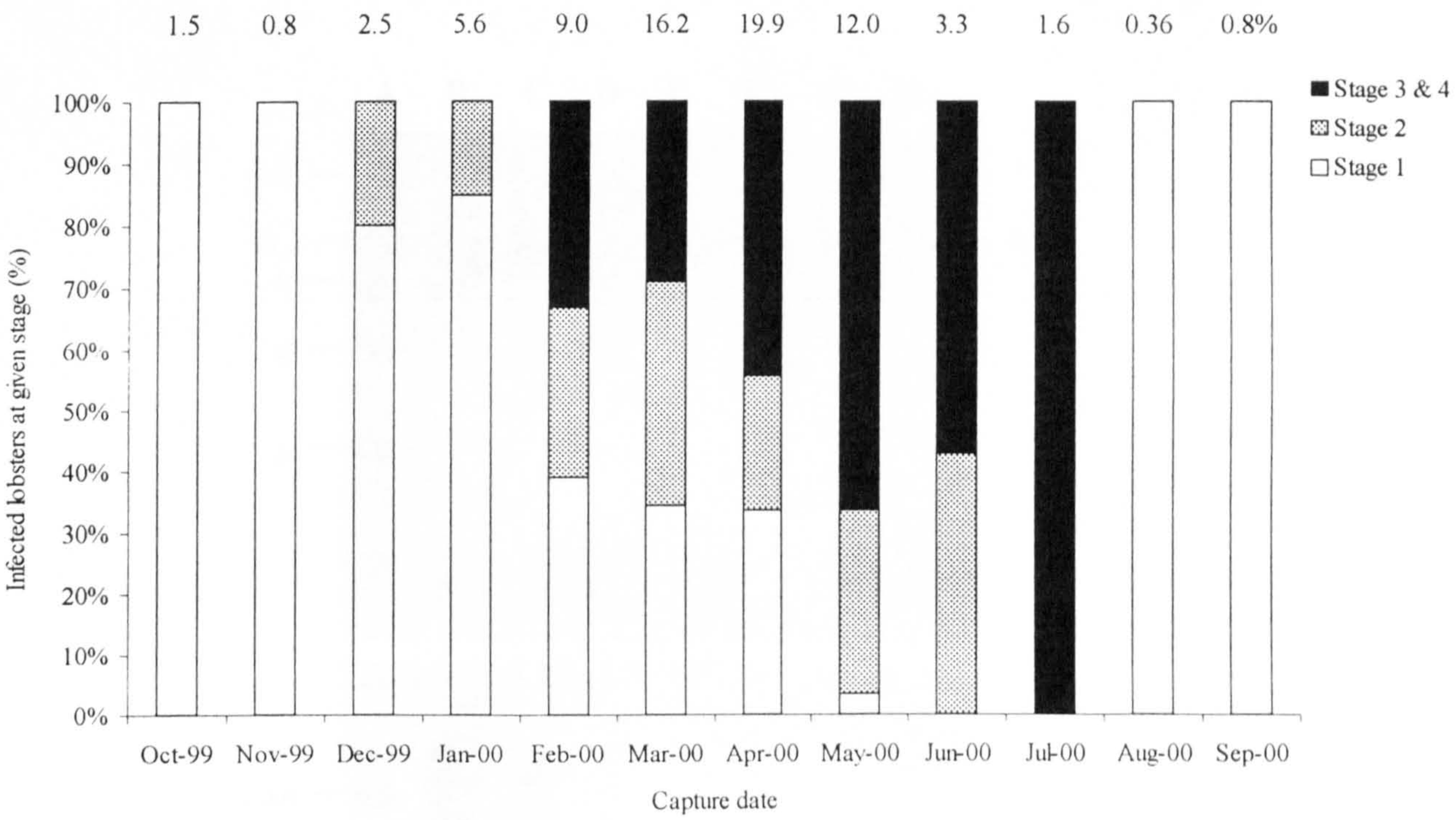


Fig. 2. (Top) 12.5 % SDS-PAGE gel of serially two-fold diluted-plasma from a *Hematodinium*-infected *N. norvegicus*. (Bottom) Western blot of the same serially-diluted infected plasma showing reaction of the separated proteins with anti-*Hematodinium* primary antibody in lanes B (815 parasites.mm^3), C (408 parasites.mm^3) and D (204 parasites.mm^3). Lack of reaction in lanes E to H (< 204 parasites.mm^3) show that parasite proteins present were at concentrations below that detectable with the assay procedure. Note the reaction of the primary antibody proteins in the molecular weight marker in lane A. Numbers on left of gel refer to approximate relative molecular weight. Hcy = plasma haemocyanin sub-units.

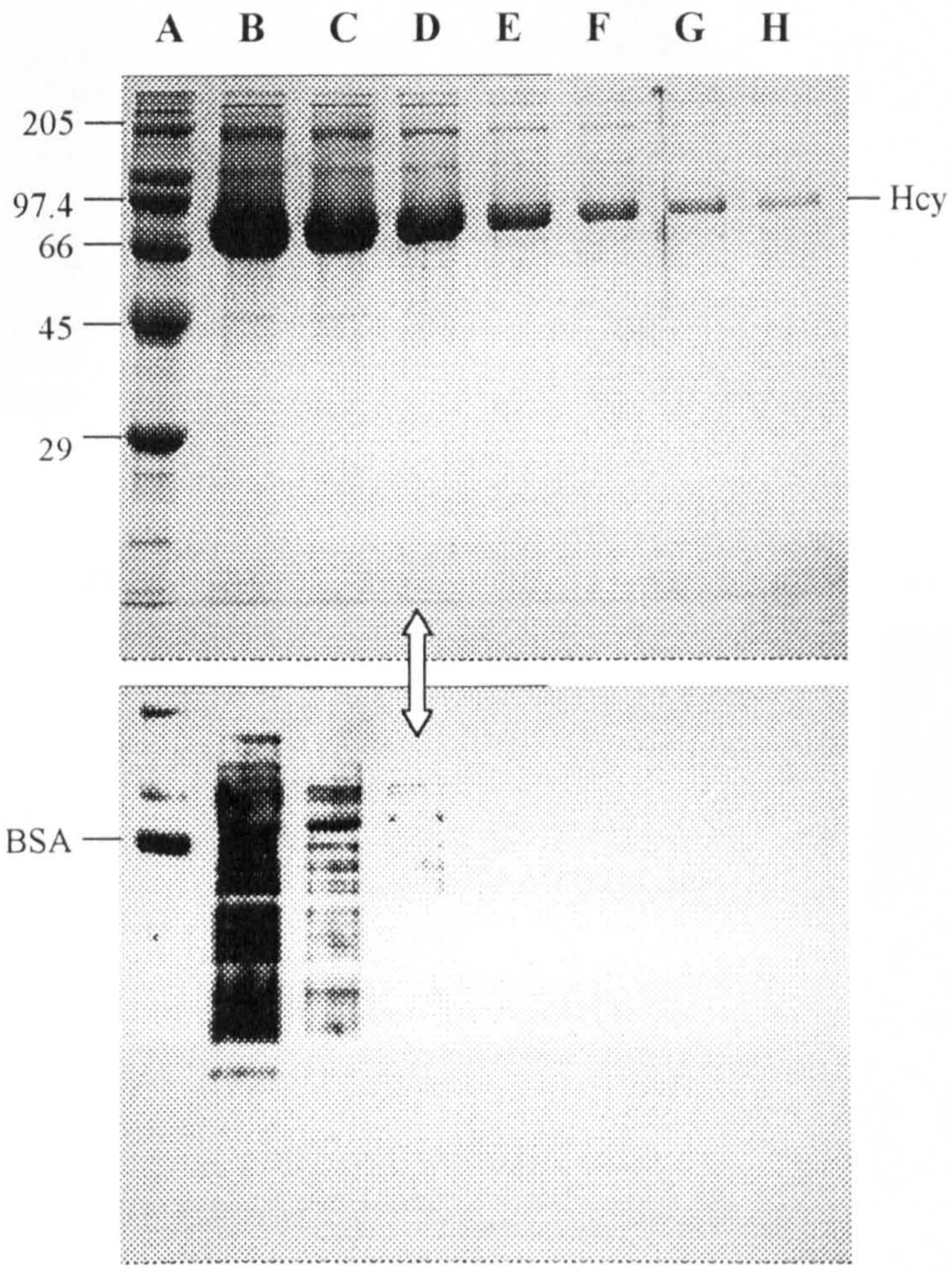


Fig. 3. Western blot of hepatopancreas samples from a typical sub-sample of pleopod-diagnosed Stage 0 lobsters. Note the multi-band or smear reaction of parasite proteins to the primary antibody in lanes 29, 33, 36, 43 and 44. Reaction of the primary antibody to proteins in the molecular weight marker mixture (HMW) also occurred (see also Fig. 2).

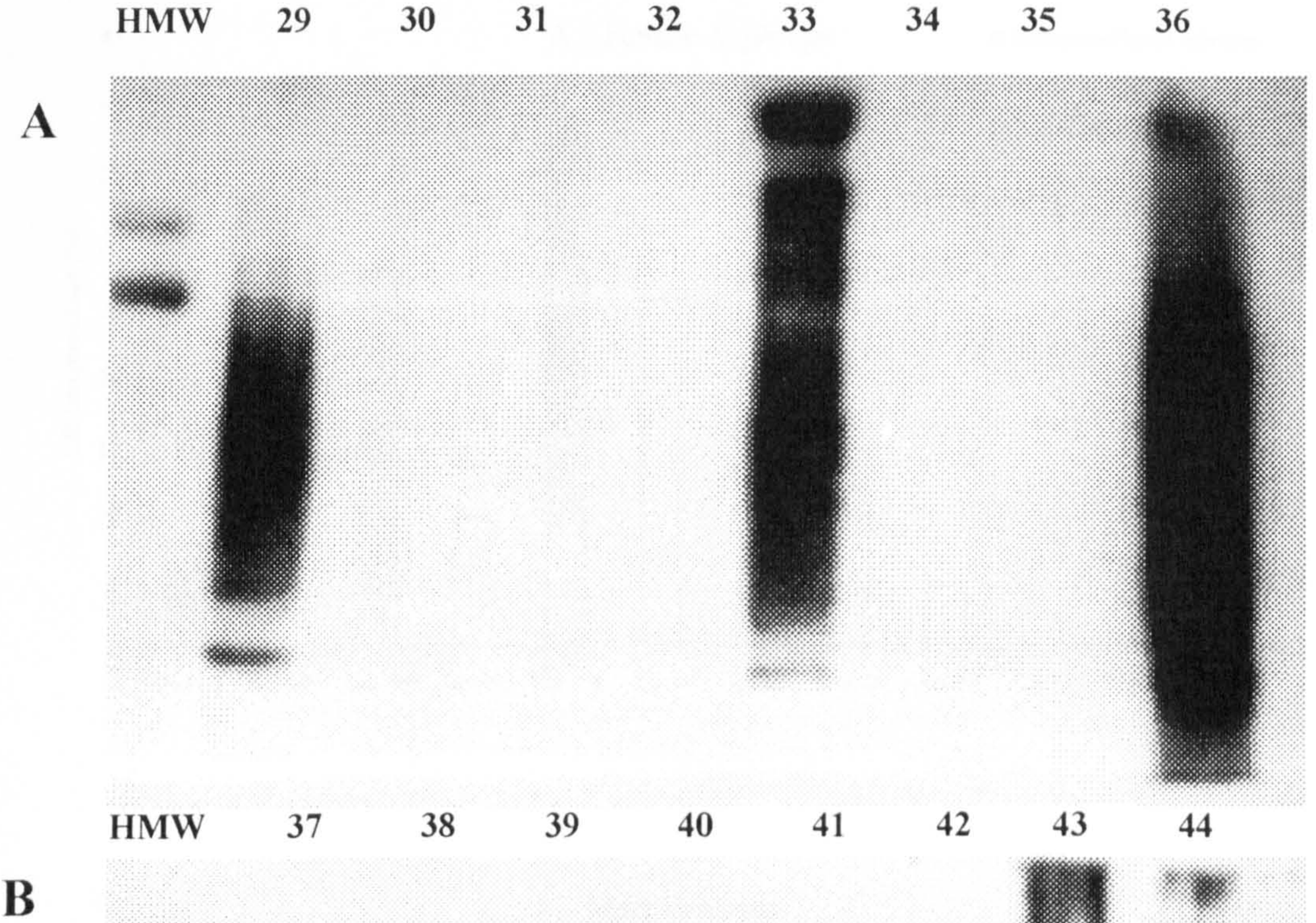


Fig. 4. Immunoassay-corrected *Hematodinium* infection prevalence estimates for female **(A)** and male **(B)** *N. norvegicus* from the Little Cumbrae site. Immunoassay data (grey bars) were collected bi-monthly (in October and December 1999 and February, April, June and August 2000). Pleopod data (white bars) were collected monthly.

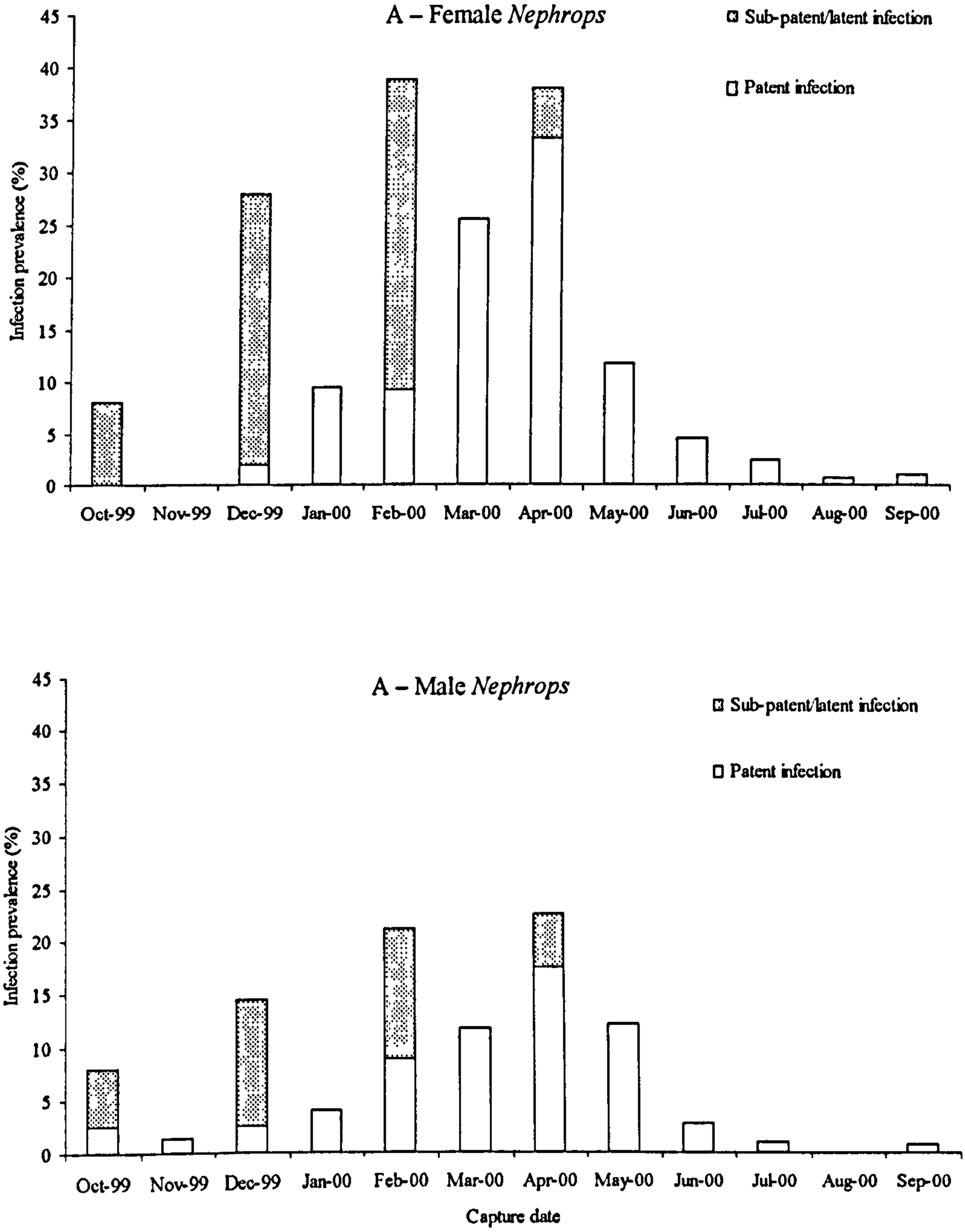


Fig. 5. Immunoassay-derived (sub-patent/latent) and pleopod-derived (patent) *Hematodinium* infection prevalence for female **(A)** and male **(B)** *N. norvegicus*. Immunoassay samples were taken bi-monthly (see Fig. 4A and B). Note the phase-shifted nature of the immunoassay-derived and pleopod-derived prevalence curves and the higher level of infection prevalence in female lobsters.

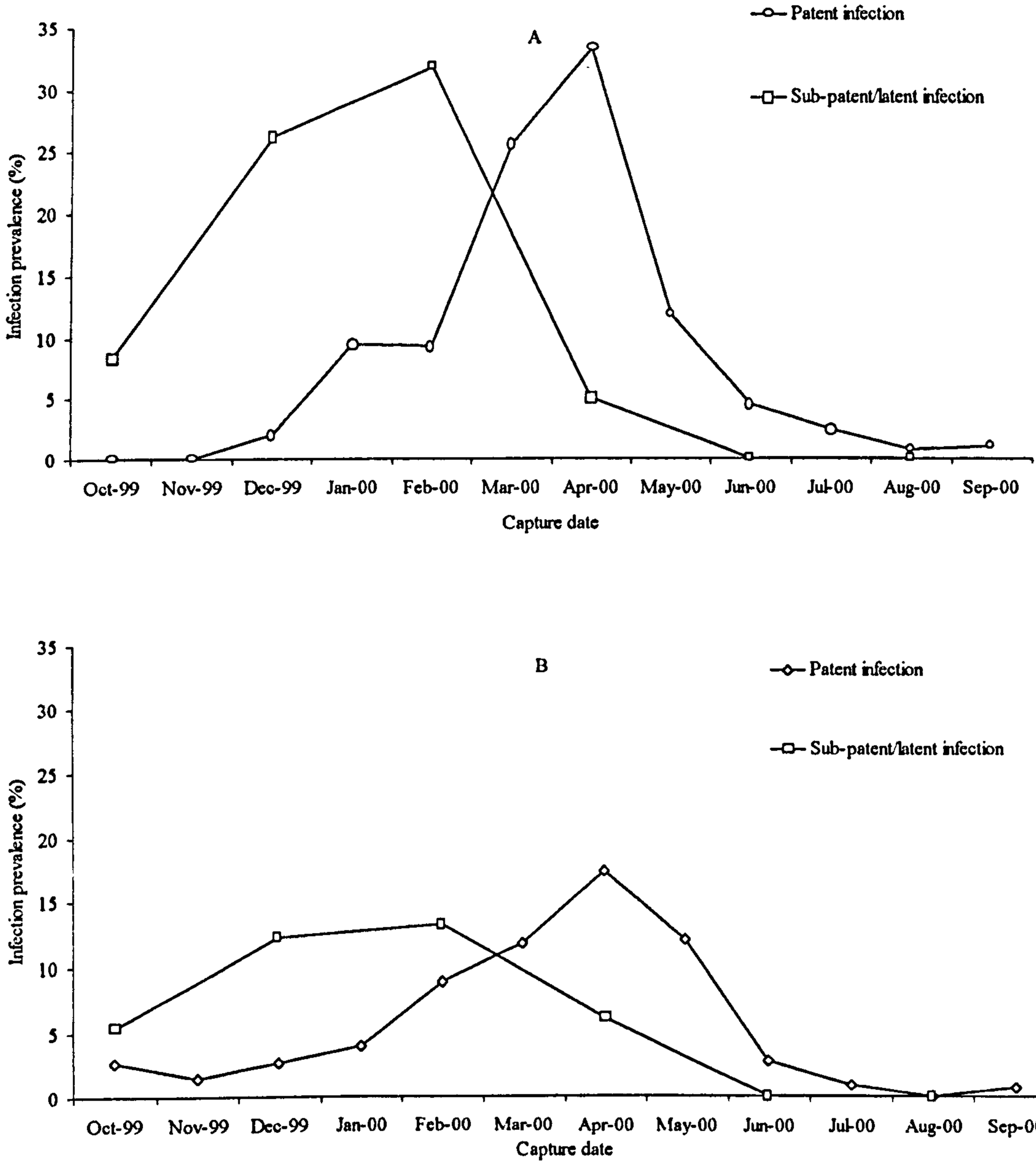
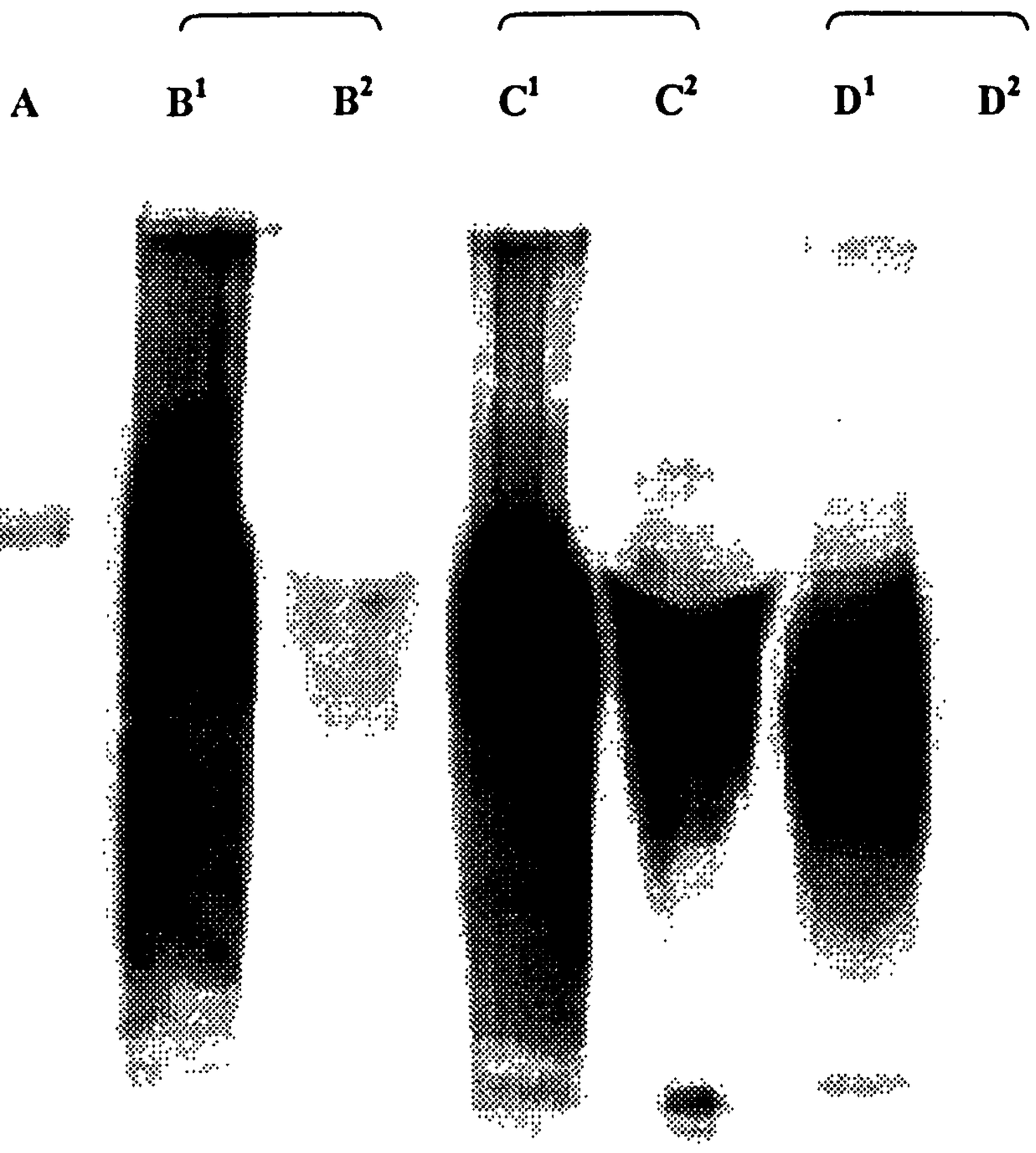


Fig. 6. Western blot of hepatopancreas (lanes B^1, C^1 and D^1) and haemolymph (lanes B^2, C^2 and D^2) samples from lobsters diagnosed as Stage 0 by the pleopod method. Note the detection of parasite material in the haemolymph and hepatopancreas in samples B and C (suggesting sub-patent infections) and the detection of parasite material in only the hepatopancreas of sample D (suggesting a latent infection). Lane A = reaction of relative molecular weight marker proteins to the primary antibody (see Fig. 2).



Chapter 4

Changes in the plasma free amino acid profile of the Norway Lobster, *Nephrops norvegicus* at different stages of infection by a parasitic dinoflagellate (Genus *Hematodinium*).

Abstract.

The haemolymph free amino acid (FAA) composition of Norway lobsters, *Nephrops norvegicus*, from the Clyde estuary, Scotland UK, at different stages of infection by the parasitic dinoflagellate *Hematodinium* sp. was determined by reverse phase High performance liquid chromatography (HPLC). In uninfected animals the total detected haemolymph FAA concentration was 3.79 $\mu mol.ml^{-1}$. Collectively, glycine, histidine/glutamine, alanine, serine, taurine and asparagine constituting 80% of the total. The concentrations of several FAAs, notably serine, were reduced in early infections (Stage 1), while at later Stages (2-4) several FAAs were increased in concentration. The most significant change was in taurine, which was increased by 13 fold (from 0.22 to 2.56 $\mu mol\ ml^{-1}$) in the final infection stage, when its relative contribution to the total FAA was 41.6%. Possible causes of these changes in the lobster haemolymph FAAs are the breakdown of host tissues including the hepatopancreas and muscle, haemocyte lysis, a host stress response and release of FAAs from the parasite cells. These results have implications for the biology of *Nephrops*, and indicate that the taurine:serine ratio in the haemolymph provides a sensitive diagnostic measure of patent *Hematodinium* infections.

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Introduction.

One consequence of some parasitic infections of invertebrates is change to the free amino acid (FAA) profile in the plasma. This occurs in oysters infected by *Bucephalus* sp. and *Minchinia nelsoni* (Feng *et al.*, 1970), honey bees infected by the microsporidian *Nosema apis* (Wang & Moeller, 1970), and mosquitoes infected with *Nosema algerae* (Henn *et al.*, 1998). There have been very few such studies on parasitized crustaceans, although alterations to the plasma FAA profile have been reported for the bacterium *Aerococcus viridans* infection in lobsters (Stewart, 1980) and the microsporidia *Thelohania maenadis* and *Ameson michaelis* infections in crabs (Vivares *et al.*, 1980; Findley *et al.*, 1981).

Populations of the Norway lobster (*Nephrops norvegicus*) from the coastal waters off the west coast of Scotland are infected with the dinoflagellate *Hematodinium* sp., with overall prevalence levels of over 70% in some years (Field *et al.*, 1992). The parasite is systemic, invading most major tissues and organs, causes considerable histological disruption (Field & Appleton, 1995) and has a major effect on the appearance and properties of the haemolymph, including extensive loss of normal haemocytes (Taylor *et al.*, 1996). However, there are no reports on the biochemical consequences of the infection. *Nephrops* is typical of marine crustaceans in having a large intracellular FAA pool (Robertson, 1961, Robertson *et al.*, 1992), while extracellular concentrations are low. With such high tissue:haemolymph FAA ratios, the extensive pathological effects of *Hematodinium* infection on the tissues and organs of *Nephrops* may be expected to cause increases in the haemolymph concentrations of amino acids when released from damaged cells There may also be compensation for the parasite-induced changes to the haemolymph.

Therefore this project was undertaken to determine the haemolymph FAA composition during infection of *Nephrops* by *Hematodinium*, to elucidate the extent of such changes, to chart their time course in relation to the onset and progress of the infection, and to determine whether the host undergoes compensatory changes in its plasma FAA profile.

Methods.

Collection and treatment of animals.

Norway lobsters (*Nephrops norvegicus*) were otter-trawled from a location south of Little Cumbrae in the Clyde Sea area, Scotland, UK, and subsequently maintained in a closed aquarium (10°C, 33ppt salinity) in the Division of Environmental and Evolutionary Biology, University of Glasgow, Scotland, UK. Animals were fed *ad libitum*, once weekly on mussels (*Mytilus edulis*) but were not fed for at least five days prior to haemolymph collection. All animals were in the intermoult state (Aiken, 1980) and were assessed for *Hematodinium* infection by the pleopod staging method of Field & Appleton (1995).

Sample collection.

Haemolymph was drawn from the base of the fifth pereiopod into a sterile syringe and centrifuged immediately at 17,000 x g for 10 min. at 10°C to remove cellular material and suspended debris. The clear plasma was deproteinized using ice cold methanol (four parts methanol to one part plasma) for 10 min. at 4°C. Protein precipitate was removed by centrifugation and the supernatant passed through a 0.22 μm filter before storage at –70°C.

High performance liquid chromatography (HPLC) analysis.

HPLC grade methanol (MeOH), tetrahydrofuran (THF) and acetonitrile were obtained from BDH Laboratory Supplies, Leicestershire, UK. Crystalline amino acids (aspartate, glutamate, asparagine, serine, histidine, glutamine, glycine, threonine, arginine, alanine, tyrosine, valine, methionine, tryptophan, phenylalanine, isoleucine and leucine), taurine, sodium acetate, mercaptoethanol (ME) and *o*-phthaldialdehyde (OPT) were from Sigma-Aldrich Co. (Poole, UK). Reagent grade Millipore-filtered water was used throughout.

Deproteinized plasma samples were analysed by HPLC using a Rainin Dyn Microsorb C18 4.6 x 150 mm column (with Microsorb C18 guard column), a Gilson HPLC system (model 302

Pumps, model 802C manometric module, model 811 dynamic solvent mixer and model 121 fluorometer) interfaced to a computer with 712 HPLC controller software. Amino acids were eluted after pre-column OPT-derivatization using the method of Zuo & Coombs (1995) by mixing equal volumes of plasma and OPT reagent. The method allowed for quantitation of 12 amino acids and taurine. Co-elution of valine/methionine and histidine/glutamine occurred in all runs and neither cysteine nor proline were detectable. Standard curves were constructed using an equimolar mixture of the above amino acids and taurine and plasma concentrations were derived from these curves. Comparison of FAA concentrations in the plasma of uninfected and infected *Nephrops* was performed either by one-way analysis of variance (ANOVA) for normally distributed data, or by a Kruskal-Wallace test for non-normal distributions. Between stage comparisons were made with a Tukey's pairwise analysis (normal distributions) and a Mann-Whitney test (non-normal distributions). Significance was considered to be at $p < 0.05$.

Results.

Analysis of the FAA concentrations in Nephrops plasma.

A total of 81 lobsters were examined for infection by *Hematodinium* using the pleopod staging method of Field & Appleton (1995). Stage 0 denotes an uninfected animal, with patent infection progressing from Stage 1 to Stage 4. Plasma FAA concentrations ($\mu mol.ml^{-1}$) were derived from peak areas on the HPLC chromatograms. The OPT-derivatized FAA compounds identified in *Nephrops* plasma under different levels of infection are shown in Table 1. In uninfected *Nephrops* (Stage 0), the total detected FAA concentration was 3.79 $\mu mol.ml^{-1}$, with glycine, histidine/glutamine, alanine, serine, taurine and asparagine collectively constituting almost 80 % of this total. The total identified FAA concentration was highest in the plasma of Stage 3 animals (6.70 $\mu mol.ml^{-1}$), this being significantly greater than that of uninfected plasma ($p < 0.001$).

The FAA profile of *Hematodinium*-infected *Nephrops* plasma was substantially altered through the course of the infection (Table 1). The concentrations of five FAA compounds were significantly less in the plasma of Stage 1 (early patent infection) animals compared with uninfected controls, resulting in a depression (though not significant) in the total concentration of detected FAA. Among the individual amino acids, the change of serine was the greatest, falling from 0.34 $\mu mol.ml^{-1}$ to 0.21 $\mu mol.ml^{-1}$ ($p < 0.001$), while other FAA's showing reduced concentrations included asparagine ($p < 0.05$), valine-methionine ($p < 0.01$), tryptophan ($p < 0.01$) and isoleucine ($p < 0.01$).

A different trend was apparent with lobsters at Stage 2 infection, in which several FAA's exhibited very significant increases in concentration. However, the most prominent increase was with taurine which increased from 0.22 $\mu mol.ml^{-1}$ in stage 0 to 1.04 $\mu mol.ml^{-1}$ in Stage 2 ($p < 0.001$). By Stage 3 of infection, taurine (at 2.20 $\mu mol.ml^{-1}$) was dominant in the plasma, and glutamate had also increased significantly from its Stage 2 concentration ($p < 0.01$). In contrast, serine, after its initial reduction, remained low in the later stages of infection and did not change significantly among successive levels of infection. Plasma arginine concentrations in Stage 3 animals were at three times the Stage 0 levels, although the increase in total plasma FAA in Stage 3 infected animals was due primarily to the increased taurine concentration.

In Stage 4 animals, most plasma FAA's had returned to concentrations not significantly different from those of stage 0 animals. The only exceptions to this were taurine, which had increased approximately 13-fold (Fig. 1a), glutamate, with a 10-fold increase (Fig. 1b), and serine, which was at approximately one half of the concentration in uninfected animals (Fig. 1c).

The importance of plasma taurine in infection.

Taurine accounted for the majority of the increase in total detected FAA during infection. It

showed significant differences between Stages 0 and 2 ($p < 0.001$), 1 and 2 ($p < 0.001$) and 2 and 3 ($p < 0.001$), but not between Stages 3 and 4 ($p > 0.05$) (Fig. 1a). However, when expressed relative to total detected FAA concentration, the difference in taurine amounts between Stage 0 (5.8% of total FAA) and Stage 1 (13.7% of total FAA) are significant ($p < 0.05$) (Fig. 2). Additional to increasing absolutely from Stage 0 to Stage 4 (0.22 $\mu mol.ml^{-1}$ to 2.56 $\mu mol.ml^{-1}$), taurine also made an increasing relative contribution to total FAA, rising from 5.8% in Stage 0 to 41.6% in Stage 4 plasma. The application of a ratiometric measure of increasing taurine concentration against decreasing serine concentration (Fig. 3) also revealed significant differences between Stages 0 and 1 (i.e. between animals that are apparently healthy and those patently infected). The taurine:serine ratio increased significantly between Stages 0 and 1 ($p < 0.01$), 1 and 2 ($p < 0.01$), and 2 and 3 ($p < 0.001$), but not between Stages 3 and 4 ($p > 0.05$). It should be noted that the difference in this ratio between Stage 0 and Stage 1 is more significant ($p < 0.01$) than the comparison of absolute concentration ($p > 0.05$) or relative contribution of taurine to the total ($p < 0.05$).

Discussion.

Several studies on invertebrates have considered alterations in FAA profile and non-protein nitrogen under conditions of infection, but few have considered changes in the FAA profile at different stages of infection (Henn *et al.*, 1998). The ability to estimate the infection severity by a simple morphological index applied to the host greatly facilitated the current study of *Hematodinium* infection in *Nephrops*, allowing the changing pattern of plasma amino acid concentrations to be tracked through the course of infection. This provides a good basis for understanding the biochemical disturbances that occur during *Hematodinium* infection.

Plasma FAA profile in uninfected and infected Nephrops norvegicus.

Of the FAAs detected in normal plasma, glycine, histidine/glutamine, alanine, serine asparagine and taurine collectively constitute almost 80 % of the total concentration (3.79 $\mu mol.ml^{-1}$). It should be noted however that this excludes proline, which is known to be high in *Nephrops* abdominal muscle (Robertson *et al.*, 1992), and cysteine, both of which are not derivatized by OPT. The total concentration in *Nephrops* haemolymph may then be similar to the 5.8 $\mu mol.ml^{-1}$ reported for *Homarus gammarus* (Camien *et al.*, 1951). The decrease in the concentrations of some amino acids early in infection (Table 1) is consistent with parasite utilisation of these compounds as the haemolymph is colonised. A reduction in non-protein nitrogen also occurs during early infection by *Aerococcus viridans* in *Homarus americanus* (Stewart, 1980). The significant reduction in the concentration of free serine that occurred even in early infection (Fig. 1c) could reflect preferential utilization of this compound by *Hematodinium*, or alternatively parasitic consumption of carbohydrate precursors or a disruption to the biosynthetic pathway of serine. Serine is considered an intermediate in the biosynthesis of taurine in lobster skeletal muscle (Finney, 1978); the observed depletion of serine may be linked to up-regulation of the synthesis of taurine during infection. Taurine is generally not metabolised, and hence the increased taurine concentration relative to total FAA (Fig. 2) could be due to a combination of increased biosynthesis and its non-utilisation relative to the other FAA compounds.

The increased total FAA concentration that occurred in later stages of infection may have several causes. There is widespread disruption in all major organs and tissues during infection of *Nephrops* by *Hematodinium*, especially at the later stages (Field & Appleton, 1995). Intracellular compounds liberated by cellular damage would be likely to appear in the haemolymph and lead to the observed increase in concentration of total FAA. However, the elevated total FAA concentration is not due to an increase in all component FAA's, but rather to

elevated levels of a few (see Table 1) which suggest either selective release or selective utilisation of released amino acids. The particular increase in plasma taurine may in part reflect the fact that its concentration is known to be high in the crustacean hepatopancreas (Van Marrewijk & Ravenstein, 1974) and skeletal muscle (Pochon-Masson *et al.*, 1984), both of which undergo considerable disruption during *Hematodinium* infection (Field & Appleton, 1995).

Another possible source of plasma FAAs is the haemolymph itself, which contains both proteins such as haemocyanin and various types of haemocytes. Haemolymph proteins serve as stores for amino acids (Pequeux *et al.*, 1979), and host mobilisation or parasitic degradation of these proteins could lead to elevated levels of some FAA in the plasma. Taylor *et al.* (1996) reported a large reduction in plasma haemocyanin in *Hematodinium*-infected *Nephrops*. Taurine, however, is not a component of proteins, and so the elevated concentrations of this amino sulphonic acid must have a source other than the catabolism of haemocyanin. The dramatic decline in the concentration of haemocytes in the host haemolymph during severe infection (Field & Appleton, 1995) suggests that haemocytes are destroyed by the parasite (although inhibition of their production may be a contributory factor), and lysis of haemocytes would result in their cellular contents being released into the haemolymph. Since taurine is concentrated within the haemocytes of certain animals (Thoroed & Fugelli, 1994), this cell lysis could also contribute to the observed elevation of the plasma taurine levels.

Since the large FAA pool in tissues of marine invertebrates such as *Nephrops* is essential in osmoregulation (Claybrook, 1983; Robertson *et al.*, 1992), the increased FAA concentration in the haemolymph of *Nephrops* during late infection could, in part, reflect an attempt by the host to compensate for osmolytes depleted by the parasite, as reported for the oyster *Crassostrea virginica* infected by *Bucephalus sp.* and *Minchinia nelsoni* (Feng *et al.*, 1970). Taurine is an important osmolyte in various animal groups, being a relatively more expendable amino compound as it is non-essential (Chamberlain & Strange, 1989). However, the finding that the

concentration of several other amino acids not thought to be principally involved in osmoregulation also increased during *Hematodinium* infection (Table 1) suggests that the changes in haemolymph FAA have multiple origins.

Finally, the possibility cannot be excluded that the elevated concentrations of taurine and other compounds are of parasite origin. To determine this an analysis of the FAA composition of media from *in vitro* cultures of *Hematodinium* (Appleton & Vickerman, 1998) and of the parasites themselves is required.

Implications of plasma FAA changes to the ecology of Nephrops.

A number of studies have implicated taurine as a neurotransmitter or neuro-modulator (Oja & Kontro, 1978; Kuriyama *et al.*, 1978). In crustaceans, taurine mimics the inhibitory actions of gamma-aminobutyric acid (GABA) and affects cardiac rhythm (Zatta, 1987; Payen *et al.*, 1981). Glutamate also has pharmacological activity at low concentrations (Lin & Cohen, 1973), and is maintained at trace concentrations (0.04 $\mu mol.ml^{-1}$) in the plasma of uninfected *Nephrops* (Fig. 1b). However, during infection the concentration of free glutamate in *Nephrops* plasma increased to values that exceed the threshold for stimulating crayfish muscle (0.053 $\mu mol.ml^{-1}$, see Lin & Cohen 1973). Due to the open nature of the crustacean circulatory system, these elevated concentrations of neuro-active compounds could affect the behaviour and locomotory ability of *Hematodinium*-infected *Nephrops.* This could significantly alter their ability to evade capture, both by predators and by trawlers, and so has both practical and economic consequences since *Nephrops* is currently the United Kingdom's most valuable shellfish resource. Animals with late stage infections are moribund. This could result from the actions of neuro-active compounds, although the increased respiratory demands due to the high parasitic burden will also contribute to this state (Taylor *et al.*, 1996).

Elevated levels of FAAs in infected plasma may also have implications for predator attraction. Amino acids play an important part in prey detection in crustaceans (Voigt *et al.*, 1996) and many species are particularly sensitive to taurine, with behavioural responses being elicited at concentrations as low as 10^{-13} M (Johnson & Atema, 1986). The increased plasma concentration of taurine together with damage to the excretory antennal gland (Field & Appleton, 1995) implies that the excretion of taurine and other amino compounds is increased during infection. This could attract benthic scavengers, which rely on chemical cues (Wong & Moore, 1995). If this is confirmed, then the possibility should be investigated that these scavenging species can act as alternative hosts for *Hematodinium*. This may help to elucidate the full life cycle of *Hematodinium* (Appleton & Vickerman, 1998).

Diagnosis of Hematodinium-infection in Nephrops.

Infection staging by observing the pleopod under low-power microscopy (Field & Appleton, 1995) is currently the primary method for field diagnosis of patent *Hematodinium* infection, while the use of a polyclonal antibody allows for accurate detection of sub-patent and latent infections (Field & Appleton, 1996). The pleopod staging method requires laboratory facilities, is somewhat subjective and can result in mis-diagnoses of infection severity and failure to detect light infections. Another limitation is that it provides only a 'parasitic index', and not an assessment of host condition. Many studies on invertebrate responses to various stressors have suggested that the use of biochemical indices may provide more consistent and reproducible measures of host condition (Livingstone, 1982; Fossi *et al.*, 1997). The current study has shown that the taurine concentration alone can diagnose infection from stage 2 onwards (Fig. 1a), while the reduced concentration of serine (Fig. 1c), the increased percent contribution of taurine to the total FAA (Fig. 2), and particularly the taurine:serine ratio (Fig. 3) provide powerful measures of early patent infection. The ratiometric measure also demonstrates that the distinction between stage 3 and stage 4 infections is an artificial one. A measure based upon the

haemolymph taurine-serine ratio may provide a better estimate of the progression of infection than the pleopod method, and could be a valuable tool for surveys of disease prevalence in commercial *Nephrops* stocks. The development of a rapid, biochemical field assay using information gained in this study may be particularly useful in mapping *Hematodinium* infection along the Scottish coastline and would provide fisheries managers with a better insight into disease epidemiology in the valuable *Nephrops* fishery.

The finding of the increased concentration of taurine during *Hematodinium* infection of *Nephrops* may also have relevance to other host-parasite interactions. As taurine is involved in stress responses and occurs at high concentrations in the tissues of many invertebrates, alterations in its plasma concentration may be both an important mediator and also a useful index in a number of infection pathologies and stress-related effects.

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Amino acid	Haemolymph Free Amino Acid (Concentrations in μmol.ml^{-1})									
	Stage 0 (n = 23)		Stage 1 (n = 11)		Stage 2 (n = 28)		Stage 3 (n = 13)		Stage 4 (n = 6)	
	Conc.	*±SE*	*Conc.*	*±SE*	*Conc.*	*±SE*	*Conc.*	*±SE*	*Conc.*	*±SE*
Aspartate	0.01	0.002	0.03	0.01	0.03	0.008	0.05	0.02	0.06	0.03
Glutamate	0.04	0.007	0.06	0.01	0.11***	0.02	0.19***	0.03	0.40***	0.21
Asparagine	0.16	0.01	0.12*	0.01	0.13*	0.01	0.11**	0.03	0.09	0.04
Serine	0.34	0.03	0.21***	0.05	0.17***	0.01	0.18**	0.03	0.19*	0.04
Histidine/Glutamine	0.56	0.04	0.52	0.11	0.42**	0.04	0.37**	0.07	0.44	0.09
Glycine	1.17	0.15	0.93	0.17	1.43	0.14	1.90*	0.23	1.24	0.30
Threonine	0.11	0.01	0.10	0.02	0.10	0.01	0.09	0.02	0.06	0.03
Arginine	0.11	0.007	0.12	0.02	0.23**	0.03	0.31**	0.03	0.16	0.03
Alanine	0.55	0.05	0.41	0.05	0.52	0.05	0.55	0.07	0.60	0.16
Taurine	0.22	0.03	0.42	0.09	1.04***	0.1	2.20***	0.21	2.56***	0.33
Tyrosine	0.06	0.006	0.06	0.007	0.08	0.008	0.12*	0.02	0.07	0.02
Valine/Methionine	0.14	0.009	0.10**	0.01	0.14	0.01	0.20	0.05	0.15	0.02
Tryptophan	0.02	0.003	0.00**	0.00	0.01**	0.003	0.03	0.01	0.02	0.01
Phenylalanine	0.05	0.005	0.04	0.006	0.06	0.006	0.11**	0.02	0.08	0.02
Isoleucine	0.10	0.007	0.05**	0.01	0.08*	0.007	0.09	0.01	0.09	0.02
Leucine	0.14	0.008	0.11	0.02	0.14	0.01	0.20*	0.03	0.20	0.05
Total Identified FAA	3.79	0.28	3.29	0.41	4.68	0.33	6.70***	0.60	6.41*	0.93

Table 1. OPT-derivatized free amino acids from the haemolymph of *Nephrops norvegicus*. Significant differences from Stage 0 concentrations are denoted by *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).

Figure 1a-c. Mean (±SE) plasma Taurine (a), Glutamate (b) and Serine (c) in the haemolymph of *Nephrops norvegicus* at different stages of infection by *Hematodinium*. For statistical significance among groups see Table 1 and text.

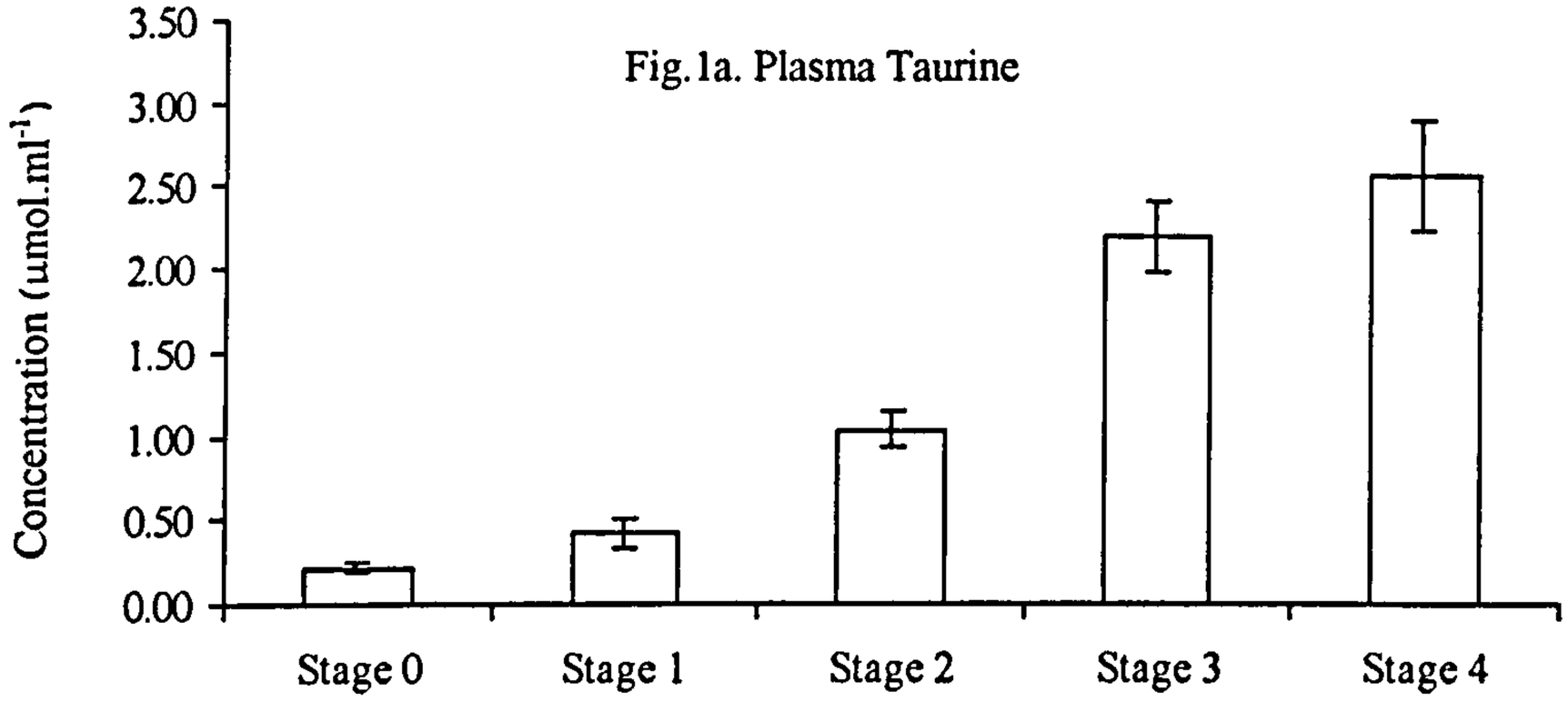


Figure 2. Mean (±SE) contribution of taurine to total detected FAA in the haemolymph of *Nephrops norvegicus* at different stages of infection by *Hematodinium*, plus between-group significance table. Mean taurine % contributions are given above each bar.

Infection stage	1	2	3	4
0	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.001$
1		$P < 0.01$	$P < 0.001$	$P < 0.001$
2			$P < 0.001$	$P < 0.001$
3				$P > 0.05$

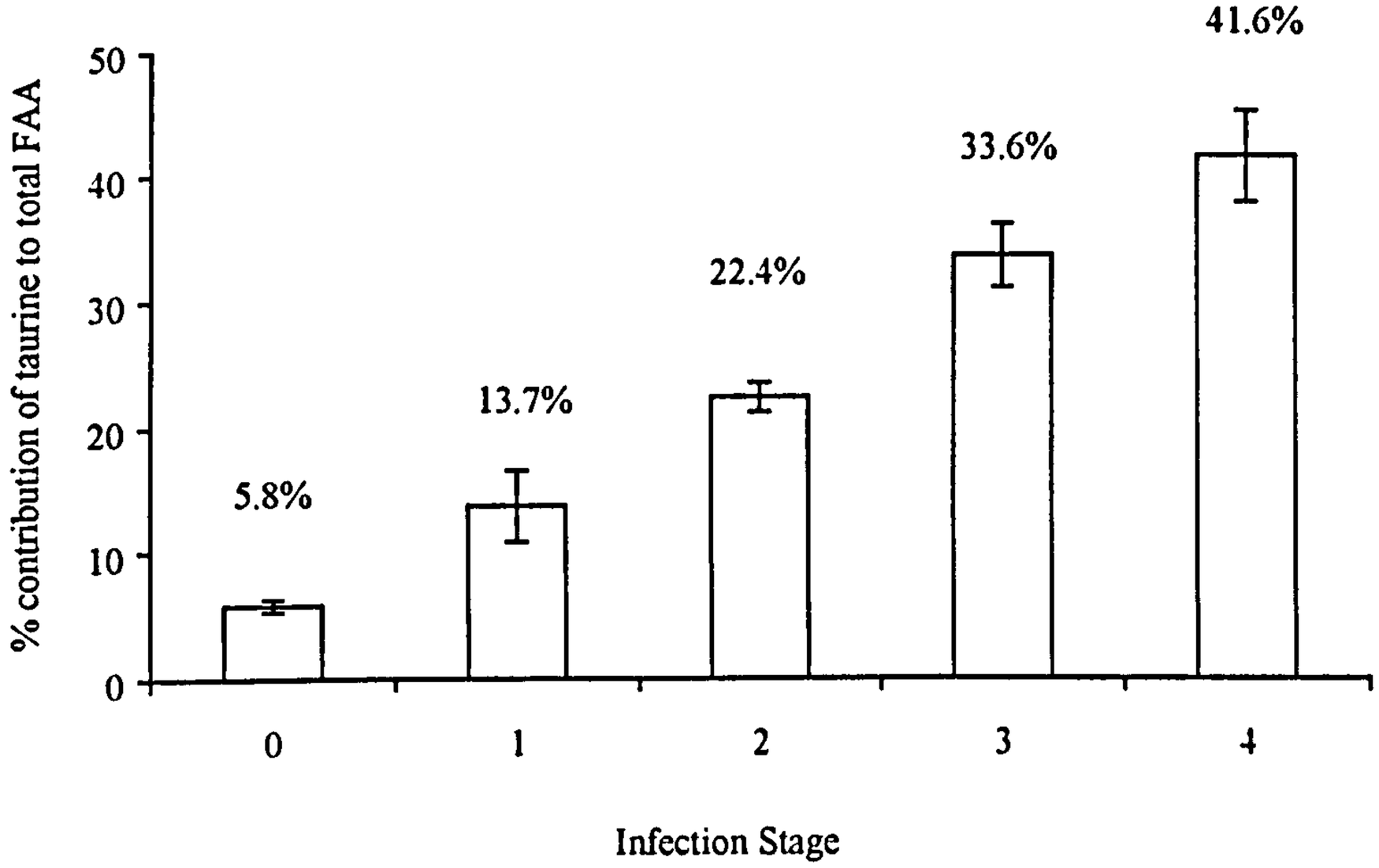
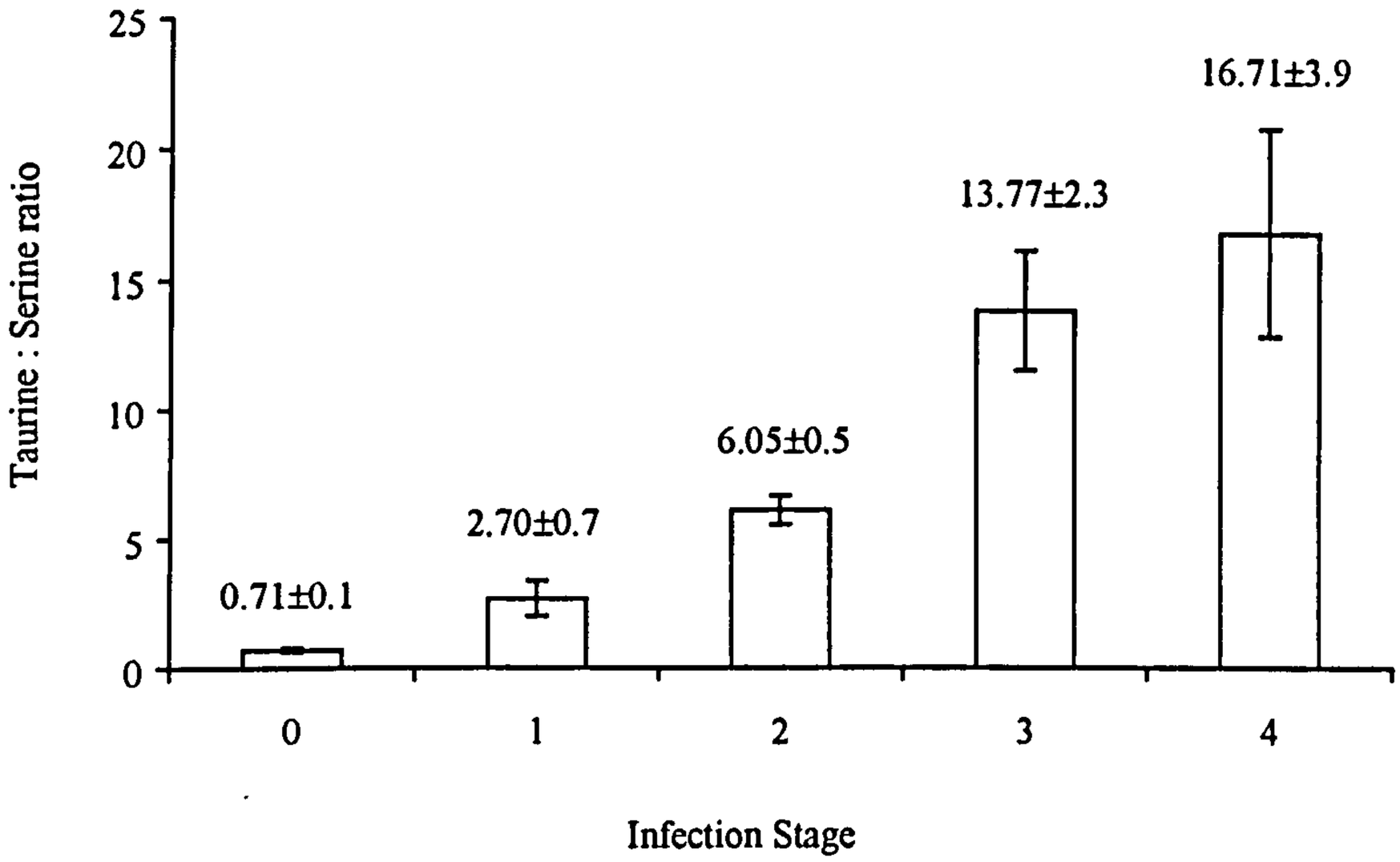


Figure 3. Mean (±SE) taurine-serine concentration ratios in haemolymph of *Nephrops norvegicus* under different degrees of infection by *Hematodinium*, plus between-group significance table. Mean ratio ± standard error of mean given above each bar.

Infection Stage	1	2	3	4
0	P < 0.01	P < 0.001	P < 0.001	P < 0.001
1		P < 0.01	P < 0.001	P < 0.01
2			P < 0.001	P < 0.01
3				P > 0.05



Chapter 5

Alterations in the biochemistry and ultrastructure of the deep abdominal flexor muscle of the Norway lobster, *Nephrops norvegicus* (L.) during infection by a parasitic dinoflagellate of the genus *Hematodinium*.

Abstract.

Changes in various biochemical and ultrastructural characteristics of the deep abdominal flexor (DAF) muscles were studied in Norway lobster (*Nephrops norvegicus*) from the Clyde estuary, Scotland, UK, at different stages of infection by a parasitic dinoflagellate of the genus *Hematodinium*. Abdominal DAF muscles from infected lobsters showed slight, significant increases in total water content, along with greatly depleted glycogen reserves and an altered free amino acid profile. However, protein concentration and composition remained unchanged. Ultrastructurally, parasitic infection of DAF muscle fibres caused alterations in sarcolemmal structure, and localized disruption of myofibrillar bundles around the periphery, but not throughout the centre of the fibres. Overall, the reduction in swimming performance previously reported for *N. norvegicus* during *Hematodinium* infection reflect an alteration in carbohydrate supply to the active muscle and some subtle disruption of muscle structure. The altered carbohydrate titre could reflect the *Hematodinium* parasites acting as a carbohydrate sink in the haemolymph, a disruption of normal tissue glycogenesis, or some alteration in the host's hormonal regulation. These changes could also adversely affect the taste, texture and marketability of infected meat.

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Introduction.

Damage to crustacean muscle has been widely reported from various infective agents, including microsporidians (Findley *et al.*, 1981; Langdon, 1991; Kabre, 1992; Dennis & Munday, 1994; Childers *et al.*, 1996), ciliates (Cawthorn, 1997), bacteria (Stewart, 1980) and dinoflagellates (Shields, 1994). Other studies have described muscle wastage with unknown etiology (Lindqvist & Mikkola, 1978), while host-induced (idiopathic) muscle necrosis can occur with natural or artificial stress (Akiyama *et al.*, 1982; Anderson *et al.* 1990, Stentiford & Neil, unpublished data). Such changes in the biochemical composition of muscle tissue can have economic consequences, as in the case of Tanner crabs (*Chionoecetes bairdi* and *C. opilio*) infected by a parasitic dinoflagellate (*Hematodinium* sp.) which causes the so-called 'Bitter Crab disease'. In this case infected muscle tissue tastes bitter (Meyers *et al.*, 1987) and is sometimes deemed to be unmarketable (Eaton *et al.*, 1991; Taylor & Khan, 1995). Similar observations have also been reported for *Hematodinium* sp. infections of other commercially important crustaceans, including the sand crab *Portunus pelagicus* (Hudson, 1995) and the blue crab *Callinectes sapidus* (Messick, 1994).

Populations of the Norway lobster (*Nephrops norvegicus*), from the west coast of Scotland are seasonally infected with a dinoflagellate of the genus *Hematodinium*, with prevalence levels of over 70% in some years (Field *et al.*, 1992). Several studies have established the progression, diagnosis and pathology of *Hematodinium* infection in *N. norvegicus* (Field & Appleton, 1995, 1996), and its effect on host physiology (Taylor *et al.*, 1996) and haemolymph biochemistry (Stentiford *et al.*, 1999 – Chapter 4).

During late infections the deep abdominal flexor (DAF) muscles of *N. norvegicus*, which are mainly responsible for the rapid abdominal flexion in 'tail flip' swimming in these lobsters (Newland & Neil, 1990), are partially invaded by the *Hematodinium* parasite (Field & Appleton, 1995). The abdominal musculature may also be altered in water content

during *Hematodinium* infection in *N. norvegicus*, similar to *Hematodinium* sp. infections in other commercially important crustaceans (Meyers *et al.*, 1987; Messick, 1994; Hudson, 1995). Such changes in the structure and composition of DAF muscles could lead to altered contractile behaviour (Field *et al.*, 1995). Deficits in the swimming performance have been measured in *Hematodinium*-infected animals (Stentiford *et al.*, 2000 – Chapter 8). This has important consequences for the success of infected lobsters in escaping from predators (Neil & Ansell, 1995; Arnott *et al.*, 1998) and in evading capture by trawl nets (Newland *et al.*, 1992). Additionally, the texture and taste quality of the tail meat, the marketable product of a large Scottish fishery (£57M in 1999) (FRS, 1999), may also be altered.

Despite their potentially damaging effect, changes in the biochemistry and ultrastructure of the DAF muscles in *Hematodinium*-infected *N. norvegicus* have never been studied systematically (Field *et al.*, 1992, 1995). Therefore in this study the water content, total protein, glycogen and free amino acids of DAF muscle fibres were measured in *N. norvegicus* at different stages of *Hematodinium* infection, and related to changes in the ultrastructure of the muscle fibres.

Materials and Methods.

Animals.

Norway lobsters (*Nephrops norvegicus* L.) were caught by otter-trawl from a location south of Little Cumbrae in the Clyde Sea area, Scotland, UK, and subsequently maintained in a closed aquarium (10°C, 33 ppt salinity) in the Division of Environmental and Evolutionary Biology, University of Glasgow, Scotland, UK. Animals were fed *ad libitum*, once weekly on mussels (*Mytilus edulis*) but were not fed for at least five days prior to tissue collection. All animals were in the intermoult state (Aiken, 1980) and were assessed for *Hematodinium* infection by the pleopod staging method of Field and Appleton (1995). Individual pleopods were removed and viewed under low power microscopy. The

accumulation of agglutinated parasite and haemocyte material was used to assign the appropriate stage, whereby Stage 0 is apparently uninfected and Stages 1 to 4 are patently infected (stages of the parasite evident in the haemolymph).

Tissue removal and treatment.

The deep abdominal flexor (DAF) from each of 128 lobsters was dissected out under physiological saline, blotted to remove excess moisture and weighed (wet weight). Muscle tissue was then frozen in liquid nitrogen and freeze-dried for 36 h (Edwards *Modulyo* freeze drier). Tissue was then re-weighed (dry weight) and samples were individually ground using a standard mortar and pestle. Ground samples were stored at –20°C until analysis. For SDS-PAGE analysis of the DAF muscle, individual muscle fibres were dissected out in calcium-free physiological saline and placed into 200 µl of SDS-sample buffer, denatured at 95°C for 4 min and stored at –20°C until electrophoretic protein separation (Neil *et al.* 1993).

For electron microscopy, muscles were fixed for 2 h at room temperature in a solution containing 4 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, with 2 % sucrose and 1.5 % sodium chloride. Fixed samples were rinsed in 0.1 M phosphate buffer with 4 % sucrose and post-fixed in 1 % osmium tetroxide in phosphate buffer for 1 h. Specimens were washed in several changes of distilled water and stained in 0.5 % uranyl acetate for 1 h. Following dehydration through an ethanol series, specimens were embedded in Spurr resin (Spurr, 1969). Thick sections (1 µm) were stained with toluidine blue and suitable areas were thin-sectioned and mounted on uncoated copper/palladium grids and stained with uranyl acetate and lead citrate. Thin sections (60-70 nm) were examined in a Zeiss 902 transmission electron microscope.

Chemicals.

For protein and glycogen analyses, sodium hydroxide (NaOH), trichloroacetic acid (TCA), potassium hydroxide (KOH), ethanol (EtOH) and the anthrone and Folin-Ciocalteu's phenol reagents were obtained from Sigma-Aldrich Co. (Poole, UK). For high performance liquid chromatography (HPLC) analysis, reagent-grade methanol (MeOH), tetrahydrofuran (THF) and acetonitrile were obtained from BDH Laboratory Supplies (Leicestershire, UK). Crystalline amino acids (aspartate, glutamate, asparagine, serine, histidine, glutamine, glycine, threonine, arginine, alanine, tyrosine, valine, methionine, tryptophan, phenylalanine, isoleucine and leucine), taurine, sodium acetate, mercaptoethanol (ME) and *o*-phthaldialdehyde (OPT) were also from Sigma-Aldrich Co. and reagent-grade Millipore-filtered water was used throughout. For SDS-PAGE, acrylamide-bis acrylamide (30%), sodium dodecylsulfate (SDS), ammonium persulfate (APS), N,N,N',N'- tetramethylethylenediamine (TEMED), brilliant-blue R250, TCA and molecular weight (M_r) standards were purchased from Sigma-Aldrich Co.

Muscle water and total protein assays.

The muscle water content was determined for 128 lobsters as the difference between the wet weight and dry weight measurements. For determination of total muscle protein, 10 mg of freeze-dried DAF from 117 lobsters was homogenised in 10 ml 0.1N NaOH, left for 12 h and centrifuged at 17,000 x g for 5 min. One ml of the supernatant was added to 2 ml of 10 % TCA and left for 12 h at 4°C to allow for complete precipitation of the protein. Samples were centrifuged as above and the protein pellet re-dissolved in 1 ml of 0.1 N NaOH. Aliquots of 0.5 ml were then assayed for total protein using the Folin-Ciocalteu's phenol reagent (Lowry *et al.*, 1951).

Muscle glycogen assay.

For determination of muscle glycogen, 400 µl of 30 % KOH was added to each of 45 10 mg freeze-dried samples from lobsters at different stages of infection. The samples were boiled for 20 min in a water bath at 95-100°C, cooled and added to 700 µl absolute ethanol before being placed on ice for 2 h. Following precipitation, samples were spun at 17,000 x g for 10 min and the supernatant discarded. One ml of distilled H_2O was added to each sample before sonication, after which 50 µl was incubated at 95-100°C in 1ml of anthrone reagent before glycogen assessment (Carroll *et al.*, 1956).

Free amino acid (FAA) measurement of muscle.

For determination of muscle FAA concentrations, each of 44 10 mg samples of freeze-dried DAF from lobsters at different stages of infection were sonicated in 1 ml MeOH for 1 min (De Vooys, 1991). Sonicated samples were deproteinized for 30 min at 4°C, following which the protein precipitate was removed by centrifugation at 17,000 x g for 10 min. The supernatant was passed through a 0.22 µm syringe filter before storage at –70°C until analysis. Reverse phase HPLC was carried out as described in Stentiford *et al.* (1999) using 20 µl of the DAF extract derivatized with 180 µl OPT.

SDS-PAGE conditions.

Samples of DAF muscle fibre were prepared as described above. Discontinuous SDS-PAGE was performed according to the method of Laemmli (1970) with 12.5 % acrylamide separating gel and a 4 % acrylamide stacking gel. Electrophoresis running conditions were as described by Neil *et al.* (1993), followed by overnight incubation in 10 % TCA and staining for 30 minutes with Coomassie-blue solution. Stained gels were later examined with an Appligene™ gel imager.

Data analysis.

Comparisons of the water content, protein, glycogen and FAA concentrations in the DAF muscle of uninfected and infected *N. norvegicus* were performed either by one-way analysis of variance (ANOVA) for normally distributed data, or by a Kruskal-Wallace test for non-normal distributions. Comparisons between stages were made with a Tukey's pairwise analysis (normal distributions) and a Mann-Whitney test (non-normal distributions). Significance was considered to be at $p < 0.05$.

Results.

Water assay of DAF muscle.

Percentage of water content in DAF muscle at all stages of infection showed small, but consistently elevated mean values, which were significantly different from the mean value for Stage 0 (76.66 %) for Stage 1 (77.57 %) ($p < 0.05$), Stage 2 (77.46 %) ($p < 0.01$) and Stage 3 (77.77 %) ($p < 0.01$). At Stage 4, an increase in DAF water was also observed, but was not significant (77.43 %) ($p = 0.095$) (Fig.1).

DAF muscle protein assay.

The protein concentration ($mg.g^{-1}$ dry weight) of DAF muscle showed no significant difference between uninfected (632.77 $mg.g^{-1}$ dry weight DAF) and *Hematodinium*-infected animals ($p = 0.943$). When expressed as $mg.g^{-1}$ wet weight, the p-value was reduced ($p = 0.443$), but differences between uninfected and infected animals were still not significant.

DAF muscle SDS-PAGE analysis.

Myofibrillar proteins from DAF muscle fibres were separated using standard SDS-PAGE procedures. Figure 2 shows that the protein banding pattern of *Hematodinium*-infected

DAF is similar to that of the uninfected control, with no apparent qualitative differences in the relative content of the major myofibrillar proteins. Electrophoresis using high percentage acrylamide gels (20 %) confirmed this absence of extra bands in the low molecular weight region, while low percentage acrylamide gels (5-7 %) revealed no loss of bands in the high molecular weight region (data not shown).

DAF muscle glycogen assay.

The glycogen concentration in the DAF muscle of uninfected *N. norvegicus* had a mean value of 13.59 $mg.g^{-1}$ dry weight. This was progressively reduced in successive stages of *Hematodinium* infection to 8.36 $mg.g^{-1}$ dry weight in Stage 1 ($p < 0.001$), 2.29 $mg.g^{-1}$ dry weight ($p < 0.001$) in Stage 2, 3.10 $mg.g^{-1}$ dry weight ($p < 0.001$) in Stage 3 and 1.92 $mg.g^{-1}$ dry weight ($p < 0.001$) in Stage 4. When different infection stages were compared, significant differences were found between Stage 1 and all other stages ($p < 0.001$), but not between Stages 2 and 3 ($p = 0.273$) or Stages 3 and 4 ($p = 0.194$) (Fig. 3).

FAA analysis of DAF muscle.

Concentrations ($\mu mol.gfw^{-1}$) of FAA in DAF muscle of *N. norvegicus* were derived from the areas of the various peaks on the HPLC chromatograms. The OPT-derivatized FAA compounds identified in the DAF muscle of animals with different levels of infection are shown in Table 1. In uninfected *N. norvegicus* (Stage 0), the total FAA concentration was 410.64 $\mu mol.gfw^{-1}$, with glycine, arginine, alanine, taurine and histidine/glutamine collectively constituting over 90 %. The total FAA concentration were highest in late stage infections, but was significantly different from uninfected DAF muscle only for Stage 4 animals (486.39 $\mu mol.gfw^{-1}$) ($p < 0.05$).

The concentrations of several individual FAA compounds were altered significantly during *Hematodinium*-infection (Table 1). The histidine/glutamine concentration was reduced in early infection, while both aspartate and taurine reached their highest concentrations at Stage 2. Taurine is particularly noteworthy, being significantly higher than the control DAF at all stages of *Hematodinium* infection. Serine and phenylalanine shared a similar pattern, with increasing concentrations that peaked in Stage 3. Serine made the largest contribution to the increased total FAA concentration, changing from 5.99 $\mu mol.gfw^{-1}$ in uninfected animals to 24.06 $\mu mol.gfw^{-1}$ in Stage 4 of infection. Glycine, which accounted for over 70 % of the total detected FAA in uninfected DAF, rose in concentration during infection, although the changes within stages were not significant (Table 1).

Ultrastructure of DAF muscle fibres

When infected *N. norvegicus* were examined ultrastructurally, *Hematodinium* parasites were often observed in close association with fibres of the DAF muscle (Fig. 4c). Compared to normal muscle fibres (Fig. 4a), the sarcolemma of *Hematodinium*-infected muscle fibres was often disrupted with apparent disorganisation at the fibre periphery, separation of myofibrillar bundles and expansion of the tubular systems (Fig. 4b). The majority of the myofibrillar bundles located more centrally in *Hematodinium*-infected DAF muscle fibres appeared normal in sarcomeric organisation, tubular systems and organelles (nuclei and mitochondria).

Discussion.

There are considerable alterations in the biochemistry and some changes in the ultrastructure of the abdominal musculature of *Nephrops norvegicus* during *Hematodinium* infection. There is a slight (up to 1.1 %), but significant increase in water content of DAF

muscle during infection. The implications for this increase in DAF water for normal muscle function are not known, though it is possible that the dynamics of normal muscle contraction are altered (Field *et al.* 1995). An alteration in the water content may also affect the cooked meat texture and nutritional quality of infected *N. norvegicus* muscle.

Measurements of total DAF muscle protein ($mg.g^{-1}$ dry weight) provide an indirect indication of gross alterations that may occur during *Hematodinium* infection in *N. norvegicus*. The data presented here show that, although there may be some association of the parasite with muscle bundles during heavy infections (Fig. 4c) (Field & Appleton, 1995), the total muscle protein concentration remains unaffected. The representation of the data as $mg.g^{-1}$ wet weight emphasises the effect of the increase in DAF water on the total DAF protein concentration. The similarity between the total protein in the DAF muscle of infected and uninfected lobsters suggests that the general structure of this muscle is relatively unaffected during *Hematodinium* infection.

SDS-PAGE is a convenient tool for studying the expression of different contractile protein isoforms in crustacean muscle (Mykles, 1985; Yoshinaka *et al.*, 1989; Youlin & Mykles, 1990; Neil *et al.*, 1993; Ishimoda-Takagi *et al.*, 1997). Fast muscle contains characteristic isoforms of paramyosin, troponin-C, I and T (Mykles, 1985) and a 75kDa protein that is cross-reactive with tropomyosin antibodies (Mykles, 1997). In the current study, the DAF myofibrillar protein profile is largely unaltered during patent *Hematodinium* infection of *N. norvegicus* (Fig.2) and additional bands, indicative of breakdown fragments of muscle proteins, are also absent. These SDS-PAGE results are consistent with the measures of total protein, indicating that there is no significant alteration in muscle protein content during patent *Hematodinium* infection.

Ultrastructurally, most myofibrillar bundles in the interior of muscle fibres retain their normal organisation, even during heavy infection (Fig. 4a-c) and the contractile machinery may produce normal levels of force, if activated. Alternatively, it cannot be excluded that

the extensive disruption of sarcolemmal membrane structure of the DAF fibres which occurs during infection could prevent normal activation by affecting the distribution of ions across the sarcolemma. Fibres with lowered membrane resting potential would have reduced ability to respond to synaptic transmission with membrane depolarisations, and the subsequent steps in excitation-contraction coupling could then be suppressed. Thus, infected DAF muscle fibres may fail to contract in a normal way, although their contractile machinery is intact.

The presence of all major contractile proteins in *Hematodinium*-infected DAF fibres suggests that normal tail flexion (at least the part reliant on the proteins themselves) should still be possible. However, Stentiford *et al.* (2000) (Chapter 8) demonstrated that most parameters of the swimming performance of *N. norvegicus* (including number of flips to exhaustion, total distance travelled and flip velocity) are reduced during progressing *Hematodinium* infection. This reduction in swimming performance does not reflect gross change in the total amount of contractile protein present, or to the breakdown of contractile machinery of *Hematodinium*-infected DAF muscle, but is related to more subtle factors either within the muscle itself, or in the supply of energy to support muscle contraction.

The most obvious change occurring in *Hematodinium*-infected DAF muscle is the reduction in the concentration of glycogen to approximately 15 % of its normal value in Stage 4 of infection. Similar reductions in muscle glycogen concentration have been noted with bacterial infection by *Aerococcus viridans* var. *homari* in the lobster, *Homarus americanus* (Stewart, 1980) and also with several nematode infections of insects; in locusts (*Locusta migratoria* and *Schistocerca gregaria*) (Jutsum & Goldsworthy, 1974 and Rutherford & Webster, 1978 respectively) and the mosquito *(Culex pipiens*) (Schmidt & Platzer, 1980). In these cases, the parasites may become a carbohydrate sink by consuming haemolymph glucose (the precursor for glycogenesis). Perhaps the parasites inhibit glycogen synthesis in the host, thereby allowing them to consume the host's glucose

reserves (Rutherford & Webster, 1978). Interestingly, Schmidt & Platzer (1980) have suggested that the condition of the host may establish the degree to which the parasites develop, with the growth of the parasites proceeding as the nutritional state of the host deteriorates. Such a feature in *Hematodinium*-infection in *N. norvegicus* would explain why infection prevalence is maximal during the spring, when animals are reaching their peak physiological condition before the onset of the main moulting season, while infection prevalence during the summer and autumn months is low (Field *et. al.*, 1998).

The reduction in the concentration of glycogen in DAF muscle during *Hematodinium* infection in *N. norvegicus* may reflect changes in normal regulatory hormone function. Hyperglycaemia is a response to various stresses, and in crustaceans is mediated, at least in part, by the crustacean hyperglycaemic hormone (CHH) (Chang *et al.*, 1999). CHH released by the X-organ/sinus gland complex acts on the main glycogen reserve tissues (hepatopancreas, abdominal muscle and possibly hemocytes) to stimulate glycogenolysis, which increases extracellular glucose availability (Santos & Keller, 1993). A disruption, or up-regulation of CHH release during *Hematodinium* infection may be partly responsible for depleting the DAF stores of muscle glycogen and liberating glucose, which is then utilized by the developing parasites.

Depletion of the DAF muscle glycogen reserve may also impact upon the swimming performance of *Hematodinium*-infected *N. norvegicus* (Stentiford *et al.*, 2000 – Chapter 8), with the reduced carbohydrate reserve causing a shortfall in the re-supply of ATP to the contractile machinery. In this study, the main reduction in DAF glycogen was seen at Stage 2 of *Hematodinium* infection, and Stentiford *et al.* (2000) (Chapter 8) reported that the main reduction in swimming performance also occurred at Stage 2. The progressive reduction in carbohydrate reserves during *Hematodinium* infection may thus be the major factor in the reduced swimming performance. However, other features, such as the

increased respiratory demand (Taylor *et al.*, 1996) and loss of integrity of DAF muscle fibre membranes may also be important, especially during severe infections.

Like many marine invertebrates, crustaceans maintain high free amino acid (FAA) concentrations within their cells for osmoregulation (Lange, 1963) and alterations in the FAA profiles of muscles occur in various parasitic infections (Vivares *et al.*, 1980; Findley *et al.*, 1981). The total FAA concentration reported in this study for the DAF muscle of uninfected *N. norvegicus* is comparable to that reported for the same species by Robertson *et al.* (1992), considering that proline, which was found by Robertson (1961) to be high in *N. norvegicus* muscle, was not derivatized using OPT in this study. During *Hematodinium* infection, the concentration of several FAA compounds was significantly altered. The largest changes were in serine and phenylalanine, which increased during progressing infection, and in taurine, which made the largest single contribution to the increase in total DAF muscle FAA. We have previously reported that plasma taurine increases significantly with severity of *Hematodinium* infection in *N. norvegicus* (Stentiford *et al.*, 1999 – Chapter 4). Up-regulation of taurine in the DAF muscle or a leakage of FAA compounds via the damaged sarcolemma of infected *N. norvegicus* may be partly responsible for this plasma increase (Finney, 1978). If up-regulation in taurine synthesis is occurring, it may be the result of some form of stress reaction due to the large plasma parasite burden (Stentiford *et al.*, 1999 – Chapter 4).

Free amino acids significantly affect the taste of crustacean meat, with differences in extractive components between species underlying their characteristic flavours (Hayashi *et al.*, 1981; Shirai *et al.*, 1996). Changes in the composition of FAAs and other compounds (nucleotides, sugars, organic acids and minerals) in the DAF may be implicated in the anecdotal changes in taste of *Hematodinium*-infected *N. norvegicus* meat and also in the characteristic taste change accompanying 'Bitter Crab' infection in Tanner crabs (*Chionoecetes bairdi*) (Meyers *et al.* 1987). Detailed study of the extractive components

from the muscles of *N. norvegicus* and *Chionoecetes bairdi* is necessary to identify the chemical basis of these taste alterations.

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FAA	DAF FAA Concentrations (+/- SE) in µmol.gfw^{-1}									
	Stage 0 (n = 10)		Stage 1 (n = 10)		Stage 2 (n = 10)		Stage 3 (n = 9)		Stage 4 (n = 5)	
	Conc.	*SE.*	*Conc.*	*SE.*	*Conc.*	*SE.*	*Conc.*	*SE.*	*Conc.*	*SE.*
Asp	1.05	*0.15*	2.46***	*0.42*	3.21***	*0.35*	1.91*	*0.35*	1.75	*0.45*
Glu	3.88	*0.39*	5.54**	*0.42*	3.76	*0.39*	4.66	*0.74*	4.27	*0.83*
Asn	4.22	*0.85*	3.98	*0.29*	3.61	*0.52*	4.75	*0.60*	4.62	*0.99*
Ser	5.99	*1.45*	14.57*	*3.51*	11.53	*2.75*	26.06**	*5.01*	24.06**	*4.56*
His/Gln	19.51	*2.89*	11.49*	*0.90*	9.18**	*1.56*	11.82*	*1.45*	13.40	*3.20*
Gly	300.81	*13.23*	361.55	*26.56*	329.71	*26.30*	329.33	*39.21*	336.97	*34.28*
Thr	3.20	*0.72*	3.49	*0.35*	2.66	*0.62*	5.00	*0.82*	3.54	*1.03*
Arg	29.86	*2.38*	19.00*	*3.03*	26.08	*3.47*	35.76	*3.01*	35.40	*4.65*
Ala	20.14	*3.55*	23.01	*3.40*	25.24	*2.11*	33.25*	*4.60*	29.14	*5.29*
Tau	12.17	*1.18*	20.11**	*2.03*	27.17***	*3.61*	24.91**	*3.13*	19.15*	*2.91*
Tyr	1.65	*0.27*	1.10	*0.15*	0.94*	*0.16*	2.00	*0.52*	2.19	*0.99*
Val/Met	3.24	*0.44*	2.54	*0.18*	2.26	*0.41*	3.97	*0.73*	4.52	*1.00*
Phe	0.38	*0.17*	0.87*	*0.16*	0.58	*0.09*	1.96**	*0.49*	1.41	*0.73*
Ile	1.65	*0.42*	1.62	*0.24*	0.83	*0.16*	2.66	*0.59*	2.39	*0.70*
Leu	2.91	*0.50*	2.76	*0.31*	2.18	*0.55*	3.95	*0.55*	3.51	*0.72*
Total FAA	410.64	*15.21*	474.11	*30.74*	448.95	*28.79*	492.02	*45.72*	486.39*	*36.59*

Table 1. Summary of free amino acid concentrations in the deep abdominal flexor muscle of uninfected (Stage 0) and *Hematodinium*-infected (Stages 1-4) *Nephrops norvegicus*. Key; * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). Full names of amino acids are given in methods section.

Fig.1. Percentage of water content in deep abdominal flexor muscle from uninfected (Stage 0) and *Hematodinium*-infected (Stages 1-4) *Nephrops norvegicus*. Total N = 128 (Stage 0 – n24, Stage 1 – n23, Stage 2 – n61, Stage 3 – n15 and Stage 4 – n5).

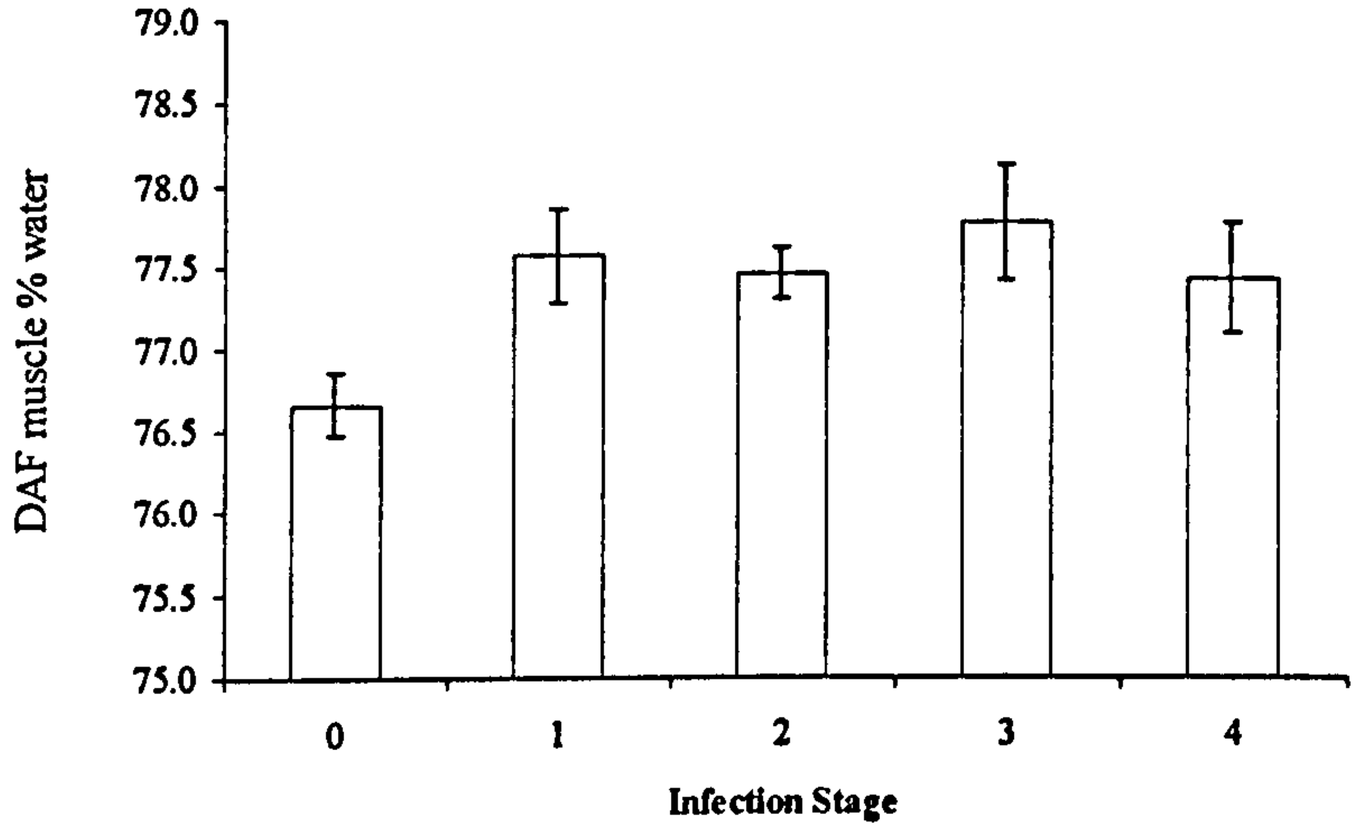


Fig.2. 12.5% SDS-PAGE gel showing separation of contractile proteins from DAF fibres from uninfected and *Hematodinium*-infected *Nephrops norvegicus*. Key; Lane A (Stage 0), B (Stage 1), C (Stage 2), D (Stage 3), E (Stage 4), F (molecular weight marker), MHC (myosin heavy chain), P1/2 (paramyosin 1 and 2), 75kDa (unnamed fast muscle protein), TN-T (troponin-T), A (actin), TM (tropomyosin), TN-I (troponin-I isoforms), Mα-LC (myosin alpha light chain). Numbers on right indicate molecular weights in kDa.

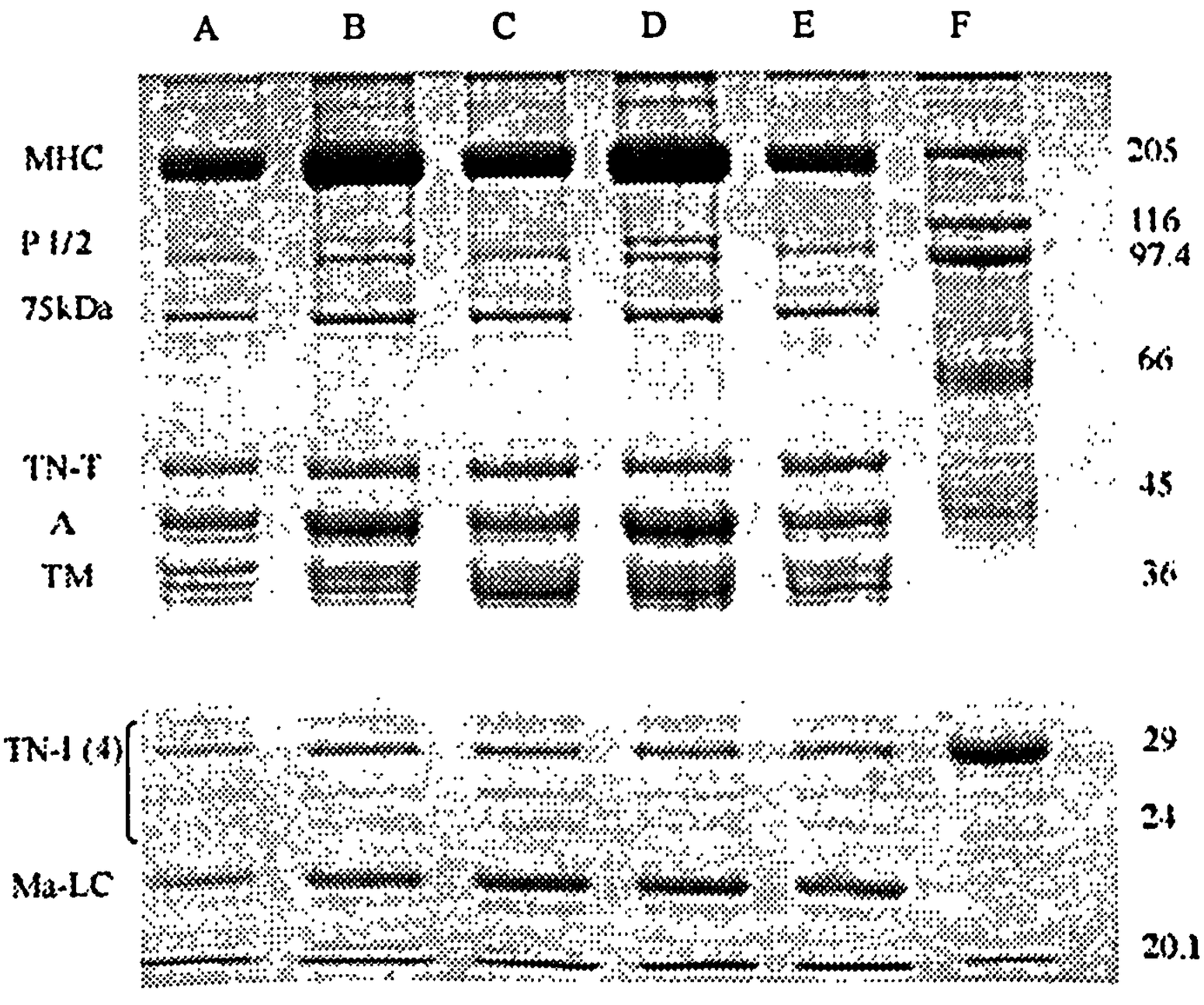


Fig.3. Concentrations of glycogen ($mg.g^{-1}$ dry weight) in deep abdominal flexor muscle from uninfected (Stage 0) and *Hematodinium*-infected (Stages 1-4) *Nephrops norvegicus*. Total N = 45 (Stage 0 – n10, Stage 1 – n10, Stage 2 – n10, Stage 3 – n10 and Stage 4 – n5).

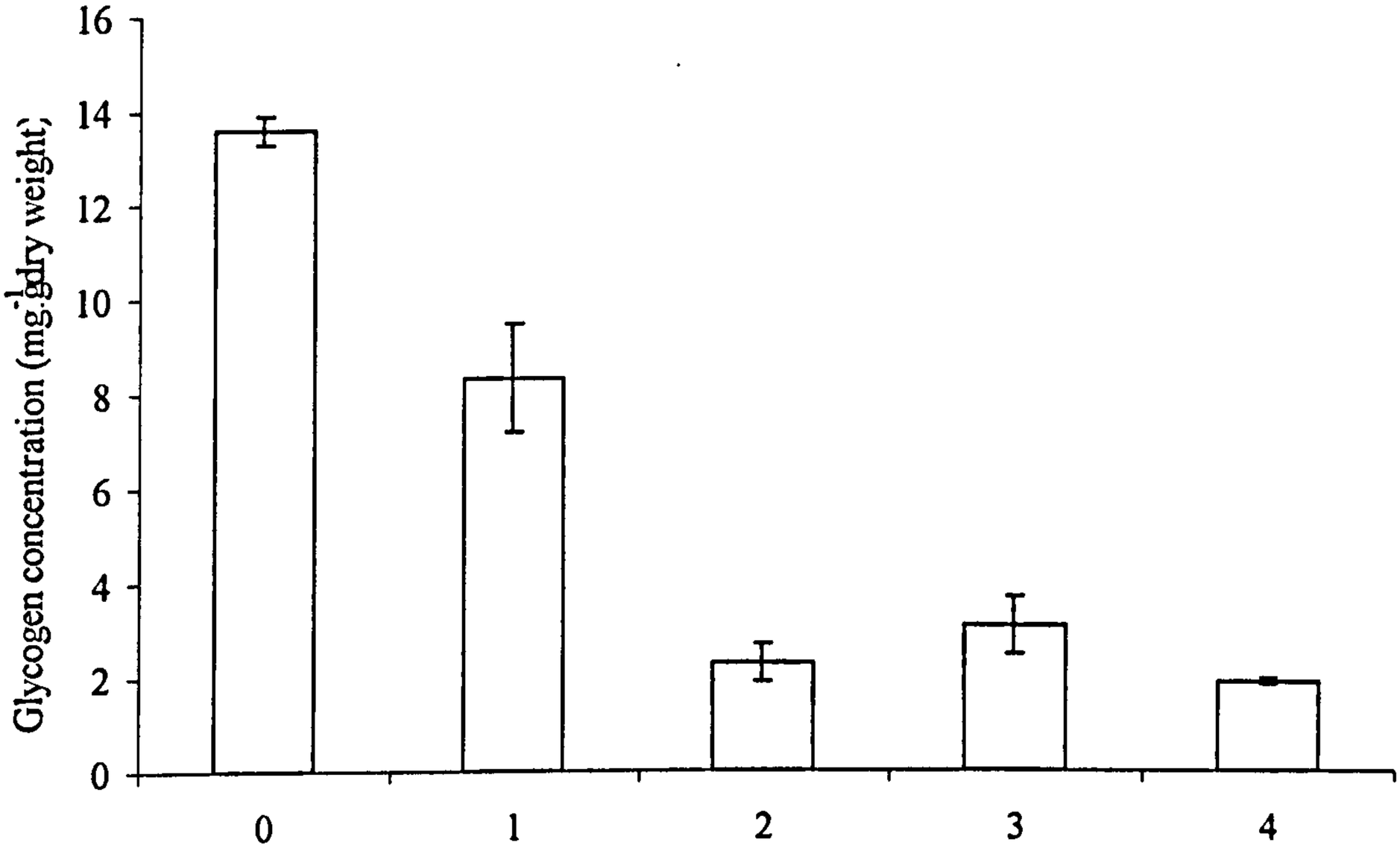
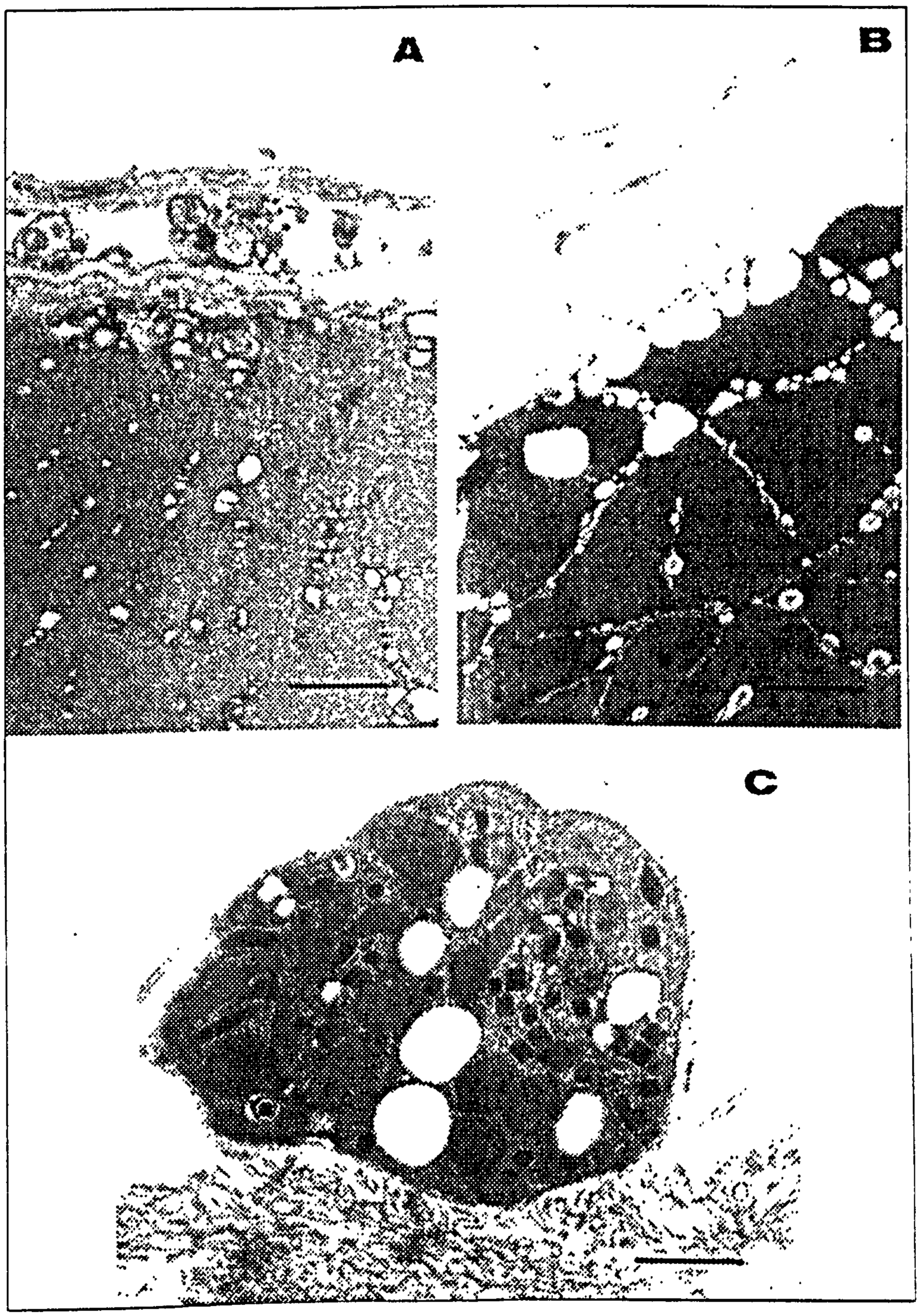


Fig 4 (A-C). (A). DAF muscle fibre from uninfected *N. norvegicus* showing an intact sarcolemma, tight junctions between adjacent myofibre bundles and a well-developed tubular system. Scale bar = 1μm. (B). DAF muscle from *Hematodinium*-infected *N. norvegicus* showing a disrupted sarcolemma and expansion of inter-bundle spaces in peripheral fibres. Scale bar = 1μm. (C). *Hematodinium* parasite cell on the outer surface of a DAF muscle fibre. Note the disorganisation and fibrous appearance of the sarcolemma. Scale bar = 1μm.



Chapter 6

Carbohydrate Dynamics and the Crustacean Hyperglycaemic Hormone (CHH): Effects of Parasitic Infection in Lobsters

Abstract.

The effects of a dinoflagellate parasite (*Hematodinium* sp.) on carbohydrate metabolism were examined in the Norway lobster *Nephrops norvegicus*. Five stages of infection were observed. These included uninfected (Stage 0), sub-patently infected, lightly infected (Stage 1), and moderately and heavily infected (Stages 2 and 3-4, respectively). We observed that lobsters that were in Stages 1-4 of infection had significantly lower levels of hemolymph glucose compared to uninfected or sub-patently infected animals. These results were accompanied by significantly lower levels of hepatopancreatic glycogen in Stages 2-4 compared to Stages 0-1. Due to the disruption of the normal feedback loops which control the release of crustacean hyperglycaemic hormone (CHH) from the sinus gland, plasma concentrations increase with infection severity. The increase in CHH concentrations occurs concomitantly with reduced concentrations of plasma glucose and tissue glycogen. We discuss these data with the perspective that the parasite places a heavy metabolic load on the host lobster.

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Introduction.

Stress responses occur in all animals when regulated physiological systems are extended beyond their normal range by external stressors. Indicators of such stress responses may therefore be useful in assessing the short-term well-being or long-term health status of an animal (Fossi *et al.*, 1997; Paterson and Spanoghe, 1997). Such indicators have received considerable attention in commercially important decapod crustacean species (Paterson and Spanoghe, 1997; Chang *et al.*, 1999) which, following capture, often require transportation to market. Post-capture economic losses in the North American lobster (*Homarus americanus*) fishery are estimated to be between $50 and $75 million annually (Cawthorn, 1997). Every step in the capture and post-capture processes has the potential to impose stress on captive animals, and under extreme conditions loss of the homeostatic regulation, by failure of all or part of the integrated response, may lead to increasing physiological disturbance and ultimately death (Morris and Airriess, 1998).

Another important stressor is the infection of an animal by parasites (see Thompson, 1983 for review). The Norway lobster (*Nephrops norvegicus*), which is the subject of an important commercial fishery in Scotland, is seasonally infected by a dinoflagellate of the genus *Hematodinium*, with prevalence levels reaching 70% in populations of *N. norvegicus* from the inshore waters off the west coast of Scotland (Field *et al.*, 1992). A number of studies have established the basic characteristics of *Hematodinium* infection in *N. norvegicus* in terms of its progression, diagnosis and pathology (Field and Appleton, 1995, 1996), its effect on host physiology (Taylor *et al.*, 1996), haemolymph and tissue biochemistry (Stentiford *et al.*, 1999 – Chapter 4, 2000b – Chapter 5) and locomotion (Stentiford *et al.*, 2000a – Chapter 8). The ability to estimate the infection's severity by a simple morphological index (Field and Appleton, 1995) and by an immunoassay for sub-patent infection (Field and Appleton, 1996) has greatly facilitated these epidemiological studies of *Hematodinium* infection in *N. norvegicus* (see Stentiford *et al.*, 1999 – Chapter

4). It therefore offers a very useful model system in which to investigate the stresses imparted by parasitic infection in crustaceans.

A number of researchers have suggested different methods for quantifying the stress reactions in crustaceans; these include the measurement of different hemocyte types in the haemolymph (Jussila *et al.*, 1997), physiological, biochemical (Paterson and Spanoghe, 1997; Stentiford *et al.*, 1999 – Chapter 4), and molecular changes in tissue and haemolymph (Fossi *et al.*, 1997) and the elevated transcription of heat shock proteins during periods of stress (Chang *et al.*, 1999). A response that has received recent attention is that of the change in the plasma titre of the crustacean hyperglycaemic hormone (CHH) in relation to imposed stressors (see Chang *et al.*, 1999).

The occurrence of some 'diabetogenic' factor from the eyestalks of crustaceans has been known for many years (Abramowitz *et al.*, 1944), as has evidence for the elevation of plasma glucose levels in crustaceans undergoing stress (Telford, 1968). The CHHs from several species have now been well described and in some cases, their amino acid profiles sequenced (see Keller *et al.*, 1985; Kegel *et al.*, 1989, 1991; Lacombe *et al.*, 1999 for review). These 8-9 kDa neuropeptides produced by perikarya in the medulla-terminalis X-organ, can be selectively released from the sinus gland into the blood stream (Santos and Keller, 1993b) after which they are known to target the hepatopancreatic plasma membranes (Kummer and Keller, 1993), the abdominal musculature (Santos and Keller, 1993a) and the hemocytes (Santos and Stefanello, 1991), with the liberated glucose either moving to the extracellular fraction by diffusion, or being converted intracellularly to lactate via glycolysis (Santos and Keller, 1993a). The advantage of such a system is not entirely understood, but due to the predictable elevation of plasma CHH during certain stresses (notably hypoxia) in crustaceans (Santos and Keller, 1993a; Webster, 1996; Chang *et al.*, 1999), it is envisaged that hyperglycaemia may be involved in the so-called 'flight or fright' response in these animals. An interesting feature of this process is that the lactate

resulting from enhanced glycolysis, released to the extracellular medium, could serve as a positive feedback mechanism for CHH release, with the increased CHH in turn stimulating glycogenolysis which then increases glucose availability (Santos and Keller, 1993b).

Variations in blood glucose have been observed in crustaceans under several different environmental and physiological conditions (such as extremes of temperature, salinity, anoxia, starvation and emersion – see Hall and van Ham, 1998) and also during parasitism (Stewart, 1980), and it is presumed that these changes may be at least partly mediated by alterations in plasma CHH. It has been shown that the plasma CHH titre is consistently increased during emersion and hypoxia and that this leads to elevated plasma glucose concentration (Santos and Keller, 1993a; Webster, 1996; Chang *et al.*, 1999). However, it has not yet been shown whether the plasma CHH titre is altered in relation to parasitism.

Therefore we have investigated the effect of progressive infection of the Norway lobster by *Hematodinium* parasites on the plasma CHH titre, as a possible sensitive indicator of stress, and assessed whether these changes may be implicated in the altered plasma glucose and tissue glycogen concentrations that often accompany these infestations.

Materials and Methods.

Collection and Treatment of Animals.

Norway lobsters were collected with an otter trawl from a location south of Little Cumbrae in the Clyde Sea area, Scotland, UK. Lobsters were maintained in a closed aquarium (9°C, 33 ppt salinity) at the University of Glasgow. Animals were allowed to settle in the aquarium for one week and were fed *ad libitum* on squid (*Loligo* spp.) and mussel (*Mytilus* spp.) tissue. Animals were not fed for three days prior to the experiment to avoid any effects of differential feeding and all animals were in the intermoult state as defined by Aiken (1980). Lobsters were staged for patent *Hematodinium* infection using the pleopod staging method of Field and Appleton (1995). According to the pleopod

staging method, Stage 1 denotes a lightly infected animal, with infection progressing through to Stage 4 (late stage infection). Stages 3 and 4 (heavily infected) animals were grouped in all analyses as animals in these groups show very similar disease characteristics (see Stentiford *et al.*, 1999 – Chapter 4). For diagnosis of true Stage 0 (uninfected) and sub-patent/latent infection, samples of hepatopancreas from previously diagnosed Stage 0 animals underwent western blot analysis using a polyclonal antibody against the *Nephrops norvegicus* isolate of *Hematodinium* sp. (see Field & Appleton, 1996 for details of antibody production). All animals showing a positive reaction to the anti-*Hematodinium* antibody in hepatopancreas tissue were diagnosed as sub-patent (i.e. *Hematodinium*-infected, but below the limit of detection using the pleopod staging method). Animals showing no reaction to the antibody were diagnosed as uninfected (Stage 0).

Chemicals.

For glycogen analyses, potassium hydroxide (KOH), ethanol and the anthrone and Folin-Ciocalteu's phenol reagents were obtained from Sigma-Aldrich Co. (Poole, UK). For glucose analysis, perchloric acid (PCA), sodium phosphate (dibasic), glucose and orthophosphoric acid (85 %) were obtained from Sigma-Aldrich Co., while the glucose oxidase assay kit was obtained from Boehringer-Mannheim (kit no. 124028). For CHH analysis, bovine serum albumin (fraction V), EDTA, glycine ethyl ester, Tween 20, and 2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid were obtained from Sigma-Aldrich Co., while the streptavidin-peroxidase solution was obtained from Boehringer-Mannheim.

Haemolymph and Tissue Preparation.

A total of 100 haemolymph samples from uninfected and *Hematodinium*-infected lobsters were drawn from the base of the fifth pereiopod into sterile syringes and centrifuged immediately at 17,000 x g for 10 min at 10°C to remove cellular material and suspended

debris. For the CHH assay, aliquots of plasma were frozen immediately in liquid nitrogen before freeze-drying for 24 h. Dried samples were stored at –20°C until transport to, and analysis at, the Bodega Marine Laboratory. For the glucose assay, the clear plasma was deproteinized using an equal volume of 0.6 M PCA for 10 min at 4°C. Protein precipitate was removed by centrifugation and the supernatant was stored briefly at -20°C until analysis at the University of Glasgow.

The hepatopancreata from 45 lobsters at different stages of *Hematodinium* infection were dissected out in physiological saline. Excised samples were blotted to remove excess moisture and weighed (wet weight) before rapid freezing in liquid nitrogen and freeze-drying for 36 h. Tissue was then re-weighed (dry weight) and samples were individually ground using a standard mortar and pestle. Ground samples were stored at –20°C until analysis of total glycogen content at the University of Glasgow.

Measurement of Plasma Glucose Concentration.

Glucose concentration in the haemolymph of uninfected and infected lobsters was measured using the glucose oxidase method (Boehringer-Mannheim) in a microplate method as described by Webster (1996). Briefly, 50 µl samples of deproteinised plasma were added to 450 µl of 0.2 M phosphate buffer (pH 7.4) and 100 µl samples of this solution were used in the assay with 200 µl of the enzyme chromogen reagent. Concentrations of plasma glucose were read from a standard curve constructed for glucose.

Measurement of Plasma CHH Titre.

The production of an antibody against purified CHH-A from *H. americanus* has been described by Chang *et al.* (1998) and previous studies have shown that *N. norvegicus* CHH can be detected using this antibody (G.D. Stentiford and E.S. Chang, unpublished data). In the current study, freeze-dried plasma samples were re-suspended to their original volume

with ddH_2O and assayed for CHH-A using the ELISA method of Chang *et al.* (1998).

HPLC-purified CHH from the crayfish *Orconectes limosus* (Kegel *et al.*, 1991) was used as the standard. Since purified *N. norvegicus* CHH was not available, it was not possible to quantify absolute concentrations of *N. norvegicus* CHH. The data were therefore presented as *O. limosus* equivalents, which allow relative quantification.

Measurement of Hepatopancreatic Glycogen Content.

For determination of muscle and hepatopancreatic glycogen, 400 µl of 30 % KOH was added to 20 mg of the freeze-dried samples from lobsters at different stages of infection, following which samples were boiled for 20 min in a water bath maintained at 95-100°C. Samples were cooled and added to 700 µl absolute ethanol before being placed on ice for 2 h. Following precipitation, samples were spun at 17,000 x g for 10 min and the supernatant discarded. One ml of ddH_2O was added to each sample before sonication. Fifty µl of each sonicated sample were incubated at 95-100°C in 1 ml of anthrone reagent before measurement of total glycogen (see Carroll *et al.*, 1956).

Data Analysis.

Comparisons of plasma glucose concentration, plasma CHH titre and hepatopancreatic glycogen content of uninfected and infected *N. norvegicus* were performed either by one-way analysis of variance (ANOVA) for normally distributed data, or by a Kruskal-Wallace test for non-normal distributions. Comparisons between stages were made with a Tukey's pairwise analysis (normal distributions) and a Mann-Whitney test (non-normal distributions). Significance was considered to be at $p < 0.05$.

Results.

Plasma Glucose Concentrations.

The mean concentration of glucose in the plasma of uninfected *N. norvegicus* was 180.0 $\mu g.ml^{-1}$ plasma. The glucose concentration in sub-patently infected lobsters (180.1 $\mu g.ml^{-1}$ plasma) was not significantly different to that of Stage 0 animals ($p = 0.979$), but patently infected animals of all stages showed significantly lower concentrations of plasma glucose than Stage 0; in Stage 1 (41.4 $\mu g.ml^{-1}$ plasma) ($p < 0.001$), Stage 2 (47.8 $\mu g.ml^{-1}$ plasma) ($p < 0.001$) and Stage 3-4 (25.3 $\mu g.ml^{-1}$ plasma) ($p < 0.001$). All patently infected animals also showed significantly lower concentrations of plasma glucose than sub-patently infected lobsters (all $p < 0.001$), but different stages of patently infected lobsters showed no significant difference in glucose concentration (all $p > 0.05$) (Fig.1).

Hepatopancreatic Glycogen Concentrations.

The mean glycogen concentration of the hepatopancreas of uninfected *N. norvegicus* at 16.19 $mg.g^{-1}$ dry weight was slightly higher than that of the deep abdominal flexor muscle (Stentiford *et al.*, 2000b – Chapter 5). Hepatopancreatic glycogen concentration was reduced during patent *Hematodinium* infection. The reduction was not significant in Stage 1 lobsters (14.71 $mg.g^{-1}$ dry weight, $p = 0.574$), but was highly significant in both Stage 2 (2.01 $mg.g^{-1}$ dry weight, $p < 0.001$) and Stage 3-4 (0.84 $mg.g^{-1}$ dry weight, $p < 0.001$) *Hematodinium* infection. When different stages of infection were compared, significant reductions in hepatopancreatic glycogen were found between Stage 1 and Stage 2 ($p < 0.001$) and Stage 1 and Stage 3-4 ($p < 0.01$), but not between Stage 2 and Stage 3-4 ($p = 0.098$) (Fig. 2).

Plasma CHH concentrations.

The mean concentration of CHH in the plasma of uninfected *N. norvegicus* was 32.2 fmol.ml^{-1}. The mean concentration of CHH in the plasma of sub-patently infected lobsters (107.65 fmol.ml^{-1}) was significantly higher than that of Stage 0 animals ($p < 0.05$), while at Stage 1 (light patent infection), the mean concentration was higher (though not significantly, $p = 0.057$) than Stage 0, and lower (though not significantly, $p = 0.070$) than sub-patently infected lobsters. In later stages of patent *Hematodinium* infection, the plasma CHH concentration was significantly higher than that of uninfected animals; in Stage 2 (77.2 fmol.ml^{-1}, $p < 0.001$) and Stage 3-4 (106.6 fmol.ml^{-1}, $p < 0.001$). The significant increase in plasma CHH concentration between Stage 1 and Stage 3-4 animals ($p < 0.05$) and almost between Stage 1 and Stage 2 animals ($p = 0.080$) suggests that the titre of CHH increases with severity of patent infection. It is interesting to note however that the mean plasma CHH titre of sub-patently infected lobsters is not significantly different to that of Stage 3-4 lobsters ($p = 0.997$) (Fig. 3).

Discussion.

Carbohydrate Dynamics in Hematodinium-infected N. norvegicus.

The current study has identified large alterations in the carbohydrate profile in the plasma and tissues, and in the crustacean hyperglycaemic hormone (CHH) titre in the plasma, of *N. norvegicus* infected by the parasitic dinoflagellate *Hematodinium* sp. The concentration of glucose in the plasma remains relatively unchanged in sub-patently infected lobsters, but is significantly reduced during all stages of patent infection. In Stage 1 infected lobsters the plasma glucose is already reduced to approximately 30 % of its normal value, and this reduction in early infection suggests that glucose provides a readily usable substrate for the growth of the *Hematodinium* parasite in the haemolymph of *N. norvegicus*. Similar reductions in the concentrations of simple carbohydrates in the host tissues have been reported for other parasitic infestations in crustaceans (Stewart, 1980; Cawthorn, 1997) and

insects (Schmidt and Platzer, 1980), and in these cases it has been suggested that the parasites may be acting as a 'carbohydrate sink', absorbing haemolymph glucose and thereby forcing the host to re-supply glucose to the haemolymph to maintain carbohydrate homeostasis. The re-supply of glucose to the plasma occurs mainly via tissue-based glycogenolysis, and the main storage tissues for such polysaccharides in crustaceans are the hepatopancreas (Dall and Moriarty, 1983), the muscle (Scwoch, 1972) and the hemocytes (Johnson *et al.*, 1971). The dramatic decrease in the concentration of glycogen in the hepatopancreas that occurs between Stage 1 and Stage 2 of infection (Fig. 2) and the similar depletion found to occur in the deep abdominal flexor muscle of *Hematodinium*-infected *N. norvegicus* (Stentiford *et al.*, 2000b – Chapter 5) are consistent with a response to an elevated burden of circulating parasites (Field *et al.*, 1992).

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). The cellular reception of CHH neuropeptide molecules elicits the mobilization of glucose from intracellular glycogen stores via glycogenolysis (Santos and Keller, 1993b), with the liberated glucose either moving to the extracellular fraction by diffusion, or being converted intracellularly to lactate via glycolysis (Santos and Keller, 1993a).

In patently infected lobsters, the plasma CHH concentration shows a steady and significant increase in relation to infection severity (Fig.3), which is mirrored by a progressive decrease in the concentration of plasma glucose (Fig.1). The co-existence of a high plasma CHH concentration and a low plasma glucose concentration, together with the significant reduction in tissue glycogen stores noted both in this study (Fig 2) and a previous study (Stentiford *et al.*, 2000b – Chapter 5), is the opposite of what may be expected in a normally-operating feedback system and is probably due to a disruption of the normal carbohydrate feedback control loop by the parasites. As infection progresses, the developing parasites will consume the glucose being liberated from the tissue glycogen

reserves, lowering circulating glucose concentrations and thereby reducing the negative feedback on CHH release from the sinus glands (see step (1) in Fig. 4 and Santos and Keller, 1993a). This, in turn, will initiate an increased release of CHH (step (2) in Fig. 4), inducing progressive glycogen depletion of the tissues (step (3) in Fig. 4). As the parasite burden increases, a steadily-reducing plasma glucose concentration, a progressive depletion of tissue glycogen stores and an increasingly elevated plasma CHH titre are all to be expected.

In addition to this change in negative-feedback control of CHH release by plasma glucose, the increased plasma lactate levels that are known to occur during *Hematodinium* infection (Taylor *et al.*, 1996) may also stimulate CHH release, with consequent effects on glycogenolysis (Santos and Keller, 1993b). The most likely cause of these elevated plasma lactate levels is a switch to anaerobic metabolism, which Taylor *et al.* (1996) have recorded in terms of increased lactate and a lowered haemolymph pH, a situation which is exacerbated by a reduction in the oxygen carrying capacity of the haemolymph. At the cellular level, pyruvate, the end product of glycolysis, is converted under anaerobic conditions to lactate, which either accumulates within the cell or is moved to the extracellular medium (step (4) in Fig. 4).

Potentially, tissue glycogen reserves could be replenished from the lactate by the process of gluconeogenesis, but this is unlikely to be significant since in invertebrates, these pathways are thought to be relatively inefficient compared with the Cori cycle in mammals (Schulman and Landau, 1992), and probably operate under aerobic conditions (Ellington, 1983). Moreover, gluconeogenesis is antagonized by CHH itself, which is known to activate cyclic nucleotide-dependent protein kinases, leading to a phosphorylation and therefore inhibition of glycogen synthase (Sedlmeier, 1985; Santos and Keller, 1993a), and is also inhibited by hyperosmolarity (Li *et al.*, 1992), which is known to occur in *Hematodinium* infection (Stentiford *et al.*, 1999 – Chapter 4). It is most likely, therefore,

that the elevated plasma CHH concentration in patent infection is due primarily to a 'functional hypoxia' in the infected lobster, which elicits a cascade response similar to that seen during the 'environmental hypoxia' caused by emersion (Santos and Keller, 1993a; Webster, 1996; Chang *et al.*, 1999).

Implications of Altered Carbohydrate Dynamics during Hematodinium Infection.

Studies on the physiological condition of crustaceans over the entire moulting cycle have shown that the carbohydrate reserves are generally highest in the late intermoult period (Icely and Nott, 1992) and are also dependent upon the reproductive cycle (Tuck *et al.*, 1997). In terms of parasitic infection, Schmidt and Platzer (1980) note that the condition of the host may establish the degree to which the parasite develops, with hosts in the best physiological condition harbouring the largest parasite burdens.

The prevalence of *Hematodinium* infection is known to be highest in *N. norvegicus* during the spring (Field and Appleton, 1995) and in females (G. D. Stentiford, unpublished data). Female crustaceans have relatively larger amounts of hepatopancreatic tissue than males as a means of supplying the nutritional requirements for egg rearing and spawning (Farmer, 1974a). In *N. norvegicus*, this egg-rearing period may last for up to 8 months of the year, during which time the female lobster remains for the most part, within the burrow (Farmer, 1974b). The higher incidence of *Hematodinium* infection in female lobsters may reflect some relative advantage of the female host to the parasite.

Recently, it has been shown that the concentration of CHH in the plasma is increased by up to 100-fold in the hours leading up to moulting, with levels returning to normal following ecdysis (Chung *et al.*, 1999). The spring peak of *Hematodinium* infection in Scottish *N. norvegicus* coincides with the onset of the main moulting period for this species (Field *et al.*, 1998) and it is tempting to suggest that a greatly increased concentration of CHH in the plasma at this time may create haemolymph conditions that are suitable for

rapid growth of the parasite population. The significantly elevated concentration of CHH in the plasma of sub-patently infected lobsters found in this study could either be responsible for, or caused by, the appearance of *Hematodinium* parasites in the haemolymph following latent infection. Further studies on the effect of elevated CHH concentration on latently infected lobsters are necessary to investigate the role of this hormone in initiation of haemolymph infection in *N. norvegicus*. An increase in plasma CHH titre due to other stressors (such as seasonal hypoxia, temperature changes or pollution) could also be implicated in infection progression.

As the main carbohydrate resources become exhausted, parasites may switch to alternative, and less efficient forms of energy such as fatty acids, proteins and free amino acids (Schmidt and Platzer, 1980; Thompson and Dahlman, 1998). Studies of plasma and tissue free amino acid and protein dynamics during *Hematodinium* infection of *N. norvegicus* have shown that the normal profile of these compounds is considerably altered as infection severity increases (Taylor *et al.*, 1996; Stentiford *et al.*, 1999 – Chapter 4, 2000b – Chapter 5). It is likely that such changes occur following exhaustion of the most accessible and high-yielding forms of nutrition: the plasma and tissue carbohydrates. Under such conditions, the host will reach a state of physiological starvation from which it cannot recover, and at this stage the post-parasitic forms emerge and the host will die (Schmidt and Platzer, 1980). Aquarium observations of *Hematodinium*-infected *N. norvegicus* have identified an apparently similar response, where swarming stages of the parasite are extruded via the integumental membranes, gills and the mouth. This leaves the host moribund and then dead within 24 h (Field and Appleton, 1995; Appleton and Vickerman, 1998). It may be suggested that sporulation of the *Hematodinium* parasite within, and exit from the host occurs when the parasite has exhausted the host's nutrient reserves. Host condition may then also play an important role in determining the timing of sporulation of *Hematodinium* in *N. norvegicus* and may explain why sporulation has been observed in

some lobsters displaying only Stage 1 and 2 infection (Appleton and Vickerman, 1998).

During the terminal stages of *Hematodinium* infection, the arthrodial membranes appear swollen and the haemolymph volume is apparently increased (Appleton and Vickerman, 1998). Increased water uptake has been associated with high concentrations of CHH in the plasma of *Carcinus maenas* preceding ecdysis (Chung *et al.*, 1999). It is possible to suggest therefore, that elevated levels of plasma CHH within, or preceding, the final stages of *Hematodinium* infection in *N. norvegicus* could be involved with the observed swelling of arthrodial membranes and may facilitate the exit of motile dinospores (sporulation) from the host lobster.

The current study has, for the first time, identified a link between the disruption of carbohydrate handling during parasitic infection of crustaceans and alterations in the expression of the crustacean hyperglycemic hormone. Whether the increase in the plasma CHH titre is caused directly by parasitic disruption of the lobster's endocrine system, or indirectly, by interfering with the positive and negative feedback loops in the plasma, requires further investigation. However, future studies which consider plasma and tissue carbohydrate dynamics in crustaceans should take into account that the relatively large variations often seen in the hyperglycemic response (Hall and van Ham, 1998), while being related to the moult stage of the test animal, may also be due to its overall health status.

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Fig. 1. Concentration of glucose ($\mu g.ml^{-1}$) in the plasma of uninfected (Stage 0) and *Hematodinium*-infected (sub-patent and Stages 1, 2. 3-4) *N. norvegicus*.

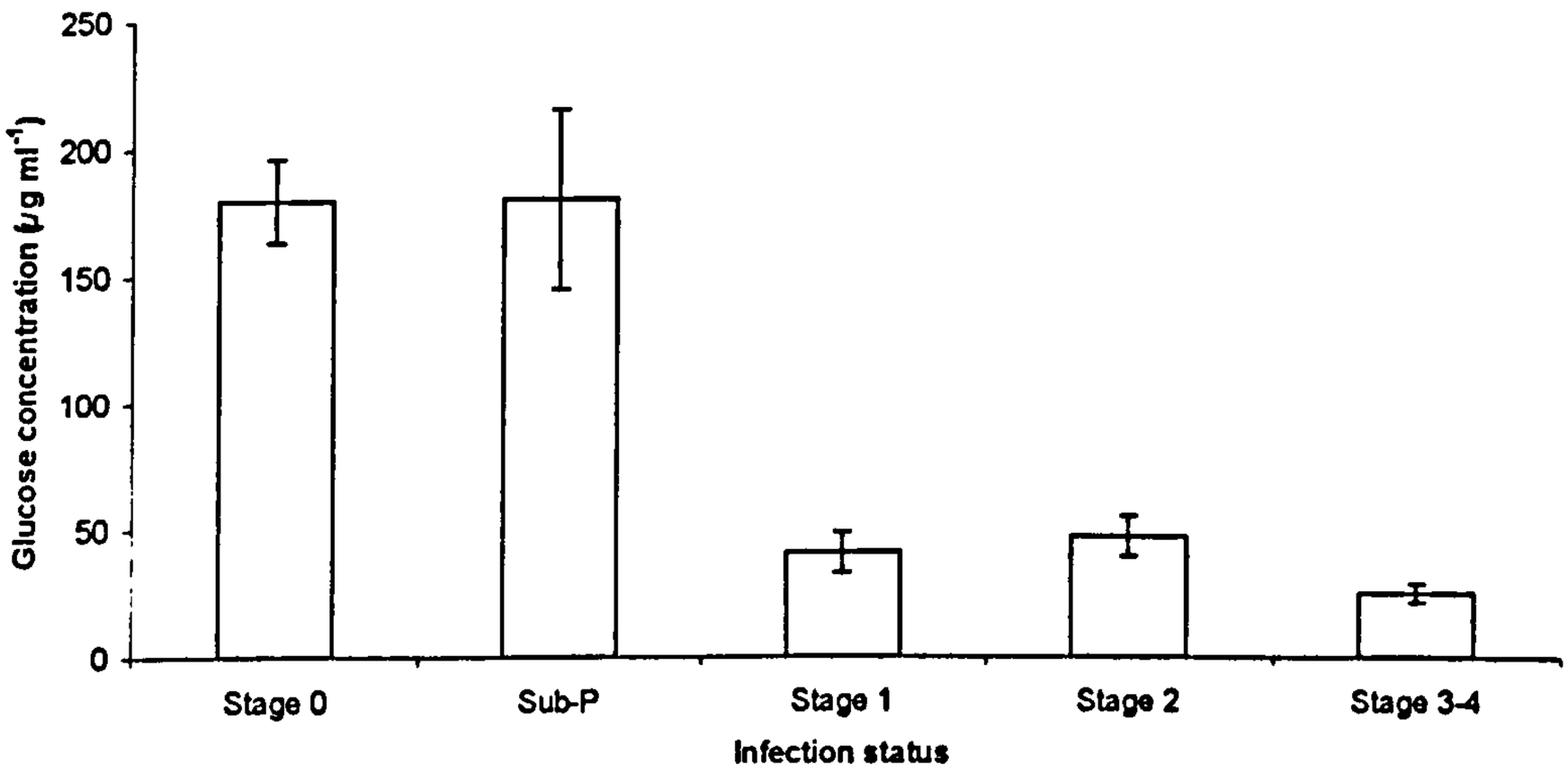


Fig. 2. Concentration of glycogen ($mg.g^{-1}$ dry weight) in the hepatopancreas of uninfected (Stage 0) and *Hematodinium*-infected (Stages 1, 2 3-4) *N. norvegicus*.

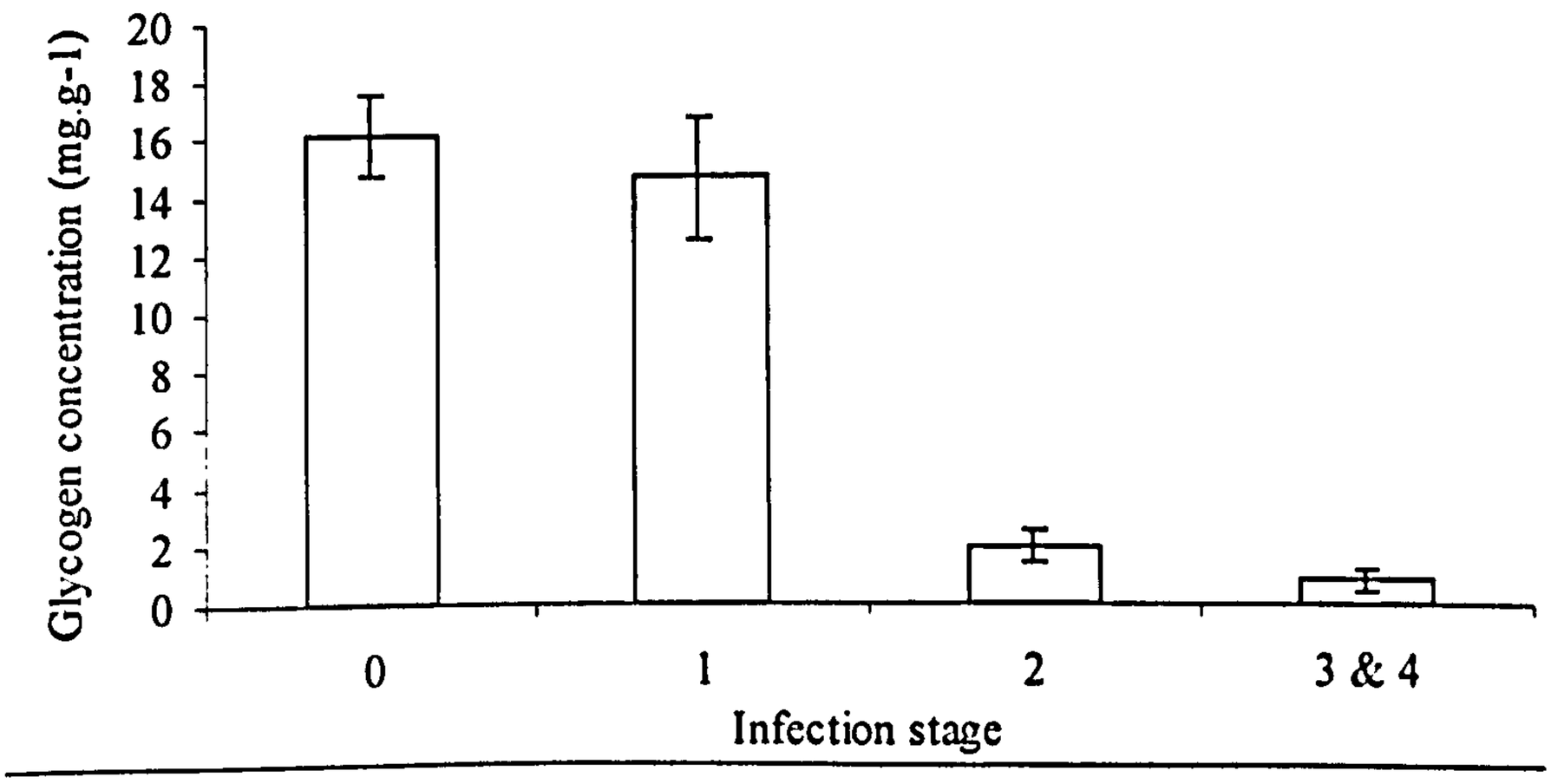
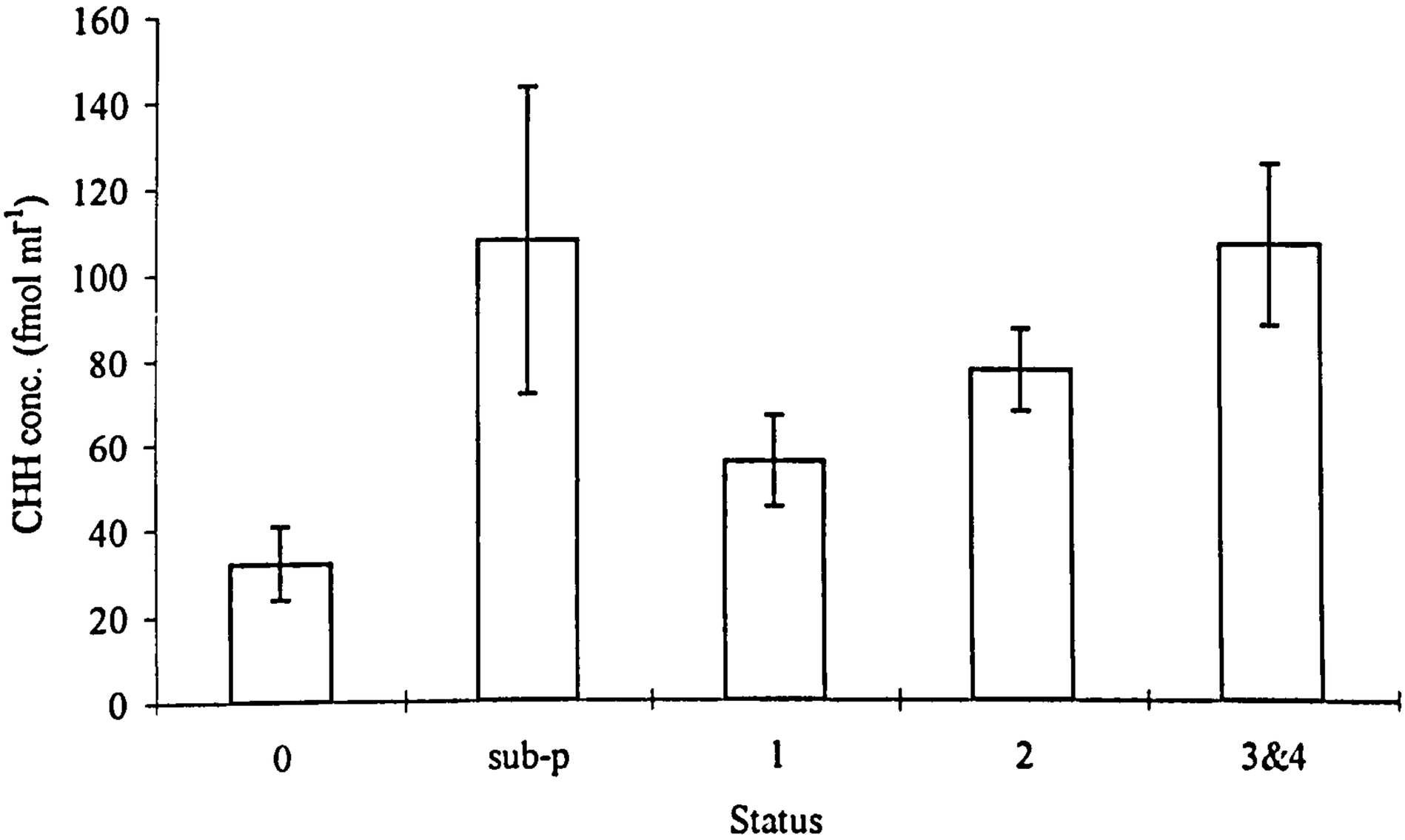


Fig. 3. Concentration of the crustacean hyperglycaemic hormone (CHH) (fmol.ml^{-1}) in the plasma of uninfected (Stage 0) and *Hematodinium*-infected (sub-patent and Stages 1, 2, 3-4) *N. norvegicus*.



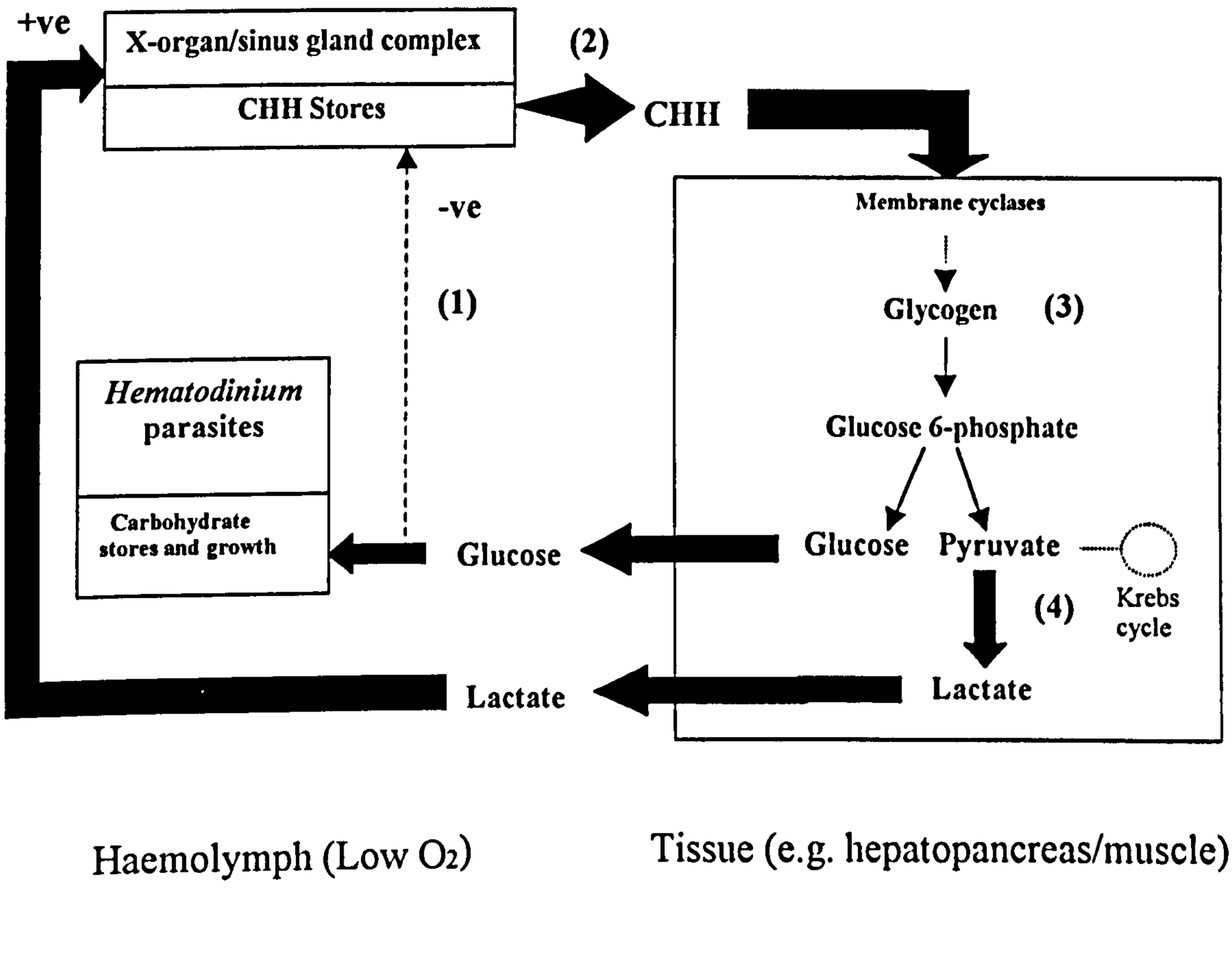


Fig. 4. Schematic diagram of the changing relationship between tissue glycogen, plasma glucose and CHH release in the presence of *Hematodinium* parasites in the plasma of *N. norvegicus* (adapted from Santos & Keller, 1993a). For description see discussion text.

Chapter 7

A rapid onset, post-capture muscle necrosis in the Norway lobster, *Nephrops norvegicus* (L.) from the west coast of Scotland, United Kingdom.

Abstract.

A post-capture, abdominal muscle necrosis of rapid onset has been identified in Norway lobsters (*Nephrops norvegicus*) captured off the west coast of Scotland, United Kingdom. Economic losses, due to mortality of these animals in transport, were encountered by Scottish wholesalers during the summer and autumn of 1999. Affected animals show a characteristic whitening of individual muscle fibres and fibre bundles of the abdomen within hours of capture, with a progression towards complete opacity of the abdominal musculature within a number of days. The pathology causes a loss of the normal function of the abdomen, thus preventing the normal 'tail flip' swimming. Electron microscopy failed to reveal any obvious causative agent but showed that affected tissue displayed a progressive disruption of sarcomere organization, loss of Z-line material, condensation of myofibrils and infiltration of necrotic regions by granulocytes. SDS-PAGE of affected muscle tissue showed that there was a great reduction of most of the major contractile proteins. The condition most closely resembles idiopathic or spontaneous muscle necrosis, a pathology previously reported from both wild and cultured crustaceans. Damage to the integument in conjunction with exposure to various stressors during and immediately following capture is the most likely cause of the pathology. The rapid onset of the pathology has implications for the post-capture handling procedure for *N. norvegicus* and their subsequent vivier transport to market. It may also be partially responsible for the high mortality rate of undersized *N. norvegicus* returned to the sea after capture and aerial emersion.

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Introduction.

Pathological damage to crustacean muscle has been widely reported and is known to have various causative agents, including microsporidians (Findley *et al.*, 1981; Langdon, 1991; Kabre, 1992; Dennis & Munday, 1994; Childers *et al.*, 1996), ciliates (Cawthorn, 1997), bacteria (Stewart, 1980), dinoflagellates (Shields, 1994; Stentiford *et al.*, 2000 – Chapter 5) and viruses (Arcier *et al.*, 1999). A number of other studies have described muscle wastage with unknown etiology (Lindqvist & Mikkola, 1978), while host-induced (idiopathic) muscle necrosis has been reported to occur in conditions of natural or artificial stress, both in the wild and under aquaculture situations (Akiyama *et al.*, 1982; Momoyama & Matsuzato, 1987; Nash *et al.*, 1987; Anderson *et al.*, 1990; Evans *et al.* 1999). Idiopathic muscle necrosis (also known as 'tail rot', muscle opacity, muscle necrosis, and spontaneous muscle necrosis) is characterized by focal to gross opaque lesions in the striated musculature of the abdomen. The lesions are composed of necrotic muscle cells with disorganized myofibrils, loss of sarcomeres, loss and atrophy of mitochondria and damage to the sarcoplasmic reticulum (Akiyama *et al.* 1982). Late stage necrosis is characterized by fragmentation and condensation of myofibrillar bundles (Nash *et al.*, 1987). Hemocytic infiltration of the damaged musculature is also commonly observed, these participating in aggregation, encapsulation and, in the late stages of necrosis, myophagia (Momoyama & Matsuzato, 1987, Nash *et al.*, 1987 and Anderson *et al.*, 1990).

Nephrops norvegicus supports a major fishery in the United Kingdom, with the Scottish fishery contributing over 76% of this (£57 million in 1999 – FRS, 1999). The majority of the landings are from trawler capture, with the animals being 'tailed' for sale as 'scampi'. Larger animals are also captured by baited creels and are often exported to continental Europe by specially-designed 'vivier' vehicles. In order to ensure sale, exported live lobsters must be in good condition on arrival at market.

During the summer and autumn of 1999, creel-caught lobsters from the Sound of Jura (west Scotland) and other west coast sites were reported to be dying or moribund during vivier transport, with some catches being refused at market due to the opaque appearance of their tail meat (Mr. G. Goldsworthy, personal communication). Signs of this condition were also noted in animals taken directly from creels, and in trawl-caught animals from the Clyde sea area (west Scotland) which were subsequently maintained in aquaria at the University of Glasgow.

This study was carried out to establish the epidemiology of this condition in the Scottish west coast *Nephrops norvegicus* fishery and to describe its pathology and progression by using histological and biochemical techniques.

Methods.

Norway lobsters showing symptoms of the described condition (opaque abdominal musculature and inability to flex the abdomen) when caught in creels in the Sound of Jura (see Fig. 1) were collected from Loch Fyne Fisheries Co., Tarbert, west Scotland, UK. Trawl-caught lobsters from the Clyde sea area (Fig. 1), were maintained post-capture in the aquarium at the Division of Environmental and Evolutionary Biology, University of Glasgow, Scotland, UK. Clyde lobsters were fed *ad libitum* on mussels (*Mytilus edulis*) until preparation of abdominal muscle for histology and SDS-PAGE, while muscle tissue from the Sound of Jura lobsters was immediately prepared for histology.

In order to establish the rate of progression of this condition, 200 lobsters captured in the Clyde sea area in January 2000 by standard otter trawling (Fig.1) were selected randomly from a sub-sample and assessed for signs of muscle opacity immediately following capture, and again four hours post-capture, after holding them on the deck in a standard fish box covered with a damp sack. In those showing symptoms of the condition, individual segments of the abdomen were visually assessed for the location of muscle opacity. In

order to investigate possible reasons for the onset of the condition within the abdomen, any damage to the integument in the vicinity of the necrosis was recorded by probing the area with a blunt dissection needle. All lobsters were also staged for infection by the dinoflagellate parasite *Hematodinium* sp., which is known to affect *N. norvegicus* in these regions (Field & Appleton, 1995). All lobsters were diagnosed as Stage 0 (uninfected). Histological samples from these animals were compared to previously collected muscle samples from *Hematodinium*-infected lobsters.

Histology

The deep flexor and medial superficial flexor muscles from the abdomen of *N. norvegicus* were exposed by removal of the abdominal cuticle and underlying extensor muscles. Small blocks of opaque muscle and muscle fibre bundles showing opacity along part of their length were removed under a dissecting microscope. Muscle tissue samples were also taken from animals diagnosed with *Hematodinium* infection. Muscles were fixed for two hours in a solution containing 4 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, with 2 % sucrose and 1.5 % sodium chloride for 2 hours at room temperature. Fixed samples were then rinsed in 0.1 M phosphate buffer with 4 % sucrose and post-fixed in 1 % osmium tetroxide in phosphate buffer for 1 hour. Specimens were washed in several changes of distilled water and block stained in 0.5 % uranyl acetate for 1 hour. Following dehydration through an ethanol series, specimens were embedded in Spurr resin (Spurr, 1969). Thick sections (1 μm) were stained with toluidine blue for viewing with a light microscope and suitable areas were cut and mounted on uncoated copper/palladium grids and stained with uranyl acetate and lead citrate. Thin sections (60-70 nm) were examined in a Zeiss 902 transmission electron microscope.

SDS-PAGE

For SDS-PAGE analysis of deep abdominal flexor muscle from lobsters showing symptoms of muscle opacity, individual muscle fibres were dissected out under calcium-free *Nephrops* physiological saline and placed into 200 µl of SDS-sample buffer, denatured at 95°C for 4 minutes and stored at –20°C until electrophoretic protein separation (see Neil *et al.* 1993). Discontinuous SDS-PAGE was performed according to the method of Laemmli (1970) with gels containing 12.5 % acrylamide separating gel and 4 % acrylamide stacking gel. Electrophoresis running conditions were as for those described by Neil *et al.* (1993), followed by overnight incubation in 10% TCA and staining for 30 minutes in Coomassie-blue solution. Stained gels were later imaged using an Appligene™ gel imager. Opaque muscle fibres were compared to fibres from lobsters showing no symptoms of muscle opacity.

Results.

In the early stages of the condition in *Nephrops norvegicus*, individual muscle fibres of the abdomen appeared opaque when viewed through the ventral membrane, with the opacity progressing to whole muscle fibre bundles and eventually the whole abdomen. In advanced cases, the abdomen did not respond to normal physical stimuli and thus affected lobsters could not initiate a normal 'tail-flip' response, while the thoracic limb system appeared to function normally. Progression of the condition was rapid, leading to death of affected lobsters within days.

Light microscopy revealed that in the abdominal superficial flexor muscles of animals showing early stages of the pathology (individual opaque muscle fibres), the peripheral areas of the muscle appeared damaged, with disruption to the sarcolemma and infiltration of the damaged regions by granulocytes (Fig.2a). However, other areas contained intact myofibrillar structure, with regular sarcomere arrangements. The deep abdominal flexor

muscles, which form the major muscle mass within the abdomen, were also progressively affected by the necrosis, and in severe cases there was a complete loss of sarcomeric structure and a condensation of myofibrillar bundles (Fig.2b). In some instances, the pathology was clearly progressing along individual myofibrillar bundles, with areas of semi-intact muscle being found immediately adjacent to areas of complete muscle degeneration, the latter being heavily infiltrated by granulocytes (Fig.2c).

Examination of the necrotic tissue using electron microcopy revealed that the normal sarcomeric structure observed in unaffected animals (Fig.3a) was initially altered by expansion of the space occupied by the tubular system and the appearance of granular material in the cytoplasm (Fig.3b). Regions of the muscle with a fairly intact myofibrillar structure also displayed some erosion of the sarcomeres, with degeneration originating in the vicinity of the Z-line (Fig.3c). Penetrating between the myofibre interstices of relatively unaffected bundles were sinuous structures, containing filaments (Fig.2d and 4a). In regions of pathological progression, the junctions between intact and degenerated muscle (see also Fig.2b) were characterized by a dissolution of myofibrillar bundles into granular material (Fig.4b).

In areas of severe muscle necrosis in *N. norvegicus*, there was a partial to complete loss of myofibrillar and sarcolemmal structure, with components of the myofibrillar membrane system often the only recognizable feature in the granular cytoplasm separating islands of densely-staining, condensed myofibrillar bundles (Fig.5a). Necrotic regions of muscle were also characterized by aggregations of atrophied mitochondria within the granular cytoplasm (Fig.5b), the appearance of myelin figures (Fig.5c) and the infiltration of the damaged tissue by granulocytes (Fig.2c and 5d). Such areas occasionally contained unidentified inclusions measuring 100-150nm in diameter (Fig.6).

A certain degree of muscle breakdown was also found to occur in the abdominal muscles of *N. norvegicus* heavily infected by the dinoflagellate parasite *Hematodinium* (Fig.3d).

However, in contrast to the effects of necrosis, this mainly involved erosion of groups of myofilaments within the sarcomeres, with the Z-line region remaining relatively unaffected.

SDS-PAGE analysis of the necrotic condition revealed a major loss of contractile proteins from opaque deep abdominal flexor muscle fibres compared with their amounts in unaffected fibres (Fig.7). The myosin heavy chain was completely absent, while paramyosin, troponin-T, actin, tropomyosin, troponin-I isoforms and the myosin α-light chain were significantly reduced in quantity.

In a study of trawled *N. norvegicus*, immediately following capture the onset of the necrotic pathology was seen in at least one abdominal muscle fibre bundle in 8 % of the 200 lobsters. When these animals were re-examined 4 hours later, after holding them in a covered fish box on deck, this prevalence had increased to 29 % (Fig.8a). In the affected lobsters, opacity was found in various abdominal segments, with those adjacent to the cephalothorax being the most commonly affected (Fig.8b). It is perhaps significant that visible damage to the abdominal integument (tearing or puncturing) was observed in the vicinity of opaque lesions in 46.5 % of the lobsters with symptoms of muscle necrosis. Small, undetectable lesions or minor damage to the main carapace may have been present in other animals, though this was not investigated.

Discussion.

Lack of causative agent

The current study has identified a post-capture, abdominal muscle necrosis of rapid onset in the Norway lobster, *Nephrops norvegicus* captured from the west coast of Scotland. Externally, the condition resembles that of a microspordian infection. These are common pathogens of crustacean tissue, often causing severe softening, opacity and general degeneration of the musculature (Breed & Olson, 1977; Findley *et al.*, 1981; Olson & Laman, 1984; Langdon,

1991; Dennis & Munday, 1994; Childers *et al.*, 1996). However, a number of features of the observed necrosis are counter-indicative of microsporidian involvement. Firstly, progression of microsporidiosis tends to be much slower than that observed for the condition in *N. norvegicus*. Breed & Olson (1977) report that progression from a light to a heavy microsporidian infection takes approximately 120 days in crangonid shrimps. The condition observed in *N. norvegicus* in this study had a much shorter time course, progressing significantly over a period of 4 hours following trawl capture, and leading to death within a further 3-4 days (a feature also noted for spontaneous muscle necrosis *in P. aztecus* – Rigdon & Baxter, 1970). Secondly, no microsporidian life stages were visible in any of the histological sections of abdominal muscle tissue, examined by either the light- or electron-microscopy.

Indeed, no other pathogenic agents were detected within the abdominal musculature, although in certain specimens some unidentified bodies of approximately 150nm in diameter were detected within severely damaged regions of muscle (see Fig.6). The possibility that these bodies are viruses cannot be ruled out at present, especially as viruses are known to cause a similar necrosis condition in post-larval stages of the freshwater prawn, *M. rosenbergii* (Arcier *et al.*, 1999). The identity of these bodies and their possible involvement in the muscle necrosis in *N. norvegicus* requires further investigation. There is also no evidence for any bacterial involvement in the pathology seen in *N. norvegicus*, though bacteria have also been implicated in a similar muscle necrosis and rapid death syndrome in *M. rosenbergii* (Cheng & Chen, 1998) and may also be a secondary consequence of necrosis in the same species (Nash *et al.*, 1987).

The pathology of necrosis

Due to absence of any obvious causative agent, the seasonal nature of the condition in creel-caught animals (appearing in the summer and autumn) and the propensity for trawled animals to become affected even during the winter months, the opaque muscle condition noted in *N. norvegicus* resembles the spontaneous, idiopathic muscle necrosis noted in a number of

crustacean species; in *Cherax terminatus* (Evans *et.al*, 1999), *M.rosenbergii* (Delves-Broughton & Poupard, 1976; Akiyama *et al.*, 1982; Nash *et al.*, 1987; Anderson *et al.*, 1990), *Penaeus aztecus* (Rigdon & Baxter, 1970, Lakshmi *et al.* 1978), *Penaeus japonicus* (Momoyama & Matsuzato, 1987), *Procambarus clarkii* (Linqvist & Mikkola, 1978) and also in the fish *Stizostedion vitreum* (Holloway & Smith, 1982).

Indeed, histological features of the condition in *N. norvegicus*, including a general loss of myofibrillar and sarcomeric structure, coupled with differential reaction of necrotic muscle tissue to histological staining, are similar to those reported in muscle necrosis of *P. japonicus* (Momoyama & Matsuzato, 1987) and *P. aztecus* (Rigdon & Baxter, 1970). The appearance of sinuous filaments with a fibrous structure is similar to that noted during muscle necrosis in *P. japonicus*, where the production of collagenous fibres was extensive in mid to late progression of the condition (Momoyama & Matsuzato, 1987). The presence of myelin figures and atrophied mitochondria in conjunction with infiltration of degenerated areas of tissue by granulocytes is consistent with the features ascribed to idiopathic muscle necrosis in *M. rosenbergii* (Nash *et al.* 1987).

Loss of Z-line material, which is a prominent feature of necrosis in *N. norvegicus*, is reported to occur in a number of pathological and physiological conditions (Kumudavalli Reddy *et al.*, 1975) and also represents an early step in pre-moult muscle atrophy in crustaceans (Mykles & Skinner, 1990a). The calcium-dependent proteases involved in pre-moult atrophy are localized in the sarcoplasm (Mykles & Skinner, 1990b) and it is conceivable that the necrotic condition observed in *N. norvegicus* involves activation of these proteases, leading to the initial breakdown of Z-line material. Interestingly, the breakdown of abdominal muscle fibres induced by *Hematodinium* infection in *N. norvegicus* does not disrupt the Z-line, but rather involves the erosion of groups of myofilaments within the sarcomeres. This suggests that the proteolytic processes that occur in this parasitic infection are different to those occurring in necrosis.

Factors which induce the onset of necrosis.

This study has shown that muscle opacity can be observed in all segments of the abdomen, but with a tendency for the condition to occur in the segments adjacent to the cephalothorax. A similar feature was noted in idiopathic muscle necrosis in *Penaeus japonicus* (Momoyama & Matsuzato, 1987) and *C. tenuimanus* (Evans *et al.*,1999), while others have reported the necrotic condition to be associated with the more distal abdominal segments in *P. aztecus* (Rigdon & Baxter, 1970, Lakshmi *et al.*, 1978) and *M. rosenbergii* (Akiyama *et al.*, 1982, Nash *et al.*, 1987). The mechanism underlying the location of lesions is not known, but it is possible that breaches in the integument (seen in almost half of those lobsters exhibiting symptoms of the pathology) may provide foci for initiation of necrosis, the progression of which is exacerbated by subsequently imposed stressors.

It is well known that crustaceans are exposed to a considerable array of stressors during and after capture (including crowding, air exposure, light exposure, heat exposure and mechanical damage) and a number of studies have considered the expression of biochemical and molecular stress indicators during commercial handling of crustaceans (see Jussila *et al.*, 1997, Paterson & Spanoghe, 1997 and Chang *et al.*, 1999). *N. norvegicus* will certainly be exposed to such stresses during trawl capture. Most studies of spontaneous muscle necrosis in crustaceans also report an induction of hyperactivity in animals prior to the onset of necrosis. Such hyperactivity in animals which are habitually sedentary is a common response to a number of stressors, and is followed by exhaustion and elevated levels in haemolymph L-lactate, which can remain elevated for 24 to 48 hours after the stressor has been removed (see Nash *et al.*, 1987). Extreme exertion in strongly glycolytic muscles such as the deep abdominal flexor muscle also causes rapid utilization of glycogen, generating local heat and lactic acid, both of which are known to have degenerative effects on the affected and the surrounding muscle fibres (Hulland, 1985). It is therefore conceivable that during capture and handling of *N. norvegicus*, the repetitive 'tail flipping' that is commonly induced could be causing these types of effect. Initiation of the

condition, possibly caused by damage to the abdominal integument, may then be exacerbated by further stressful post-capture holding conditions.

Once the condition in *N. norvegicus* muscle is established and begins to progress, it does so extremely rapidly, with complete necrosis of the tail muscle and death within days. It has been suggested that rapid progression of necrosis may be linked to the infiltration of the muscle by activated granulocytes and their subsequent production of superoxide radicals, which induce lipid peroxidation, damage membranes and kill cells (Fridovich, 1978; Di Giulio *et al.*, 1989; Nash *et al.*, 1987). However, the role of O_2^- and other reactive oxygen intermediates in the necrosis of *N. norvegicus* muscle remains to be established.

It has been noted in *P. aztecus* that the appearance of muscle necrosis can be reversed if the environmental stressors are removed soon after onset of the condition (Rigdon & Baxter, 1970, Lakshmi *et al.*, 1978). The data presented in this study for the prevalence of muscle opacity immediately after trawl capture. and 4 hours later (Fig.8a) reinforce the theory that the holding conditions of the animals in the period immediately following capture are crucial in determining whether the necrotic condition develops or regresses. This has important consequences both for the vivier transport of live *N. norvegicus*, and the quality of the meat in 'tailed' lobsters. It may also contribute to the high mortality of discarded *N. norvegicus* which are returned to the sea after trawl capture and several hours of emersion (Ulmestrand *et al.*, 1998). The apparent effect of damage to the integument and the reported reversal of the similar necrotic condition in *P. aztecus* requires further investigation in *N. norvegicus* and may lead to advisory measures for the post-capture handling of this commercially important species.

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Fig.1. Map of western Scotland showing the capture sites in the Sound of Jura (A) and the Clyde sea area (B). The University Marine Biological Station Millport is situated on the Isle of Cumbrae in the Clyde estuary.

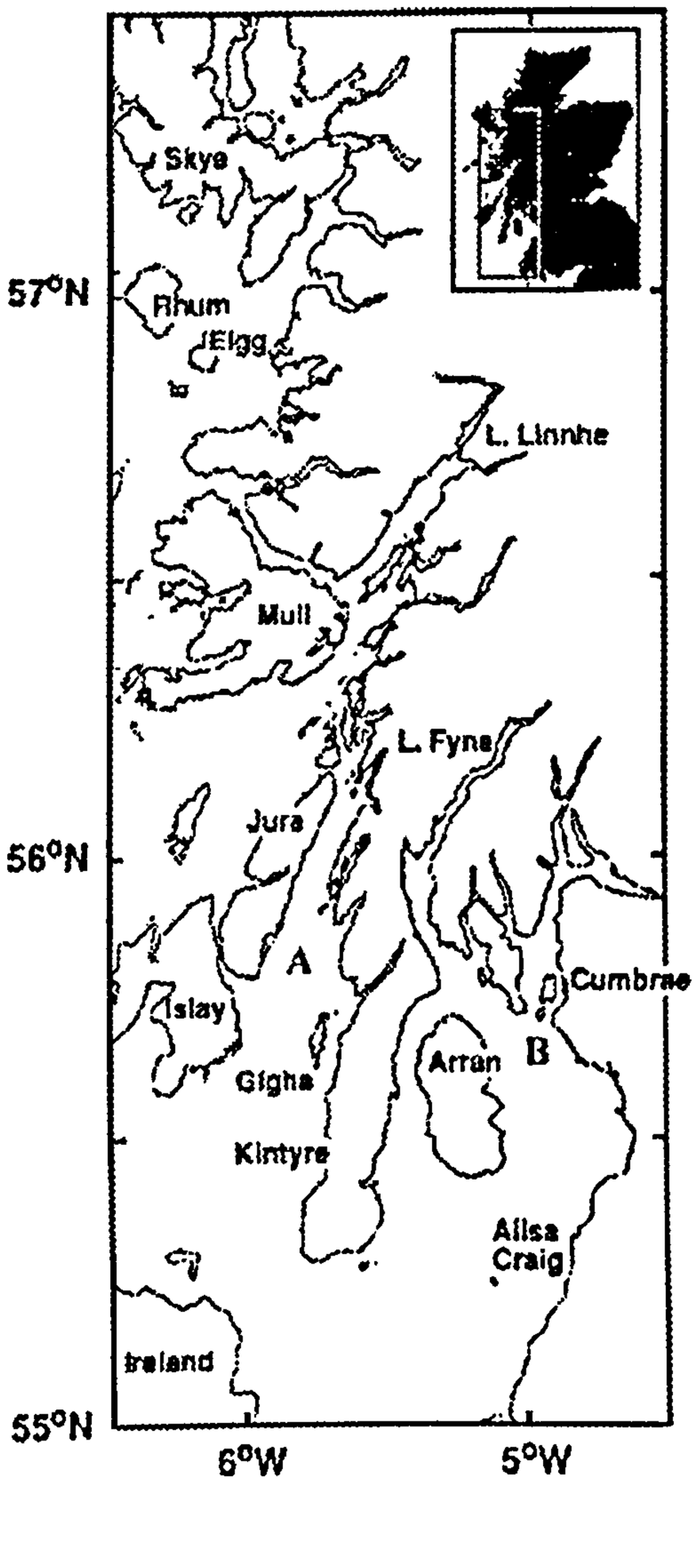


Fig.2 (A-D). (A) Superficial flexor muscle from lobster showing early symptoms of pathology. Note the intact sarcomeres and adjacent region of necrotic muscle showing infiltration by granulocytes. Scale bar = 40 μm **(B).** Deep flexor muscle from lobster showing late stage necrosis. Note the junction between relatively intact and disorganized muscle (arrow), condensed myofibre bundles and granulocyte infiltration Scale bar = 80 μm (see also Fig.2D). **(C)** Fibrous processes at the junction between intact and disorganized muscle (arrow) Scale bar = 30 μm **(D).** Infiltration of granulocytes into necrotic regions of DAF muscle. Scale bar = 20 μm.

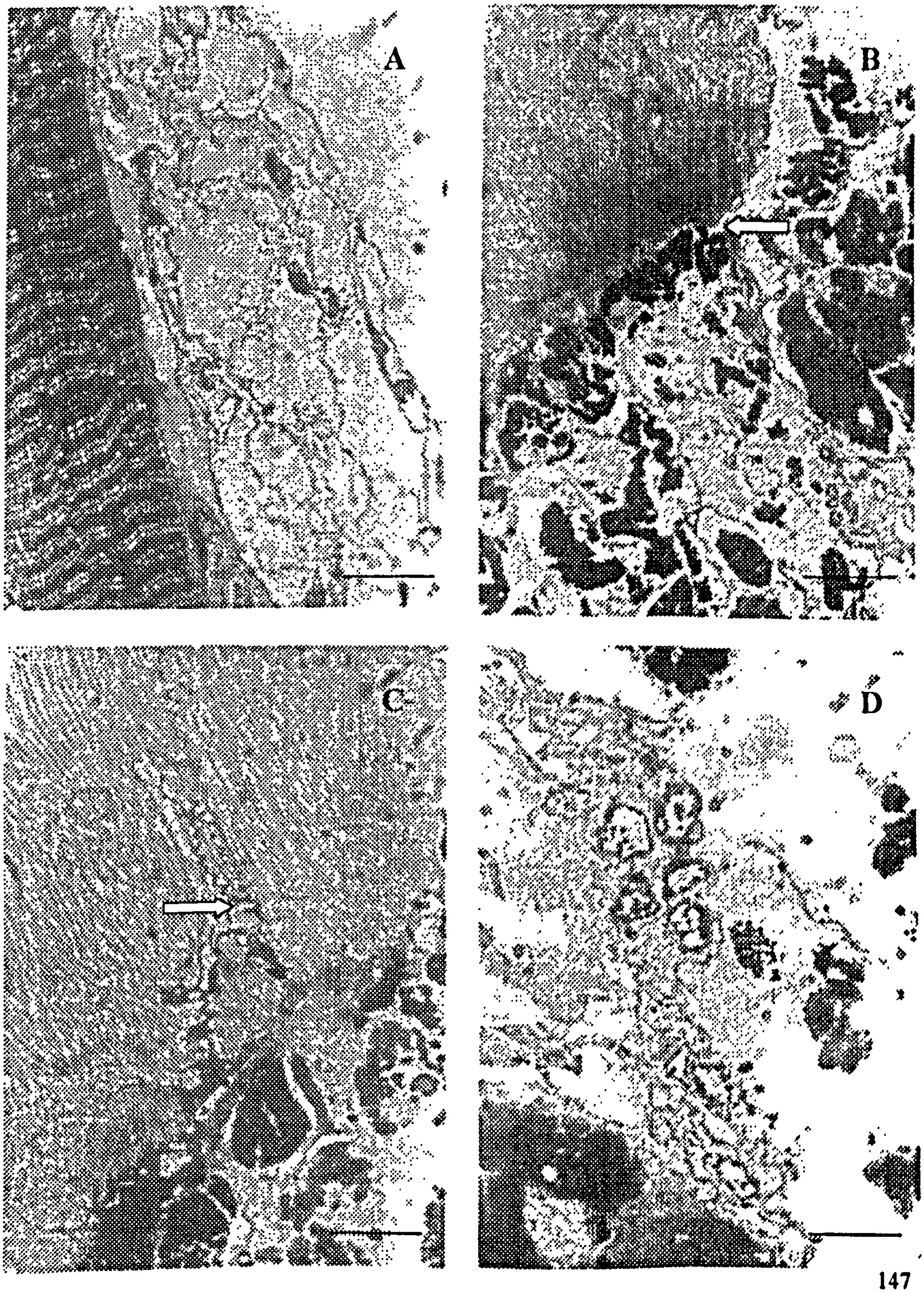


Fig.3 (A-D). (A). Electron micrograph of normal DAF muscle showing regular sarcomeric structure, clear Z-line (arrow) and well-developed tubular system (T). Scale bar = 1 μm **(B).** Electron micrograph of DAF muscle from lobster showing early symptoms of muscle necrosis. Note the expansion of the spaces between adjacent fibre bundles, disruption of the tubular system and appearance of granular material in the cytoplasm (arrow). Scale bar = 1 μm **(C).** Electron micrograph from lobster showing progressing signs of necrosis. Note the disorganized appearance of myofibre bundles, expansion of the inter-bundle spaces and degeneration originating at the Z-line (arrows). Scale bar = 1 μm **(D).** Electron micrograph of superficial flexor muscle from *Hematodinium*-infected lobster. Note the clear erosion of material from within the sarcomere (E) and the intact Z-lines (arrows) Scale bar = 1 μm.

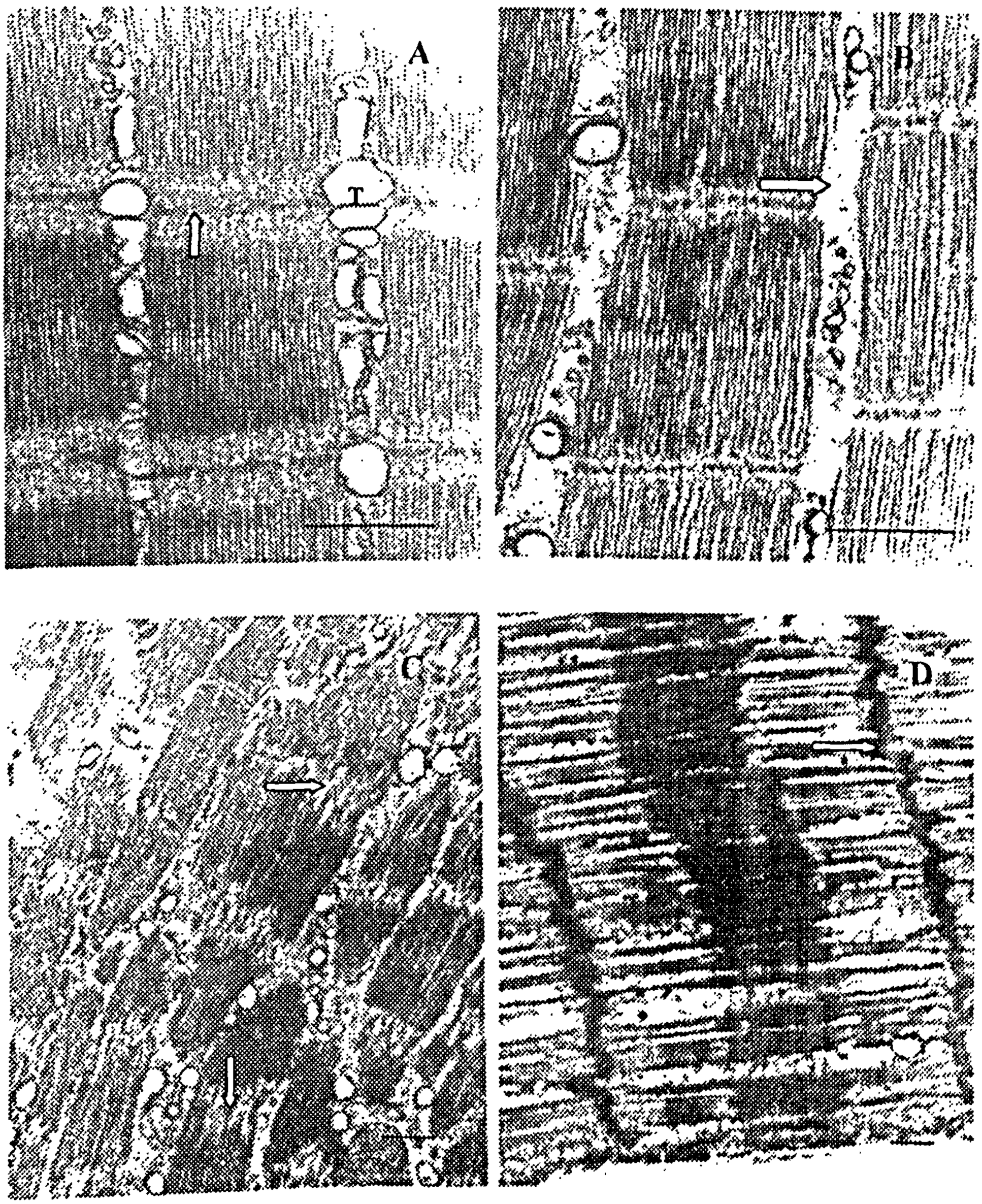


Fig.4 (A,B) (A). Electron micrograph showing fibrous protrusion in DAF muscle of lobster showing early symptoms of necrosis. Scale bar = 1 μm. **(B).** Electron micrograph of junction between relatively intact and severely necrotic DAF muscle. Note the loss of normal sarcomeric organization and appearance of granular material in the region of the junction (arrows). Scale bar = 1 μm.

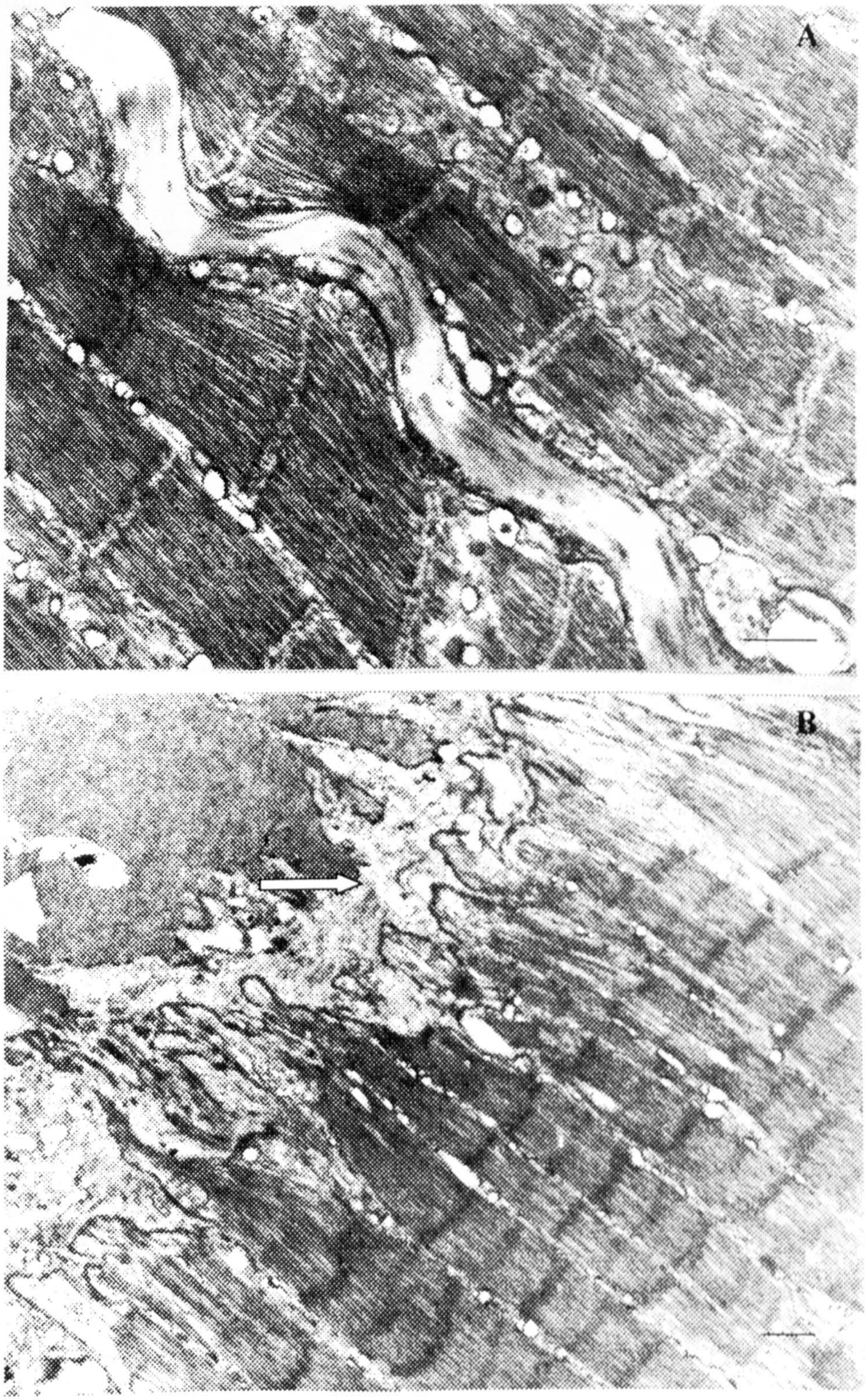


Fig.5 (A-D). (A). Electron micrograph of densely staining DAF muscle from lobsters showing severe symptoms of necrosis. Note the erosion of bundles (E) and the presence of granular material (arrow). Scale bar = 1 μm **(B).** Electron micrograph of atrophied mitochondria within the granular cytoplasm of heavily necrotic DAF muscle. Scale bar = 1 μm **(C)**. Electron micrograph of myelin figures within the granular cytoplasm of heavily necrotic DAF muscle. Scale bar = 1 μm. **(D)**. Electron micrograph of a large (L) and a small (S) granulocyte in the cytoplasm of heavily necrotic DAF muscle. Scale bar = 1 μm.

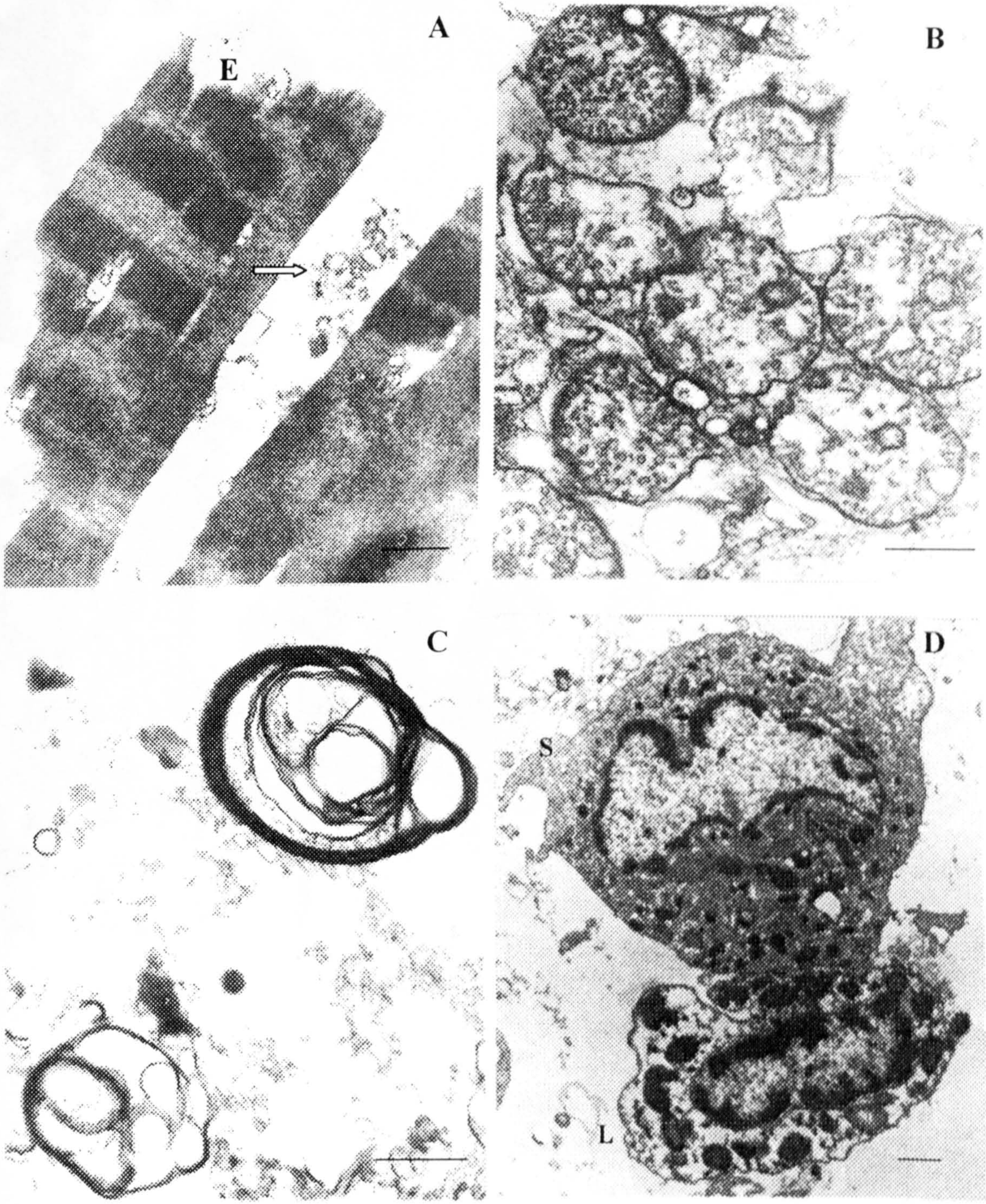


Fig.6. Electron micrograph of unidentified bodies in the cytoplasm of heavily necrotic DAF muscle. Scale bar = 1 μm.

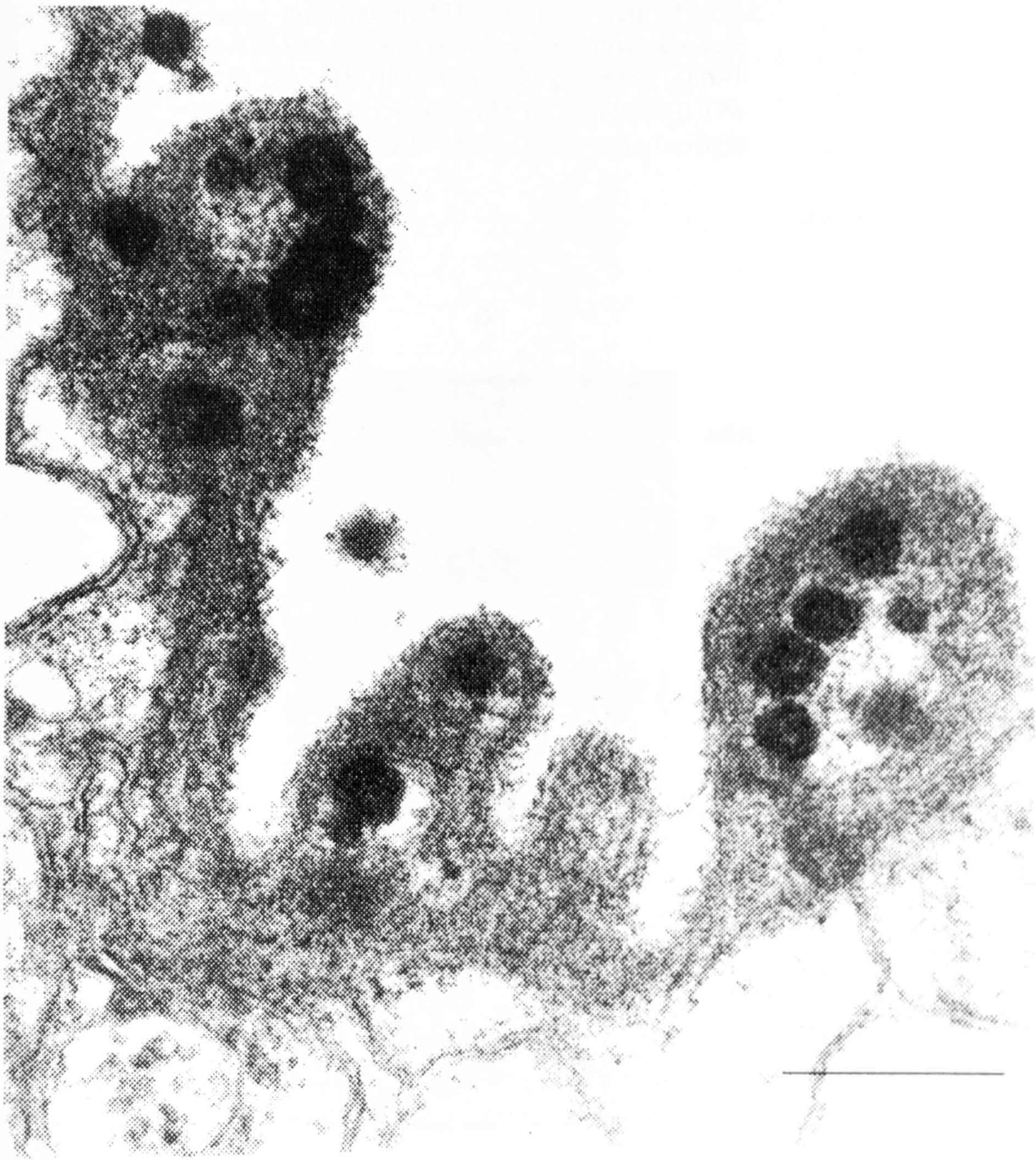


Fig.7. 12.5% SDS-PAGE gel of contractile proteins from an unaffected muscle fibre (lane B) and a necrotic muscle fibre (lane C) from *N. norvegicus*. Lane C contains a molecular weight marker (weights in kDa given on left of gel). Key: **MHC** (myosin heavy chain), **P** (paramyosin 1 & 2), **HP** (haemolymph proteins), **TN-T** (troponin-T), **A** (Actin), **TM** (tropomyosin), **TN-I** (troponin-I) and **Mα-LC** (myosin α-light chain).

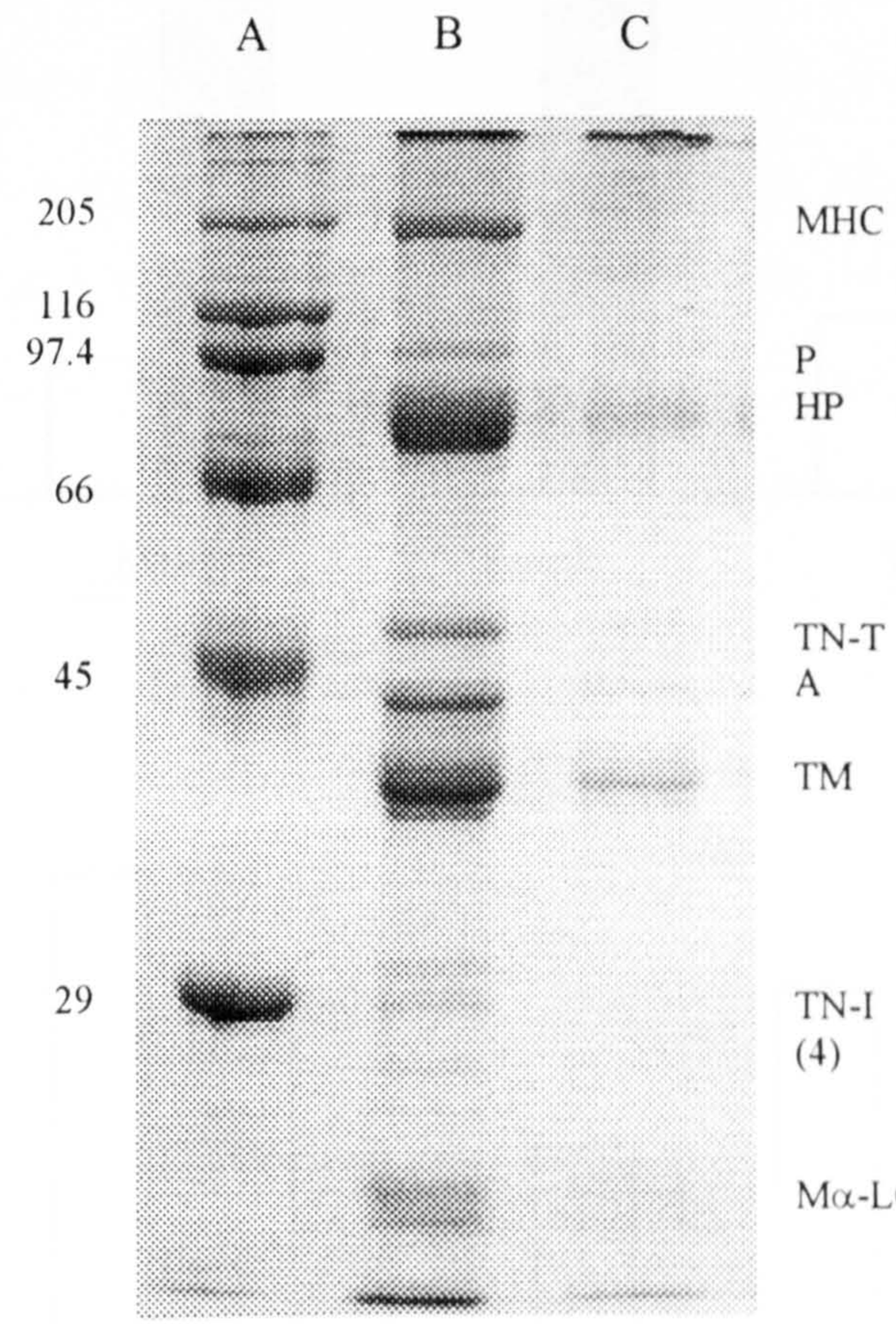
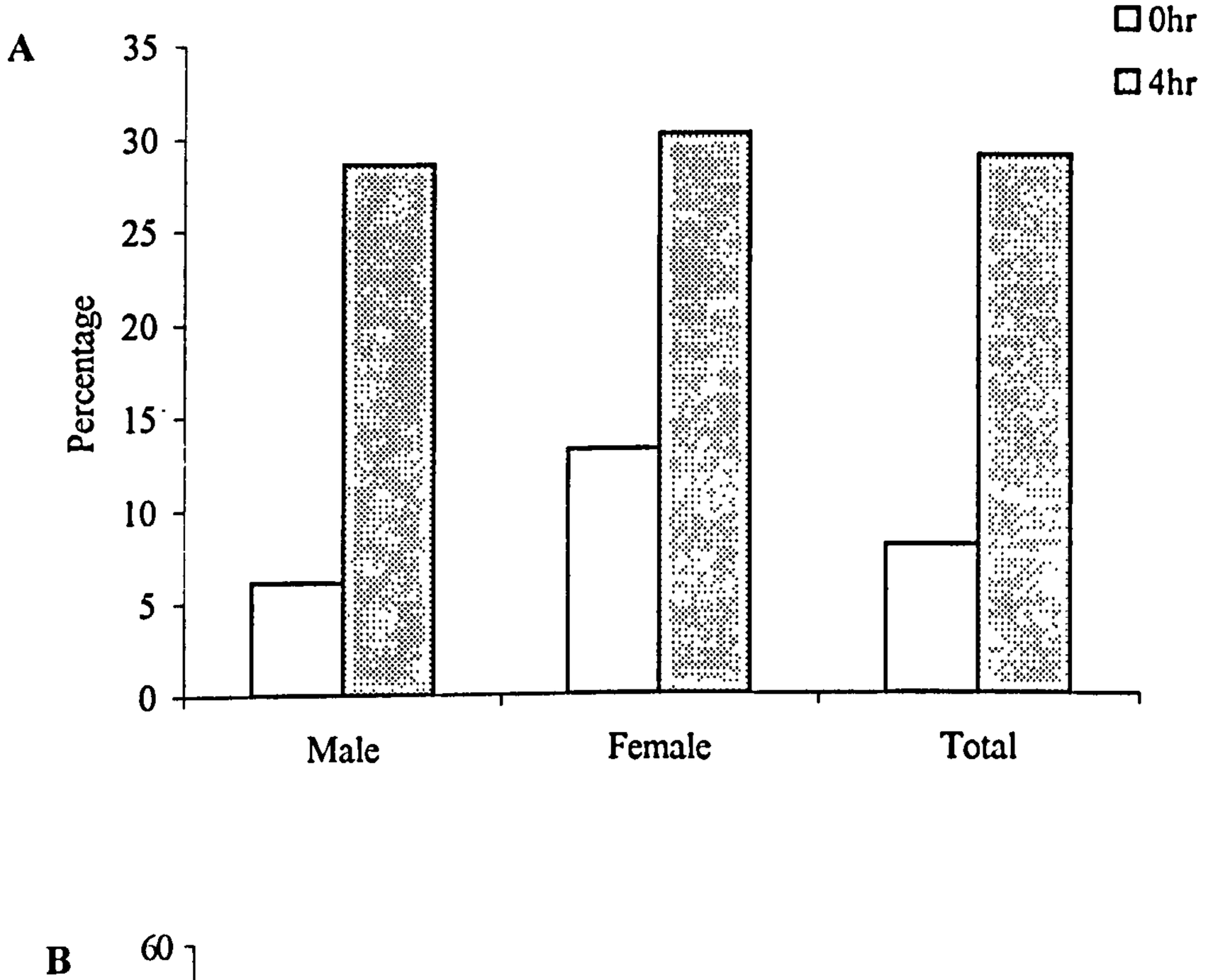


Fig. 8 (A,B). (A). Chart showing the prevalence of muscle opacity symptoms in the abdomens *of N. norvegicus* immediately following (0 hour) and 4 hours following trawl capture. **(B).**Chart showing the percentage of *N. norvegicus* exhibiting muscle opacity in different abdominal segments 4 hours following trawl capture. Addition of percentages to above 100% indicates that lobsters regularly showed muscle opacity in more than one abdominal segment.



Chapter 8

An analysis of swimming performance in the Norway lobster, *Nephrops norvegicus* L. infected by a parasitic dinoflagellate of the genus *Hematodinium*.

Abstract.

Various components of swimming performance were measured in uninfected Norway lobsters (*Nephrops norvegicus*) and compared to animals at different stages of infection by a parasitic dinoflagellate (*Hematodinium* sp.). Animals showed a progressive decline in overall swimming performance as infection severity increased, with reductions in the number of tail-flips performed, the number of swimming bouts and the total distance travelled by swimming. The velocity of the first (giant-fibre mediated) tail flip and average velocity over the swimming bout were also significantly reduced in infected lobsters. Possible reasons for this decreased swimming performance are suggested and the implications of this for predator avoidance of infected lobsters in the benthic habitat, and for capture by *Nephrops* by trawl rigs are discussed.

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Introduction.

The Norway lobster *Nephrops norvegicus*, hereafter referred to by genus alone, like other species of lobster and like crayfish, can react to a visual or tactile stimulus by undertaking a series of 'tail flips' which propel the animal backwards through the water (Neil & Ansell, 1995). The main function of such a response is to remove the animal from a threatening situation, such as a predator. However, in the case of *Nephrops*, the response may facilitate avoidance of capture by trawlers (Newland & Chapman, 1989). The general 'tail flip' response of *Nephrops* has been well studied: upon abrupt mechanical stimulation of the rostrum or telson, *Nephrops* performs a series of tail flips, the first of which is preceded by giant fibre activity in the abdominal nerve cord (Newland & Neil, 1990a, 1990b). This provides one of the best known examples of a stereotyped motor act (Newland *et al.*, 1992). Such information has been used to show that the ability of *Nephrops* to evade trawler capture depends strongly on its ability to initiate a successful 'tail flip' response, although other factors such as its orientation to the trawl gear, proximity to the burrow and its ability to endure prolonged swimming are also important (Newland & Chapman, 1989).

Nephrops supports a major fishery in the United Kingdom, with the Scottish fishery contributing over 76 % of this (£57 million in 1999 – FRS, 1999). Recently, stocks of *Nephrops* off the west coast of Scotland have been shown to harbour a seasonal infection by a parasitic dinoflagellate of the genus *Hematodinium* (Field *et al.*, 1992) which is responsible for a disease syndrome resembling the 'Bitter Crab disease' (BCD) reported in Alaskan tanner crabs (*Chionoecetes bairdi*) (Meyers *et al.*, 1987, 1990) and also *Hematodinium* infections in other decapod hosts (Newman & Johnson, 1975; Wilhelm & Boulo, 1988; Latrouite *et al.*, 1988; Shields, 1992; Hudson *et al.*, 1993). *Nephrops* showing symptoms of patent infection are seen in catches taken in spring and early summer (Field & Appleton, 1995).

A number of studies have established the basic characteristics of *Hematodinium*-infection

(Field *et al.* 1992), its progression, diagnosis and pathology (Field & Appleton, 1995, 1996) and its effect on host physiology (Taylor *et al.*, 1996). It has been noted that the parasite partially invades the main flexor muscles of the abdomen (Field & Appleton, 1995), suggesting that there may be some reduction in its ability to produce tail flips, which could in turn lead to altered swimming performance during patent infection. Reduced swimming performance during infection may alter the ability of *Nephrops* to evade capture by trawlers and predators and thus has obvious commercial and ecological implications for affected populations. Resultant adjustment of catchability, expressed as fishing mortality (F) per unit of fishing effort (E) (i.e. F/E) also has implications for the quantitative assessment of populations exhibiting significant disease prevalence. The present study was therefore carried out to determine whether changes can be observed in the swimming performance of *Nephrops* when infected by *Hematodinium*. Data are discussed in relation to the capture of infected animals.

Methods.

Collection and treatment of animals.

Norway lobsters were collected with an otter trawl from a location south of Little Cumbrae in the Clyde Sea area, Scotland, UK. Lobsters were maintained in a closed aquarium (9°C, 33 ppt salinity) in the Division of Environmental and Evolutionary Biology, University of Glasgow, before transportation to holding tanks (9°C, 33 ppt salinity) in the Fish Behaviour Unit (FBU) at the Fisheries Research Services Marine Laboratory Aberdeen (MLA), Scotland, UK. Animals were fed *ad libitum* on squid (*Loligo* spp.) on arrival at MLA and again three days later. The carapace length of animals used was between 25 and 30 mm, to avoid known size-specific differences in swimming performance (Newland *et al.*, 1988). *Nephrops* of both sexes were used as sex-specific differences in swimming performance are not seen unless the female is ovigerous (Newland *et al.*, 1988). Animals were not fed for three days prior to the experiment to

avoid any effects of differential feeding. All animals were in the intermoult state as defined by Aiken (1980).

Experimental set-up.

Experiments designed to examine the effect of *Hematodinium* infection on various parameters of swimming performance were carried out in a 12m dumbbell-shaped tank housed in the FBU of MLA (see Fig.1). A frame carrying three video cameras (D_1, D_2 and D_3) (Panasonic WV-CL350) fitted with 6.5mm auto-iris lenses was constructed along the length of tank area D (Fig.1). A hand-held integrator switch (Primebridge PVS-1) allowed for switching between the different cameras, thereby keeping the animal in the field of view. The integrator output was linked to a time and date generator (Mitsibushi TDG-10B) before being recorded on a standard VHS video recorder. A distance marker (10cm intervals) was placed along the length of tank area D and the tank was illuminated with standard halogen lighting fitted with dimmer switches.

Experimental protocol.

Animals were housed in tank area B (Fig.1) prior to being individually removed in a net and placed into tank area C (settlement area) for 10 minutes. After settlement, each animal was admitted to tank area D (recording run) and subjected to front-end stimulation (rostrum taps) using a plastic rod. The three video cameras and switching box allowed the entire swimming sequence to be recorded. At the end of a trial, as determined by a failure to respond to three successive taps to the rostrum, each animal was removed from the run, measured, sexed and staged for *Hematodinium* infection using the pleopod staging method of Field & Appleton (1995). According to this method, Stage 0 denotes an apparently uninfected animal, with patent infection progressing from Stage 1 through to Stage 4. Stage 3 and 4 (heavily infected) animals were grouped in all analyses as animals in these groups show very similar disease characteristics (see Stentiford *et al.*, 1999- Chapter 4). A 'bout'

of swimming is defined as the series of full flexions and extensions between tactile stimulation and rest and a 'flip' as one cycle of flexion and extension of the abdominal segments (Newland *et al.*, 1988)

Video Analysis.

Recordings of *Nephrops* swimming performance (73 animals) were analyzed at the Division of Environmental and Evolutionary Biology at the University of Glasgow. The total number of tail flips, total number of bouts, number of flips per bout, total distance covered, overall swimming velocity, velocity of the giant and subsequent flip and flip frequency (Hz) were measured using a Panasonic AG-5700 VCR linked to a Panasonic AG-570 single-frame editing facility controller and a Panasonic WV-CM1000 colour monitor.

Measurements of tail-flip velocity and flip frequency were made on a total of 46 animals with different stages of *Hematodinium* infection. An acetate overlay was placed on the monitor screen and the position of the rostrum at the start position and at the initiation points of subsequent tail flips (tail extension following full flexion) were marked (see Arnott *et al.*, 1998). The number of frames (20 milliseconds) between each re-extension was recorded and the distance covered between marked points was calculated from the distance marker on the tank floor. Overall velocity was measured over one complete bout within the first three bouts of the swimming sequence and from this, the velocity of the first flip (giant-fibre mediated) and subsequent flip (non-giant fibre mediated) was calculated. Tail flip frequency (Hz) was calculated by dividing the number of flips in one bout by the time taken up to the initiation of the final flip of a given bout.

Data Analysis.

Comparisons of swimming parameters from uninfected and infected *Nephrops* were

performed either by one-way analysis of variance (ANOVA) followed by between stage comparisons with a Tukey's pairwise analysis (for normal distributed data), or by a Mann-Whitney test (for non-normal distributions). Tests were considered significant if $p < 0.05$.

Results.

Video recordings from a total of 73 uninfected and infected lobsters were analysed for total number of tail flips, total number of bouts, number of tail flips per bout and total distance travelled, while swims by 46 animals were analysed for overall mean velocity, velocity of the giant flip, number of non-giant flips per bout and flip frequency.

The mean number of flips performed by animals in response to the standard stimulus regime was reduced in relation to the severity of infection (Fig. 2). Uninfected animals flipped a mean of 140.2 times, with a reduction to 26.2 times in Stage 3-4 infection ($p < 0.001$). There were significant differences between all stages of infection: Stage 1 and 2 ($p < 0.001$) and Stage 2 and 3-4 ($p < 0.05$).

The mean number of bouts was also reduced in relation to the severity of infection (Fig. 3), from 57.9 in uninfected animals to 12.2 in Stage 3-4 infection ($p < 0.001$). Differences in this measure were significant between Stage 1 (43.9) and 2 (27.9) ($p < 0.01$) and Stage 2 and 3-4 ($p < 0.01$).

Owing to these similar trends, the number of flips per bout changed less, although there was still a significant difference between uninfected (2.6 flips.bout^{-1}) and Stage 3-4 animals (1.8 flips.bout^{-1})($p > 0.05$) and between Stage 1 animals (2.7 flips.bout^{-1}) and Stage 3-4 ($p < 0.05$). The total distance travelled in the tail flip sequences also reduced with infection (Fig. 4), from 24.05m in uninfected animals to 3.24m in heavily infected (Stage 3-4) animals ($p < 0.001$). The distance travelled was not significantly reduced in Stage 1 infection (20.56m) when compared with uninfected animals ($p = 0.065$), but was significantly reduced in Stage 2 (10.13m) ($p < 0.001$) and in Stage 3-4 (3.24m) ($p < 0.001$).

There was also a highly significant reduction in distance travelled between Stage 1 and 2 ($p < 0.001$) and between Stage 2 and 3-4 ($p < 0.01$).

Figure 5 shows a typical bout velocity profile from an uninfected (Stage 0) and a heavily infected (Stage 3-4) *Nephrops*. From the start position, both animals reach peak velocity with the first (giant-fibre mediated) flip. This velocity is then maintained, albeit slightly reduced, until the final flip, following which the animal glides to rest (one bout).The bout duration and velocity profile of infected animals is almost identical to that of uninfected animals, but the flip velocity of both the giant and subsequent flips, is greatly reduced by the infection.

The overall mean velocity of a swimming bout (Fig. 6) was not significantly reduced in Stage 1 infection (0.48 $m.sec^{-1}$) when compared with uninfected animals (0.56 $m.sec^{-1}$) ($p > 0.05$). However, in Stage 2 (0.35 $m.sec^{-1}$) and 3-4 (0.24 $m.sec^{-1}$), overall velocity of the bout was reduced significantly (both $p < 0.001$). There was also a significant decrease in overall velocity between Stage 1 and 2 ($p < 0.01$) and between Stage 2 and 3-4 ($p < 0.05$).

Figure 7 shows the mean velocities of the giant flip and second (non-giant) flip for uninfected and infected animals. The mean giant flip velocity shows similar trends to that for overall mean velocity, with no significant differences between Stage 1 velocity (0.66 $m.sec^{-1}$) and Stage 0 velocity (0.75 $m.sec^{-1}$) ($p > 0.05$) but highly significant differences between Stage 0 and Stage 2 (0.52 $m.sec^{-1}$) ($p < 0.01$) and Stage 3-4 (0.32 $m.sec^{-1}$) ($p < 0.001$). The velocity of the subsequent flip, which is less than that of the giant flip in all cases, is also progressively reduced with the severity of infection, so that the relationship between the giant flip velocity and subsequent flip velocity remains unchanged (data not shown).

Any change in the ratio of the number of non-giant (NG) flips to giant (G) flips is likely to influence the overall velocity (Fig. 6) since the NG velocity has been shown to be less than that of G flip velocity (Fig. 7). However, the number of NG flips per bout is not

significantly different, even in heavily infected animals (1.76 versus 1.59 in uninfected animals) ($p > 0.05$).

The frequency of tail flips measures the rate at which repeated full tail flexions can be performed. When single bouts were analyzed, flip frequency was approximately 3 Hz in all stages of infection (Fig. 8) and no significant differences in flip frequency (Hz) were found between uninfected and infected *Nephrops*.

Discussion.

Swimming performance in relation to Hematodinium infection.

The mean swimming speed of uninfected *Nephrops* measured in the present study (0.56 $m.sec^{-1}$) is in good agreement with previously published measures made both in the laboratory and in the field (Newland & Chapman, 1989, Newland *et al.*, 1988; Newland & Neil, 1990a,b). However, *Hematodinium* infection causes significant reductions in the overall swimming capacity of *Nephrops*, which is most clearly expressed in a reduction in the total distance travelled before animals cease to respond. This progressive decline in swimming performance with increasing parasite burden involves a reduction in both the velocity of individual tail flips and the total number of flips performed, and may reflect damage to the muscle contractile proteins, a reduced ability to use available energy or a depletion of energy reserves. However, infected lobsters are still capable of initiating a giant tail flip at the start of each bout, and of performing as many subsequent non-giant flips as uninfected animals at an equivalent frequency, which suggests that the neuronal circuitry controlling both giant fibre activation and non-giant pattern generation is unaffected by the infection.

An alteration in normal muscle contraction could arise as a consequence of the parasite-induced damage of muscle fibres, as described by Field *et al.* (1992) and Field and Appleton (1995), with parasites penetrating the fibre interstices in late infection (Stage 3

and 4) and possibly lysing the peripheral regions of the fibres. A reduced swimming performance would be expected as a result of such loss of contractile machinery, since giant-fibre mediated tail flipping maximally excites these muscles, causing them to operate close to their maximum isometric condition, with little or no reserve capacity. Muscle damage of a similar nature has also been reported as a symptom of *Hematodinium*-infections of other decapods (Meyers *et al.*, 1987, Hudson & Shields, 1994 and Wilhelm & Mialhe, 1996). Muscle fibre damage could also affect the rate of muscle contraction by disrupting the steps in excitation-contraction coupling. Although tail-flip frequency remained unchanged in infection, no separate measures of flexion duration were made, and therefore it cannot be excluded that a decrease in the contraction rate of abdominal flexor muscles also contributed to the decrease in propulsive swimming thrusts. Combined measurements of the movements of the abdomen during tail flipping and the propulsive forces that are produced, as performed in the studies of Newland and Neil (1990a, b) and Baden and Neil (1998), are required to resolve these issues.

The lower number of tail flips may represent a reduced swimming endurance with progressing *Hematodinium* infection, since it has been shown that this infection is accompanied by changes in blood composition (Field and Appleton, 1995) and an increased respiratory demand on the host imposed by the large burden of *Hematodinium* parasites (Taylor *et al.*, 1996). Infected animals may therefore be in a state of persistent partial exhaustion, which would restrict their capacity to respond to the increased respiratory demands of swimming.

Elevated levels of muscle and plasma L-lactate are also known to cause a cessation of tail flipping in spiny lobsters (Vermeer, 1987), and in *Nephrops* recovery of full swimming ability is linked to recovery of near normal haemolymph L-lactate concentrations (Field *et al.*, 1991; Newland *et al.*, 1992). Heavily infected *Nephrops* have considerably elevated plasma L-lactate concentrations (Taylor *et al.*, 1996), but apparently unaltered muscle L-

lactate concentrations (Field, 1992), and it is therefore possible that this increased plasma L-lactate is a factor in reducing the swimming endurance of infected animals. Depletion of the metabolic reserves used for active swimming may also play a part in the reduced swimming endurance during *Hematodinium* infection. Although the concentration and re-synthesis of ATP in abdominal muscle appear not to limit escape swimming endurance in uninfected *Nephrops* (Field *et al.*, 1991) in the manner reported for the scallop (Grieshaber, 1978), it is possible that the reduced muscle glycogen concentrations measured during *Hematodinium* infection (unpublished observations) cause a shortfall in the re-supply of ATP to the contractile machinery, thereby degrading swimming performance.

The observed decline in the number of tail flips performed by *Nephrops* infected by *Hematodinium* is consistent with an increased rate of habituation in the giant fibre systems. Habituation of giant-fibre systems is a common feature of tail flip swimming in many crustacean species (Neil & Ansell, 1995; Arnott *et al.*, 1998; reviewed by Edwards *et al.*, 1999) including *Nephrops* (Newland *et al.*, 1988), and is known to be mediated by the neurotransmitter GABA (Krasne & Roberts, 1967; Vu & Krasne, 1993; Krasne & Teshiba, 1995). One observation that may link *Hematodinium* infection to an alteration in the action of a neuronal pathway for habituation mediated by GABA is that free amino acid concentrations in the plasma of *Nephrops* are changed during infection (Stentiford *et al.*, 1999- Chapter 4). Of these changes, an approximately 12-fold increase in the circulating taurine concentration is the most significant, and since taurine is known to have a neuromodulatory action (Oja & Kontro, 1978; Kuriyama *et al.*, 1978), and in crustaceans is known to mimic the inhibitory actions of GABA (Zatta, 1987; Payen *et al.*, 1981), it may contribute to a greater suppression of repeated giant fibre activation, and thus the reduction of tail flipping.

Reduced swimming performance in relation to trawl capture.

Commercial *Nephrops* trawls are normally towed at speeds of between 1 and 1.6 m sec^{-1} (2-3 knots) and it is suggested that a high proportion of animals directly in line with the mouth of the net will be captured. Due to the observed reduction in swimming performance, it may be suggested that *Hematodinium*-infected *Nephrops* at the net periphery and within the net itself may be less likely to escape capture, while some of those directly in-line with the ground gear may evade capture by not swimming up and over the ground gear. Differences in the swimming performance of *Nephrops* at different stages of infection may also lead to stage-specific alterations in the probability of trawl capture. Most infected animals captured in trawls are either Stage 1 or 2 (unpublished observations). It is therefore possible that the behaviour of animals in more advanced stages of infection reduces their vulnerability to capture. The use of creel sampling for *Hematodinium*-infection assessment has shown that infected animals are rarely found within the creels (Pers.Obs. Dr. R.J.A. Atkinson). However, in the Clyde sea area, only shallow waters (with larger *Nephrops*) can be fished with creels, while the deeper grounds, which tend to contain *Nephrops* within the size range commonly infected by *Hematodinium* (30-35mm carapace) are fished by trawlers. As a result, a direct comparison of trawl and creel prevalence (see Wilhelm & Mialhe, 1996) would be misleading. However, both the present work and other studies of *Nephrops* swimming performance (Newland *et al.*, 1992), suggest that infected animals are more likely to be captured by standard *Nephrops* trawling rigs than their non-infected counterparts. Thus, the prevalence of *Hematodinium* reported for sites off the west coast of Scotland (Field *et al.*, 1998) may be an overestimate of the true level of infection in these populations. The same decrease in swimming performance may also make infected animals more prone to predation, causing further imbalance in proportions of uninfected and infected animals.

Early attempts to incorporate the high observed prevalence of *Hematodinium* infection

into analytical stock assessments of the Clyde sea area (ICES, 1997), modelled the effect as an additional loading on the natural mortality rate (since animals showing symptoms of patent infection usually die). However, the exercise was not entirely successful, leading, as it did, to unrealistic estimates of recruits entering the fishery. The observations above suggest that a more subtle approach is necessary when examining the effect of the disease on the population. Reduced swimming performance implies increased catchability (F/E); in other words, a given unit of fishing effort would result in a higher increased mortality rate in populations with high prevalence of the disease. Furthermore, if infected animals are likely to be more susceptible to predation through reduced ability to escape, then the disease may not necessarily add to overall natural mortality but rather, replace a proportion of it.

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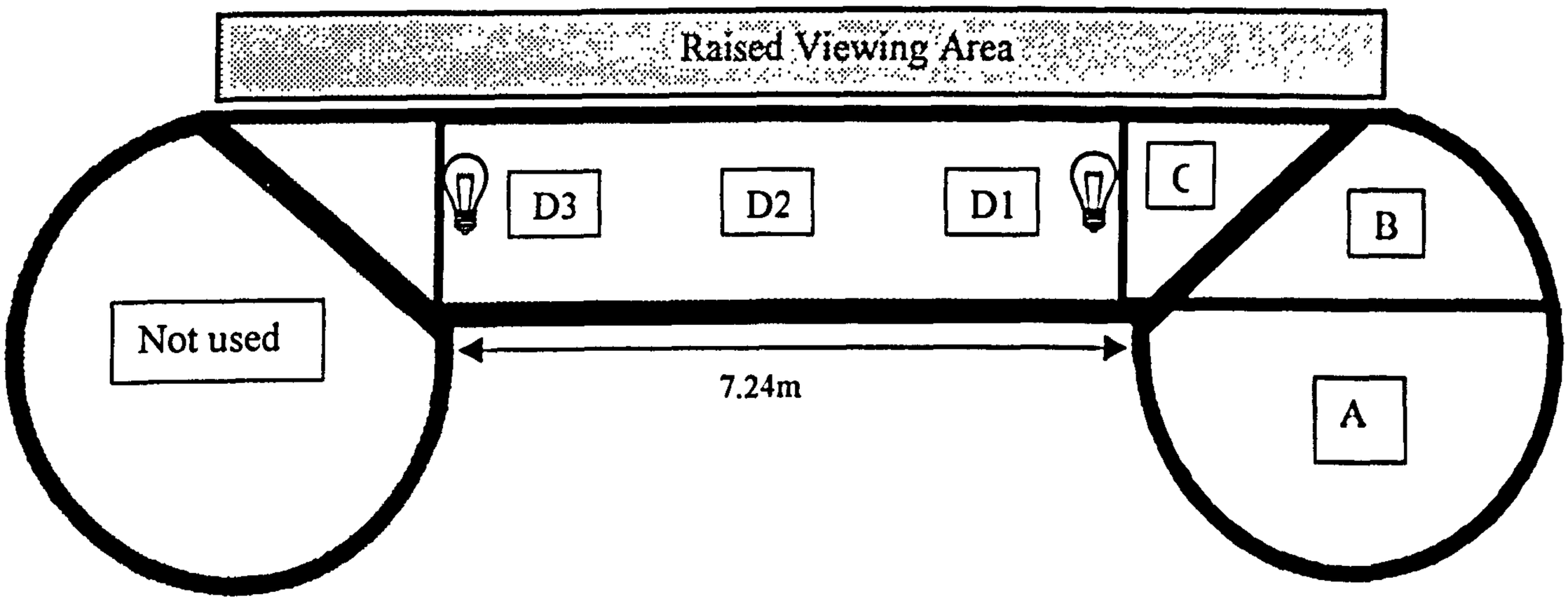
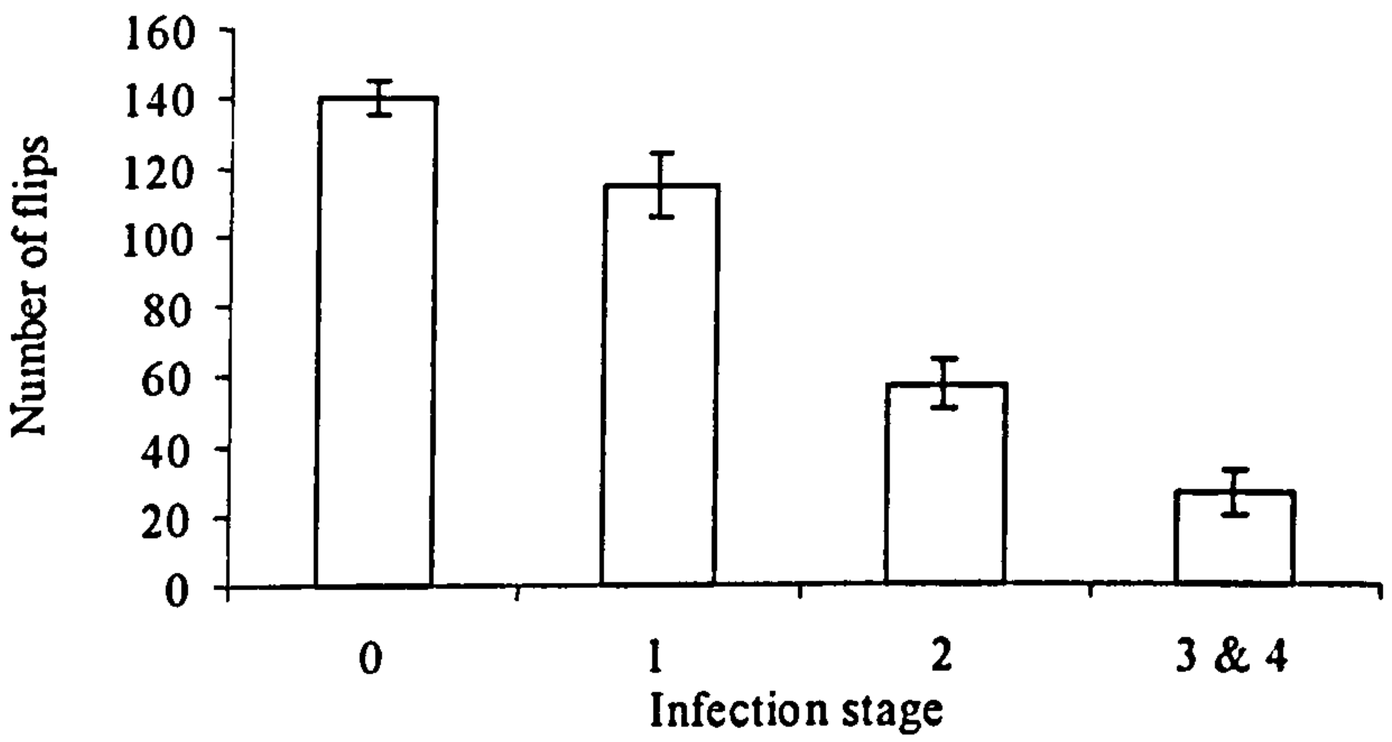
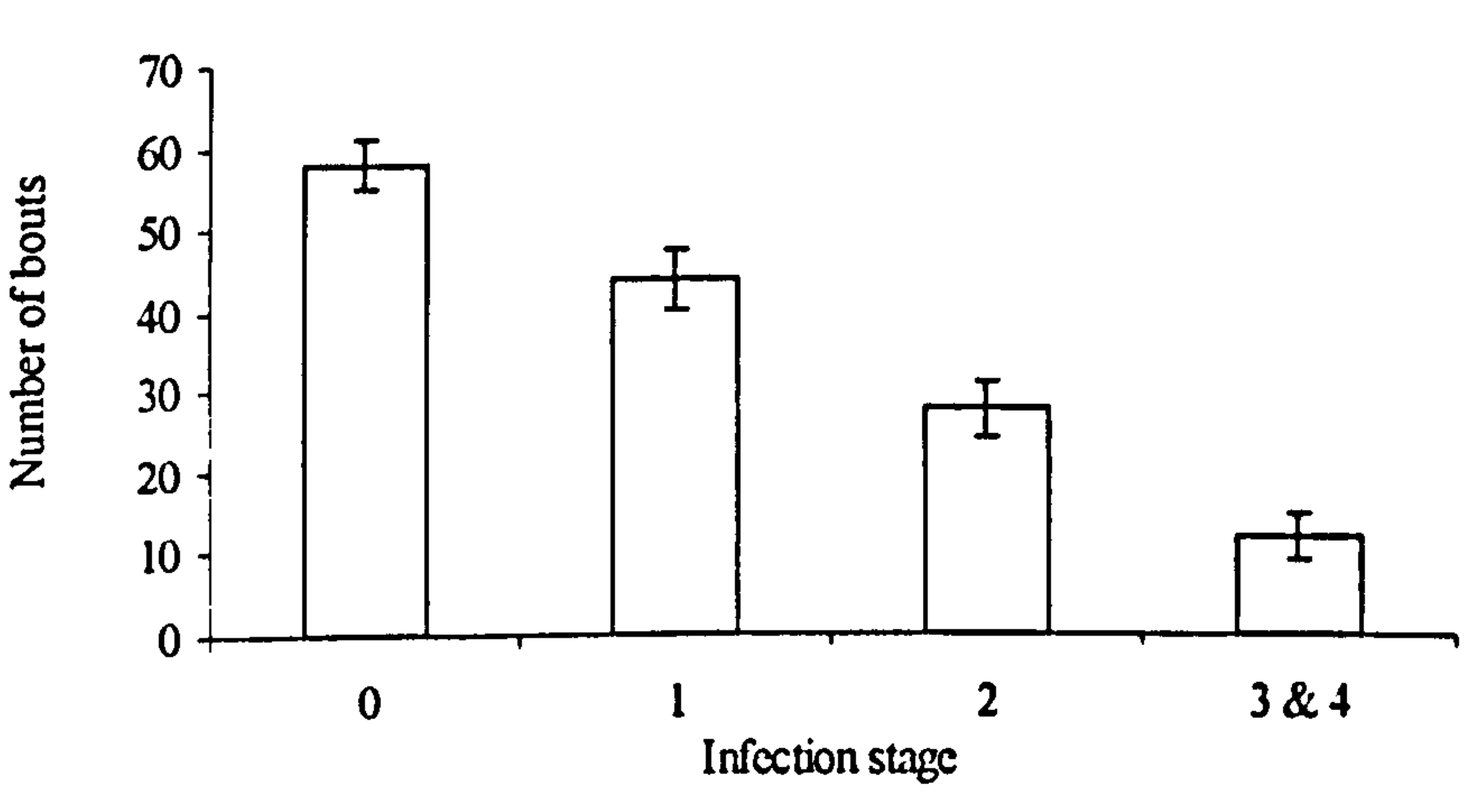
Fig. 1. Plan view of dumbbell tank at Marine Laboratory Aberdeen. Key : **Area A** (post-run animals), **Area B** (pre-run animals), **Area C** (pre-run settlement area) and **Area D** (recorded swimming run), divided into **D1** (camera 1 field), **D2** (camera 2 field) and **D3** (camera 3 field).



Figs. 2, 3 and 4. **(2).** Mean total number of tail-flips performed over entire swimming sequence (± standard error) by infection stage. **(3).** Mean total number of swimming bouts performed over the entire swimming sequence (± standard error) by infection stage. **(4).** Mean total distance travelled (m) by swimming, over the entire sequence (± standard error) by infection stage. Stage 0 denotes uninfected animals, while Stage 1, 2 and 3-4 denote patent *Hematodinium* infection.





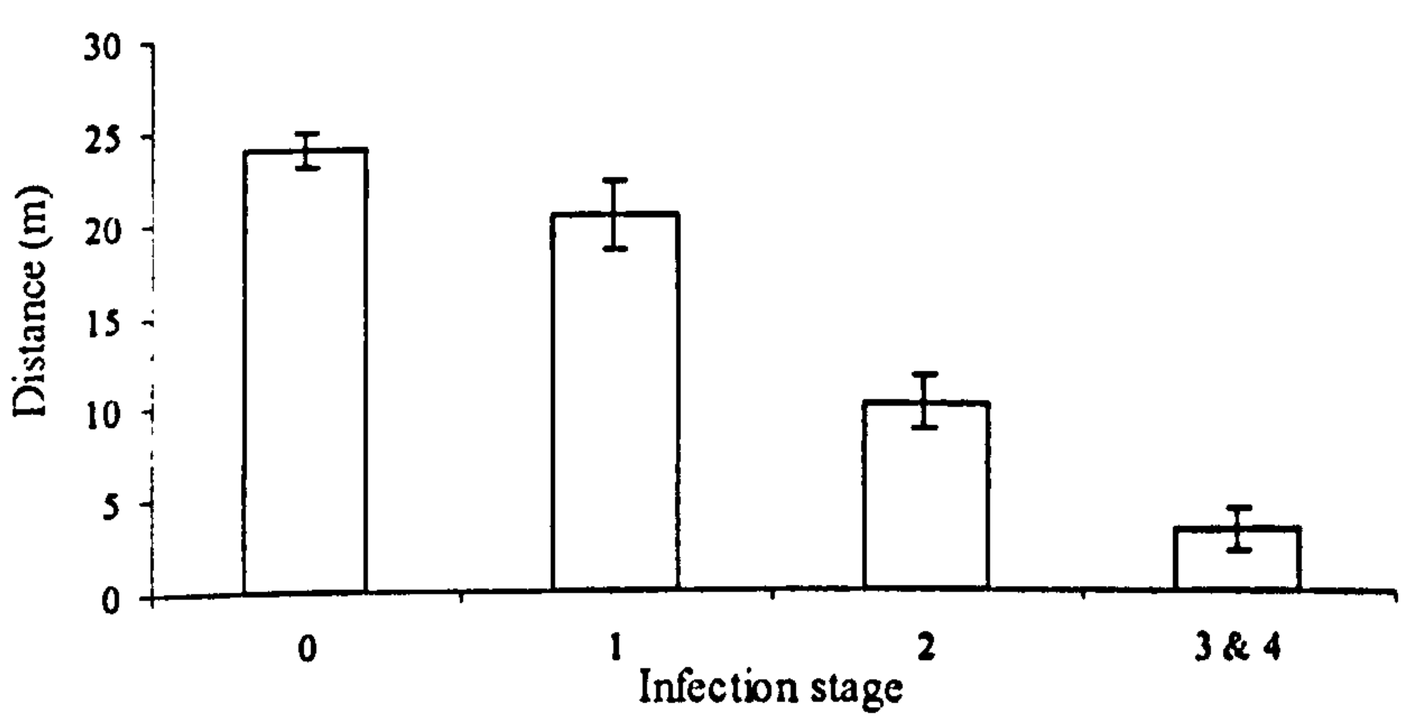


Fig. 5. Typical bout velocity profile for an uninfected (Stage 0) and heavily infected (Stage 3-4) *Nephrops*. Key : **S** (start point – zero velocity), **S to G** (point of tail extension at end of giant flip), **G to 1** (tail extension at end of flip following giant flip), **1 to 2** (tail extension of 2nd flip after giant), **2 to 3** (tail extension of 3rd flip after giant), **3 to 4** (tail extension of 4th flip after giant), **4 + Glide** (velocity of glide following fourth flip after giant).

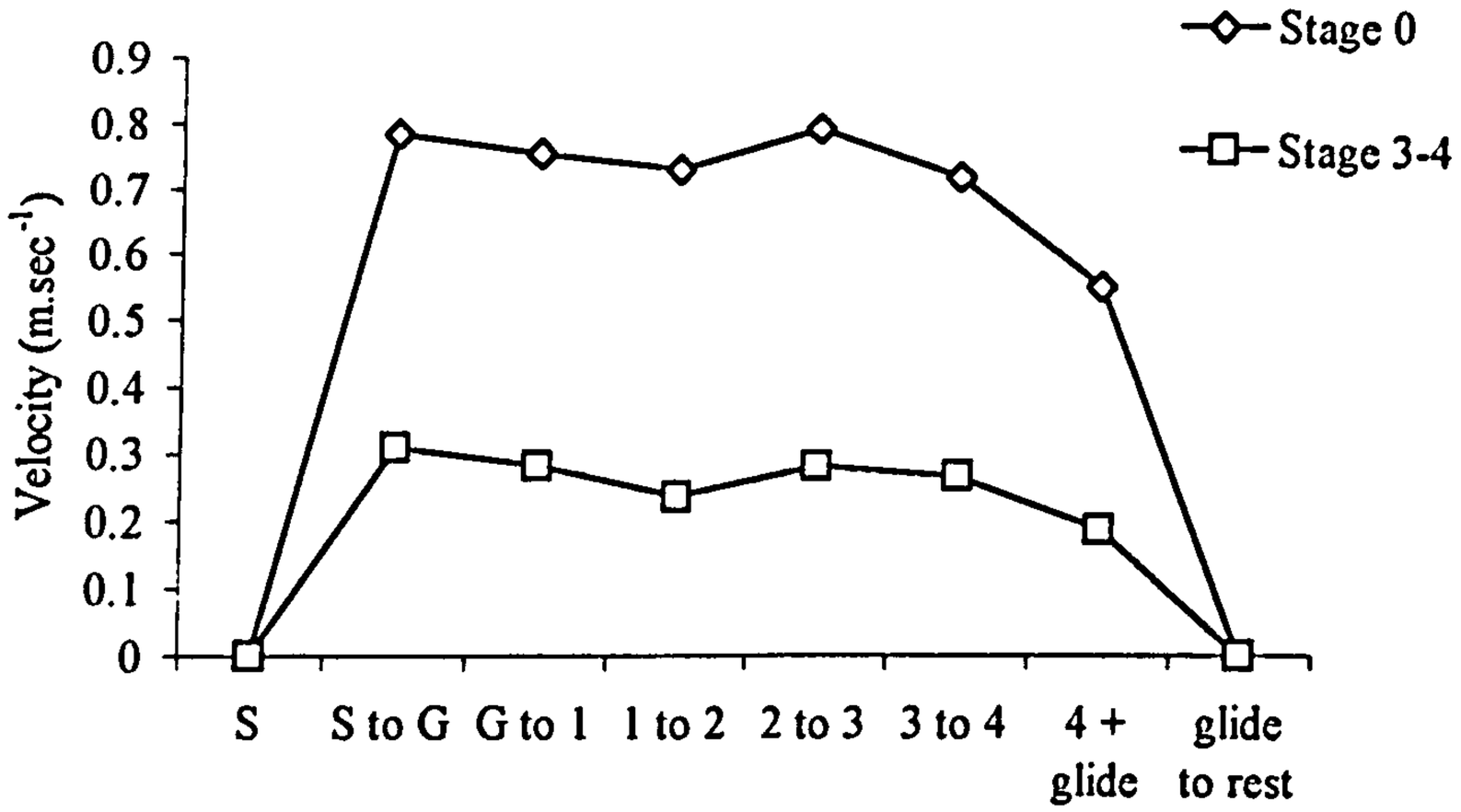


Fig. 6. Mean overall velocity ($m.sec^{-1}$) of an individual swimming bout (± standard error) by infection stage. Stage 0 denotes uninfected animals, while Stage 1, 2 and 3-4 denote patent *Hematodinium* infection.

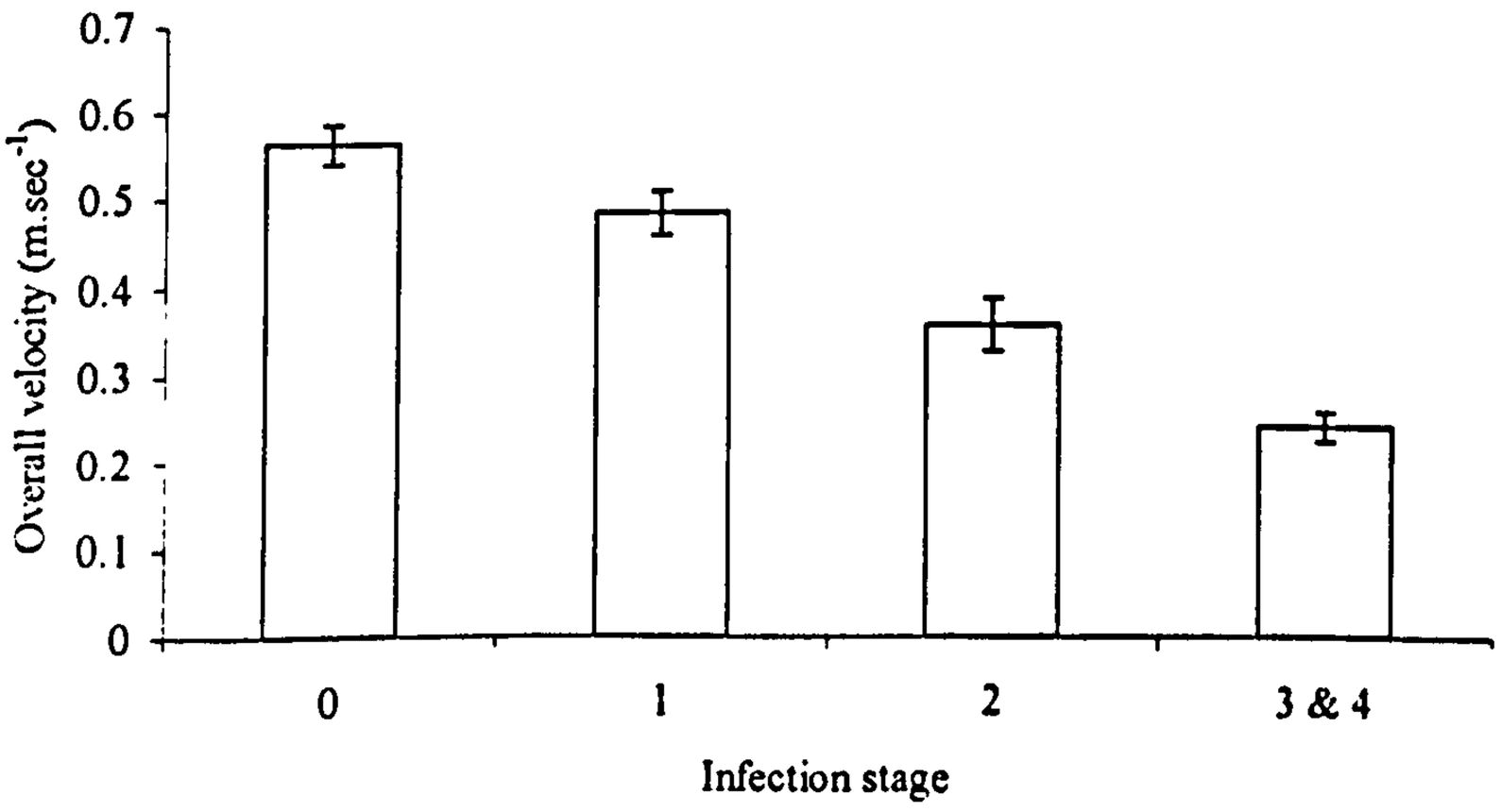


Fig. 7. Mean giant tail-flip and second tail-flip velocity comparisons ($m.sec^{-1}$) (± standard error) by infection stage. Stage 0 denotes uninfected animals, while Stage 1, 2 and 3-4 denote patent *Hematodinium* infection.

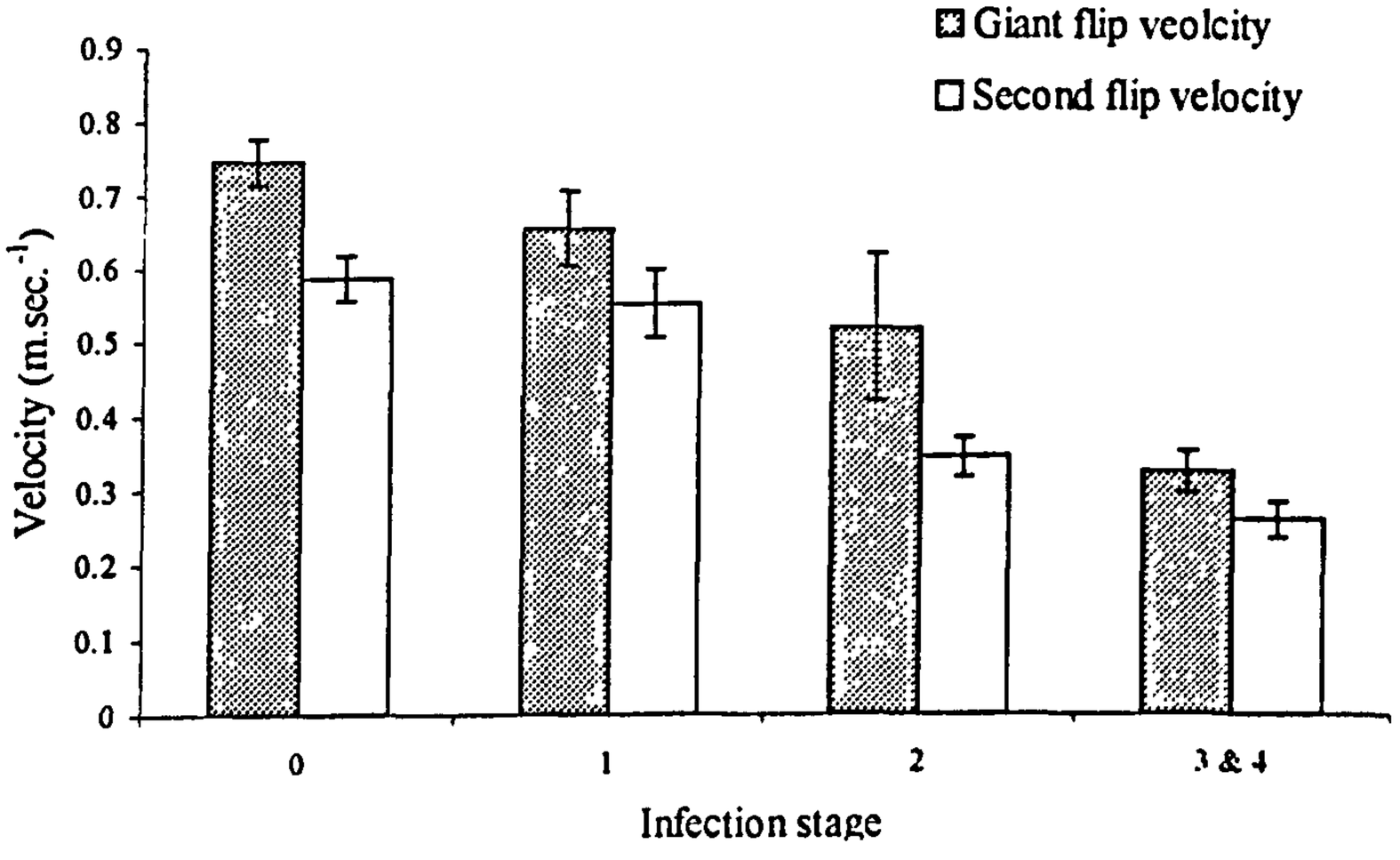
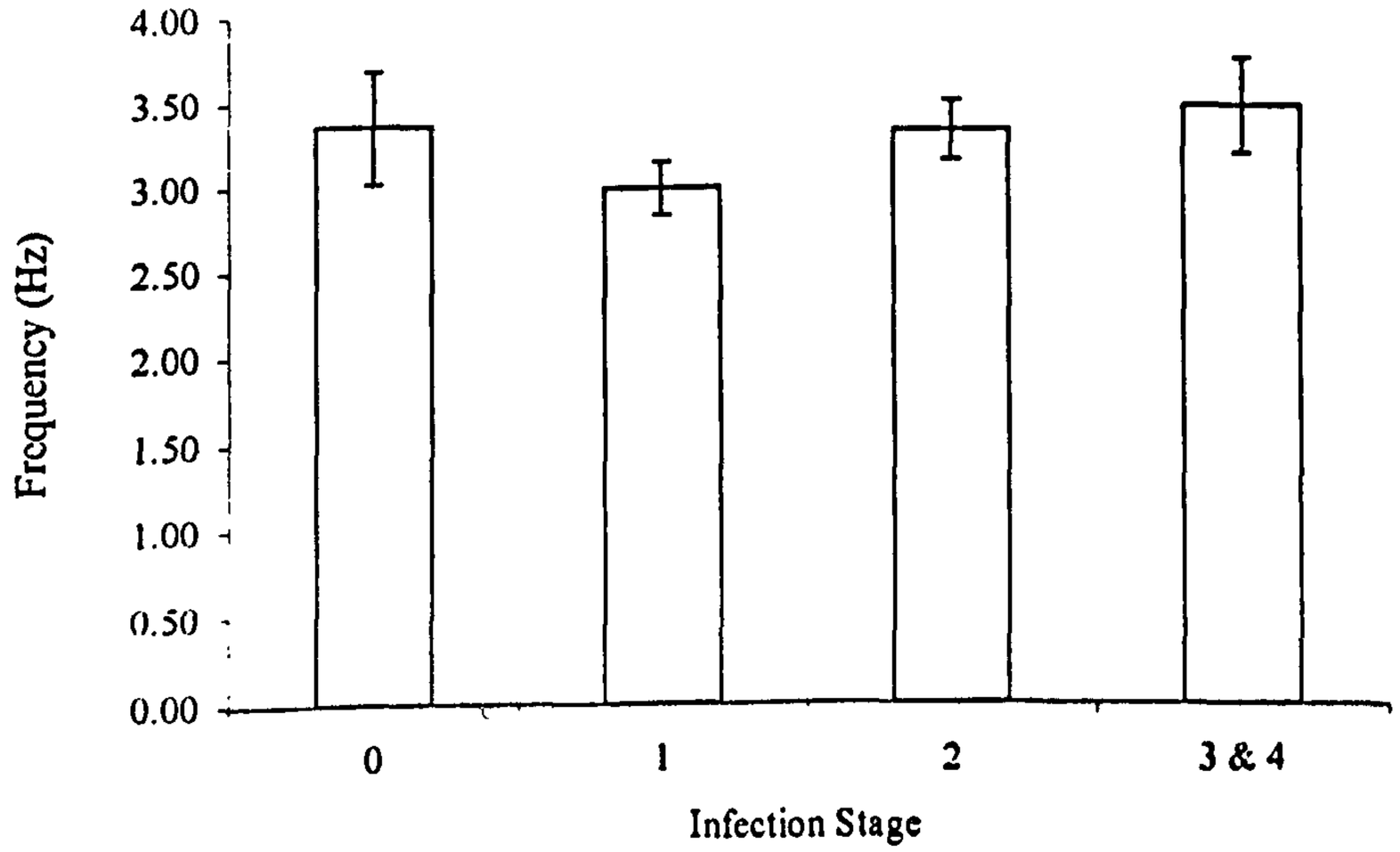


Fig. 8. Mean tail flip frequency (Hz) over a swimming bout (± standard error) by infection stage. Stage 0 denotes uninfected animals, while Stage 1, 2 and 3-4 denote patent *Hematodinium* infection.



Chapter 9

Cessation of swimming and subsequent recovery in the Norway lobster, *Nephrops norvegicus* (L.), and changes associated with infection by a parasitic dinoflagellate (genus *Hematodinium*).

Abstract.

The current study describes the pattern of tail flipping in the Norway lobster, *Nephrops norvegicus* (L.) over a complete swimming sequence, and the subsequent performance following 4 h of recovery. Data is compared to *Hematodinium*-infected *N. norvegicus*. The escape swimming response of uninfected *N. norvegicus* appears to be divided into two contiguous phases with distinct characteristics. The first phase comprises 80 to 100 relatively consistent, high power tail flips, while the variable number of second phase flips are less powerful and precede complete cessation of swimming. Lightly infected *N. norvegicus* appeared to show the same two-phase pattern, though the number of flips comprising the second phase was much less, leading to shorter swimming sequences. Heavily infected *N. norvegicus* did not show this pattern but instead produced a smaller number of weaker flips before cessation of swimming. Following 4 h recovery, uninfected *N. norvegicus* produced 84.3 % of the flips produced in the initial trial, this reduction being due to a smaller number of Phase 2 tail flips. Infected *N. norvegicus* showed a lower recovery rate than uninfected animals, this being proportional to the severity of infection. The metabolic basis of the two phase swimming pattern and the reason for less Phase 2 tail flips being produced during *Hematodinium* infection is discussed in relation to the catchability of the weakest uninfected and of infected *N. norvegicus* by trawlers and predators, especially on fishing grounds where the trawling frequency is high.

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Introduction.

The ability of many crustacean species to perform both sustainable and exhaustive locomotion has been well studied (for reviews see Hartnoll, 1971, Hargreaves, 1980). Aerobic respiration typically supplies the energy needed for prolonged exercise, as seen in crustacean species undergoing extensive locomotion (Full & Herreid, 1983) and long-distance migrations (Houlihan *et al.*, 1985). Anaerobic metabolism may also supplement the energy required during the migration of certain species (Booth *et al.*, 1984), although these pathways are more generally associated with escape responses (Phillips *et al.*, 1977). Exercise of this type is usually rapidly exhaustive, leading to increased tissue and haemolymph lactate titres, lowered tissue glycogen concentrations, reduced muscle ATP stores, alterations to acid-base status and a profound disturbance of ionic, osmotic and fluid volume homeostasis (Wood, 1991). The mechanisms involved in recovery from exhaustive exercise in crustaceans have also received considerable attention. They include the restoration of tissue phosphagen and ATP pools, the clearance of lactate and other anaerobic end products from active tissues, the recovery of intracellular and extracellular pH to pre-exercise values and the replenishment of tissue glycogen (for review see Ellington, 1983). Recovery from exhaustion in crustaceans is generally slow (up to 24 hr) (Gäde *et al.*, 1986) and it is only when energy reserves have been replenished that locomotory performance recovers to its normal level.

The Norway lobster (*Nephrops norvegicus*) is the subject of an important commercial fishery in Scotland and typifies a normally sedentary crustacean species which utilizes anaerobically-driven muscle contraction in order to elicit a rapid escape reaction in response to impending danger (Field *et al.*, 1991). This escape reaction in *Nephrops*, which comprises a series of rapid tail flexions, has been shown to be important in the avoidance of capture by trawlers (Newland & Chapman, 1989) and may also be used during aggressive conflict and escape from predators (see Arnott *et al.*, 1998).

Stocks of *Nephrops* off the west coast of Scotland have been shown to harbour a seasonal infection by a parasitic dinoflagellate of the genus *Hematodinium* (Field *et al.*, 1992), with animals displaying the symptoms of patent infection in catches taken during spring and early summer (Field & Appleton, 1995). Recently, it has been shown that many of the major features of swimming in *Nephrops* are altered during patent infection, and that this reduced swimming performance may alter the ability of infected lobsters to evade capture by trawlers and predators (Stentiford *et al.*, 2000a – Chapter 8). Any change in catchability also has implications for the quantitative assessment of populations exhibiting significant infection prevalence.

The current study was carried out to establish if *Hematodinium* infection, as well as reducing the escape swimming performance in *Nephrops* (Stentiford *et al.*, 2000a – Chapter 8) also affects its endurance and recovery rate. Data are discussed in relation to the factors that may limit the recovery of escape swimming, and to the consequences of recurrent challenge for the catchability of infected lobsters by trawlers and predators on heavily fished grounds.

Materials and Methods.

Collection and treatment of animals.

Nephrops were collected with an otter trawl from a location south of Little Cumbrae in the Clyde Sea area (55.41°N, 4.56°W), Scotland and were maintained in a closed aquarium at the University of Glasgow, before transportation to holding tanks in the Fish Behaviour Unit at the Marine Laboratory Aberdeen. Conditions in both systems were the same (9°C, 33 ppt salinity) and squid (*Loligo* spp.) was provided *ad libitum* as food on arrival at MLA and again three days later. The carapace length of animals used was between 25 and 30 mm, to avoid known size-specific differences in swimming performance (Newland *et al.*, 1988). *Nephrops* of both sexes were used since sex-specific differences in swimming performance are not seen unless the

female is ovigerous (Newland *et al.*, 1988). Animals were not fed for three days prior to the experiment to avoid any effects of differential feeding. All animals were in the intermoult state as defined by Aiken (1980).

Experimental set-up.

All experiments were carried out in a 12m dumbbell-shaped tank as described by Stentiford *et al.* (2000a) (Chapter 8). Briefly, a frame carrying three video cameras (Panasonic WV-CL350) fitted with 6.5mm auto-iris lenses was constructed along the length of the tank. A hand-held integrator switch (Primebridge PVS-1) allowed for switching between the different cameras, thereby keeping the animal in the field of view. The integrator output was linked to a time and date generator (Mitsibushi TDG-10B) before being recorded on a standard VHS video recorder. A distance marker (10cm intervals) was placed along the length of the tank floor, which was illuminated with standard halogen lighting fitted with dimmer switches.

Experimental protocol.

For the first swimming sequence, *Nephrops* were removed individually from the holding end of the tank with a net and placed into a settlement area for 10 min. After settlement, the animal was admitted to the main tank and subjected to mechanical stimulation (rostrum taps) using a plastic rod. The three video cameras and switching box allowed the entire swimming sequence to be recorded. At the end of the swimming sequence, as determined by a failure to respond to three successive taps to the rostrum, the animal was removed from the main tank, measured, sexed, numbered with permanent ink and returned to the holding area (see Stentiford *et al.* 2000a – Chapter 8). It was then allowed to recover for 4 h, after which time it was again removed from the holding area, and subjected to the same stimulus protocol as in the first session. Assessment for *Hematodinium* infection followed termination of this second swimming sequence using the pleopod staging method of Field

& Appleton (1995), according to which, Stage 0 denotes an apparently uninfected animal, with patent infection progressing from Stage 1 through to Stage 4. Stage 3 and 4 (heavily infected) animals were grouped in all analyses since animals at these stages show very similar pathologies (see Stentiford *et al.*, 1999 – Chapter 4). The swimming performance of a total of 73 *Nephrops*, both uninfected and *Hematodinium*-infected, was recorded in this way.

Video Analysis.

The recordings of *Nephrops* swimming performance were analyzed at the Division of Environmental and Evolutionary Biology at the University of Glasgow. The total number of tail flips and total distance covered were measured using a Panasonic AG-5700 VCR linked to a Panasonic AG-570 single-frame editing facility controller and a Panasonic WV-CM1000 colour monitor. In the current study, a tail 'flip' is defined as one cycle of flexion and extension of the abdominal segments (Newland *et al.*, 1988). Mean distance per flip was calculated by dividing the total number of flips by the total distance travelled during active swimming. Individual flip distances over an entire swimming sequence were recorded for a representative range of uninfected lobsters. For this, an acetate overlay was placed on the monitor screen and the position of the rostrum at the start position and at the initiation points of subsequent tail flips (tail extension following full flexion) were marked (see Arnott *et al.*, 1998). Data were compared to that from *Nephrops* at each stage of infection. These data were used to determine any changes in the swimming performance over the course of a whole swimming sequence.

Data Analysis.

Comparisons of distance per flip in rested *Nephrops* were made between uninfected and infected individuals by using a general linear regression model, followed by comparisons of

the slope and elevation of regression curves using analysis of covariance (ANCOVA). These data were then compared to those obtained after 4 h of recovery following the initial swimming sequence, using a t-test to analyze the paired differences between observations from individual animals. Comparisons between the mean percentage recovery in the number of tail flips in different stages of infection were performed using paired t-tests. All tests were considered significant if $p < 0.05$.

Results.

The swimming pattern of rested Nephrops.

The swimming sequences of uninfected *Nephrops* in the rested state ranged from 90 to 180 tail flips, and were composed of two distinct phases. During Phase 1, which comprised the first 80 to 100 flips, the distance travelled in successive flips was relatively constant, with a mean of 18.97 ± 0.42 cm (n=6 individuals). After this, animals produced a variable number of less powerful flips (Phase 2), before the swimming sequence terminated. Figure 1a-f shows this two-phase pattern of swimming for the range of six uninfected animals, and illustrates the variation in the overall length of the swimming sequence, but the relative constancy of the number of flips comprising Phase 1 (indicated by the dashed line).

When swimming sequences of representative individual *Hematodinium*-infected *Nephrops* were assessed, those with Stage 1 infection displayed a similar two phase pattern of swimming, but there was a reduction in the total number of tail flips performed (Fig. 2b). This was due almost entirely to a reduction in the number of Phase 2 flips, whereas the number of Phase 1 flips (80 - 90) remained similar to that of Stage 0 animals. Stage 2 *Hematodinium* infection resulted in a further reduction in the number of tail flips in a swimming sequence, and most individuals failed to reach the number representing the Phase 1 / Phase 2 transition of uninfected animals (Fig. 2c and Fig. 3). However, comparing the

first 50 flips performed in each case, the mean distance per flip in Stage 2 *Nephrops* (18.86 ± 0.56 cm; n=6) was not significantly different to that of Stage 0 lobsters (18.97 ± 0.42; n=6) ($p > 0.05$), indicating that these flips were equally powerful. This suggests that light infections primarily affect the number of flips performed, but have virtually no affect on either the transition between Phase 1 and Phase 2 swimming, or on the thrust produced by Phase 1 flips.

In the advanced stages of infection (Stage 3-4) the swimming response of *Nephrops* differed more substantially from that of uninfected animals (Fig. 2d). The total number of flips in a swimming sequence was further reduced to less than 40, and in addition the distance covered in a swimming sequence was also considerably lower, yielding a mean value for distance per flip of 9.4 ± 1.7 cm (n=11). This indicates that all of the tail flips made by heavily infected animals are reduced in power, compared to both uninfected or lightly-infected animals.

In uninfected animals the total distance travelled in a swimming sequence was proportional to the number of flips performed (Fig. 3 Stage 0), and the spread of values reflects the underlying variability seen in Fig. 1. This variability in the length of swimming sequences was also a feature of the data obtained from animals at different stages of infection (Fig. 3, Stages 1-4).

For animals that performed only Phase 1 swimming (Stage 2 & Stage 3-4), regression lines through their data sets have slopes approximately equal to their Phase 1 mean values (Stage 2 – $y = 0.2103x - 1.8773$; Stage 3-4 – $y = 0.182x - 0.2729$) (Fig. 3). However, for animals that exhibited both Phase 1 and Phase 2 swimming (Stage 0 and Stage 1), the distance covered over the whole swimming sequence is the average of the Phase 1 and Phase 2 contributions, which is lower than for Phase 1 alone. Since this average decreases as the number of Phase 2 flips performed increases, the regression lines for these data sets have

progressively lower slopes as the length of the swimming sequence increases (i.e. from Stage 2 to Stage 1 to Stage 0) (Fig. 3). The lower elevation of the regression line for Stage 3-4 animals ($y = 0.1388 - 0.3882$), however, reflects the reduced distance travelled per flip achieved in the later stages of infection.

The swimming pattern during recovery.

The stimulation regime applied to rested animals was repeated after 4 hours recovery from the initial swimming sequence. The major change, compared with rested animals, was in the total number of flips performed, which reduced from mean values of 140.2 to 117.9 ($p < 0.01$) for uninfected animals, from 114.5 to 79.3 flips ($p < 0.01$) for Stage 1 animals, from 57.1 to 38.3 flips ($p < 0.05$) for Stage 2 animals and from 26.2 to 15.0 flips ($p < 0.01$) for stage 3-4 animals. This represents a partial recovery of the number of flips performed towards the values obtained in the rested state: 84.3 % recovery for Stage 0 animals, and significantly lower values of 69.8 %, ($p < 0.01$) for Stage 1, 66.2 %, ($p < 0.01$) for Stage 2 and 36.8 % ($p < 0.001$) for Stage 3-4 infections (Fig. 4). These changes caused proportional reductions in the overall length of the swimming sequences (Fig. 5).

In contrast, the power of the flips performed by the recovering animals, reflected in a plot of distance travelled vs number of flips (Fig. 6), showed little change from that of the rested animals. This can be seen in the regression curves, which follow the predicted trends as the swimming sequences shorten: i.e. increasing in slope in uninfected and Stage 1 animals (due to the smaller number of Phase 2 flips being performed during recovery), but remaining virtually the same in Stages 2 and Stage 3-4 animals (which performed only Stage 1 flips).

Discussion.

The current study has described the pattern of tail flipping in *Nephrops* over a complete swimming sequence, and the subsequent performance following 4 h of recovery. It has also

shown that infection by *Hematodinium* leads to a reduction in the recovery rate of normal swimming ability and that this reduction is proportional to the plasma parasite burden.

The exhaustion response and subsequent recovery.

The escape swimming response of uninfected *Nephrops* appears to be divided into two contiguous phases with distinct characteristics. The first phase comprises 80 to 100 relatively consistent, high power tail flips, while the variable number of second phase flips are less powerful and precede complete cessation of swimming. A very similar escape response with two distinct phases has been observed in the Australian yabby *Cherax destructor* (Edwards & Baldwin, 1983).

Compared to Stage 1 and to Stage 2 *Hematodinium*-infected *Nephrops*, uninfected animals had a relatively higher proportion of their swimming sequences contained within the Phase 2 period of swimming, where distance travelled per flip is less. The incorporation of a higher number of weaker tail flips into the overall swimming sequence of uninfected animals leads to a decreased regression curve slope relative to Stage 1 and 2 infected animals (which made fewer Phase 2 tail flips). Stage 3-4 *Nephrops* showed a reduced distance per flip, which is consistent with data presented by Stentiford *et al.* (2000a) (Chapter 8) which showed a significant reduction in flip velocity during the later stages of *Hematodinium* infection.

The tail flip in crayfish and nephropid lobsters is essentially a 'single oar' propulsion system, whereby the inertial forces generated are largely attributed to movements of the tail fan (Webb, 1979, Neil & Ansell, 1995). It is also known that an additional and significant thrust is created by 'squeeze forces' at the end of the tail flexion as the abdomen is pressed against the cephalothorax, ejecting trapped water (Daniel & Meyhöfer, 1989). In the current study, the tail flips of *Nephrops* at late stage infection appeared to involve flexion of the tail fan alone, with little or no movement of the main abdomen. The reduced velocity

reported by Stentiford *et al.* (2000a) (Chapter 8) therefore seems due to both a reduction in initial contraction power (see Stentiford *et al.*, 2000b – Chapter 5) and an absence of the final 'squeeze forces' described by Daniel & Meyhöfer (1989).

Metabolic energetics of swimming.

The rapid tail flexions involved in escape swimming in *Nephrops* are produced by the deep abdominal muscles, which are phasic in type (Neil *et al.*, 1993), with high ATPase activity and little capacity for oxidative phosphorylation (Field *et al.*, 1991). These muscles are provided with high levels of phosphagen and a high glycogenolytic capacity (Gruschczyk & Kamp, 1990). It has been reported that glycogen is the principal fuel for exercise in crustaceans, with arginine phosphate being an important fuel for burst swimming (Onnen & Zebe, 1983; Raffin *et al.*, 1988; Adamczewska & Morris, 1994). In crustaceans, the main end product of glycolysis, formed from the anaerobic reduction of pyruvate, is lactate (Phillips *et al.*, 1977; Gäde *et al.*, 1986; Lallier & Walsh, 1992; Henry *et al.*, 1994; de Wachter *et al.*, 1997), which accounts for most of the tissue acidosis associated with exhaustive swimming (Booth *et al.*, 1984; Adamczewska & Morris, 1994; Kinsey & Ellington, 1996). Elevated levels of haemolymph and tissue lactate (Vermeer, 1987), depletion of the main tissue energy supplies (Henry *et al.*, 1994) and habituation of the neuronal system (Neil & Ansell, 1995; Arnott *et al.*, 1998; Edwards *et al.*, 1999) have all been implicated in the cessation of exercise in crustaceans, and Field *et al.* (1991) showed that the absolute cessation of swimming, or 'functional exhaustion' in *Nephrops* is probably influenced by both metabolic and neuronal factors. Due to the importance of lactate in the cessation of tail flipping (Vermeer, 1987, Field *et al.*, 1991), the slow clearance of lactate from the haemolymph and tissue (Phillips *et al.*, 1977; Forster *et al.*, 1989; Booth *et al.*, 1984; Henry *et al.*, 1994) and the fact that muscle contraction function has also been shown to recover from a fatigued state more slowly in an acidic extracellular

environment (Renaud & Mainwood, 1985), it is probable that lactate accumulation is a major feature underlying the cessation of the swimming response in uninfected *Nephrops*. The elevated concentrations of haemolymph lactate which accompany *Hematodinium* infection of this species (Taylor *et. al.*, 1996) may further reduce the swimming performance of *Hematodinium*-infected animals.

In addition to the absolute cessation of tail flip swimming, the two-phase swimming pattern recorded in uninfected animal in the current study is suggestive of a subtle shift in energy substrate being utilized by the active muscle before complete cessation occurs. In the escape swimming in *Cherax destructor*, which demonstrates a similar two-phase swimming pattern, arginine phosphate-derived ATP was found to fuel the initial burst of swimming (c. 30 flips), while anaerobic glycolysis was not used until the second phase of less powerful flips (England & Baldwin, 1983). It is conceivable that the onset of second phase swimming observed in *Nephrops* is controlled by similar mechanisms and that the transition point between Phase 1 and 2 swimming represents a depletion of muscle phospho-arginine reserves. It also suggests that the response to functional exhaustion in *Nephrops* is a progressive, stepwise process.

The concentration of glycogen in the deep abdominal flexor muscle of *Nephrops* is known to be reduced in proportion to the severity of *Hematodinium* infection (Stentiford *et al.*, 2000b – Chapter 5). As Stage 1 and Stage 2 *Hematodinium*-infected individuals performed progressively fewer Phase 2 tail flips than their uninfected counterparts, it is likely that it is the energy substrate fuelling anaerobic glycolysis (principally glycogen) that limits the duration of the swimming response, rather than a depletion in the concentration of muscle phospho-arginine. Further studies on the concentrations of phospho-arginine in the abdominal flexor muscles of *Hematodinium*-infected animals would resolve this issue.

In Stage 3-4 *Hematodinium*-infected *Nephrops*, additional physical and biochemical disturbances to the abdominal flexor muscles may be superimposed upon these more subtle alterations in energy metabolism, degrading the swimming response further (Stentiford *et al.*, 2000b – Chapter 5).

Repletion of muscle metabolites during recovery.

Although recovery in the muscles powering escape responses might be expected to be relatively rapid (Ellington, 1983), a number of studies have shown that in crustaceans, removal of tissue and haemolymph lactate following exhaustive swimming is rather slow, with clearance times of between 3 and 24 h (Phillips *et al.*, 1977; Forster *et al.*, 1989; Booth *et al.*, 1984; Henry *et al.*, 1994). Although crustaceans do appear to possess the capacity for gluconeogenesis from lactate (Gäde *et al.*, 1986, Lallier & Walsh, 1992, Henry *et al.*, 1994), the system is regarded to be considerably less efficient than the Cori cycle described in mammals (see Schulman & Landau, 1992).

Replenishment of glycogen reserves in the abdominal muscles of tail flipping crustaceans is important for the restoration of normal muscle function, since it allows phosphagen repletion (Ellington, 1983; Gruschczyk & Kamp, 1990; Shulman & Landau, 1992). The importance of glycogen in this tissue is probably a consequence of the relatively poor blood supply, which prevents regeneration of arginine phosphate by aerobic mechanisms (Gruschczyk & Kamp, 1990; Milligan & Girard, 1993). However, the recovery of muscle from active exercise in invertebrates occurs in conjunction with a period of supranormal O_2 consumption, indicating that at least some elements of recovery are aerobic processes (Ellington, 1983).

During recovery from exhaustive swimming, replenishment of abdominal muscle phospho-arginine from intracellular glycogen stores is probably very efficient in uninfected and lightly-infected *Nephrops*. However, as phospho-arginine is replenished at the expense

of glycogen, less glycogen will be available at this time as a substrate for Phase 2 swimming (which relies on glycolytic production of ATP). Such a response would explain why uninfected and lightly-infected animals do not perform as many Phase 2 flips during the recovery period.

Stentiford *et al.* (2000a) (Chapter 8) have noted that during *Hematodinium* infection, animals may be in a state of persistent partial exhaustion, which restricts their capacity to respond to the increased respiratory demands of swimming. In heavily infected animals a 50% reduction in the O_2 carrying capacity of the haemolymph leads to an internal hypoxia, with some tissues resorting to anaerobic metabolism, even when the animal is at rest (Taylor *et al.*, 1996). A shift towards anaerobic metabolism (Baden *et al.*, 1994), coupled with the increasing metabolic demands of the parasite is responsible for the reduction the tissue glycogen stores reported in *Hematodinium*-infected lobsters (Stentiford *et al.*, 2000b – Chapter 5). A combination of reduced tissue glycogen and parasite-induced oxygen depletion make it unlikely that sufficient energy will be available for prolonged escape swimming in heavily-infected *Nephrops*. Under such conditions, the efficient repletion of muscle phospho-arginine and glycogen following periods of exertion, is doubtful.

Additionally, the replenishment of tissue glycogen is significantly reduced during periods of starvation (Wood, 1991) and under conditions of hyper-osmolarity, due to mitochondrial shrinkage and a reduction in fatty acid oxidation (leading to increased glycolysis and a reduction in glycogen) (Li *et al.*, 1992). The large changes in the free amino acid composition of the haemolymph and muscle reported to occur during *Hematodinium* infection (Stentiford *et al.*, 1999 – Chapter 4, 2000b – Chapter 5) may be causing similar effects on the accumulation of glycogen in abdominal muscle of *Nephrops*.

The swimming response in relation to catchability.

Swimming data presented for *Hematodinium*-infected *Nephrops* in the current study, in conjunction with that previously reported by Stentiford *et al.* (2000a) (Chapter 8) and other studies on the swimming performance of *Nephrops* (Newland *et al.*, 1992), suggest that infected animals are more likely to be captured by standard trawling rigs than their non-infected counterparts. The variation in swimming endurance (number of flips performed) and the two-phase swimming pattern seen in the absence of infection also suggests that capture by trawlers may not only be dependent on the position of the individual in relation to the net mouth (Newland & Chapman, 1989), but also on physiological status – with those *Nephrops* performing the least flips being the most likely to be overtaken and captured by the trawl net. Furthermore, the reduced swimming performance may make *Hematodinium*-infected (and those uninfected individuals with the lowest swimming endurance) most prone to predation. The lower swimming performance in uninfected *Nephrops* after 4 h recovery suggests that this period was not sufficient to allow complete restoration of the normal swimming response. Recovery from exhaustion in crustaceans is generally slow (up to 24 hr) (Gäde *et al.*, 1986) and it is only when energy reserves have been replenished that locomotory performance recovers. To some extent, the slow recovery time in *Nephrops* may be mitigated by the fact that the species constructs a burrow and is able to retreat to this when threatened by using the escape response. Once within the burrow, recovery from the swimming burst could take place with relatively little interruption. In areas where *Nephrops* exhibits crepuscular emergence, a period in excess of four hours would be available between forays from the burrow. The ability to remain within shelter, however, would depend on energy intake requirements being met and eventually animals have to emerge. On heavily fished grounds therefore, where trawl frequencies can be as high as once every 24 h (Marrs *et al.*, 2000), animals which have recently evaded trawl capture may be more likely to be captured by subsequent trawls. This

effect may be enhanced in *Hematodinium*-infected *Nephrops*, which showed impaired recovery in the 4 h period and are also known to have higher energy requirements owing to the parasite loading (Taylor *et al.*, 1996); which may necessitate more frequent feeding activity. A detailed study of the metabolic limitations of escape swimming in uninfected and *Hematodinium* infected *Nephrops* is required to resolve these issues.

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Fig. 1a-f. Distance travelled by individual tails flips, up to cessation of swimming in a range of uninfected (Stage 0) *N. norvegicus*. The suggested point of transition to the 'second phase' of swimming (see text) is represented by a dashed line.

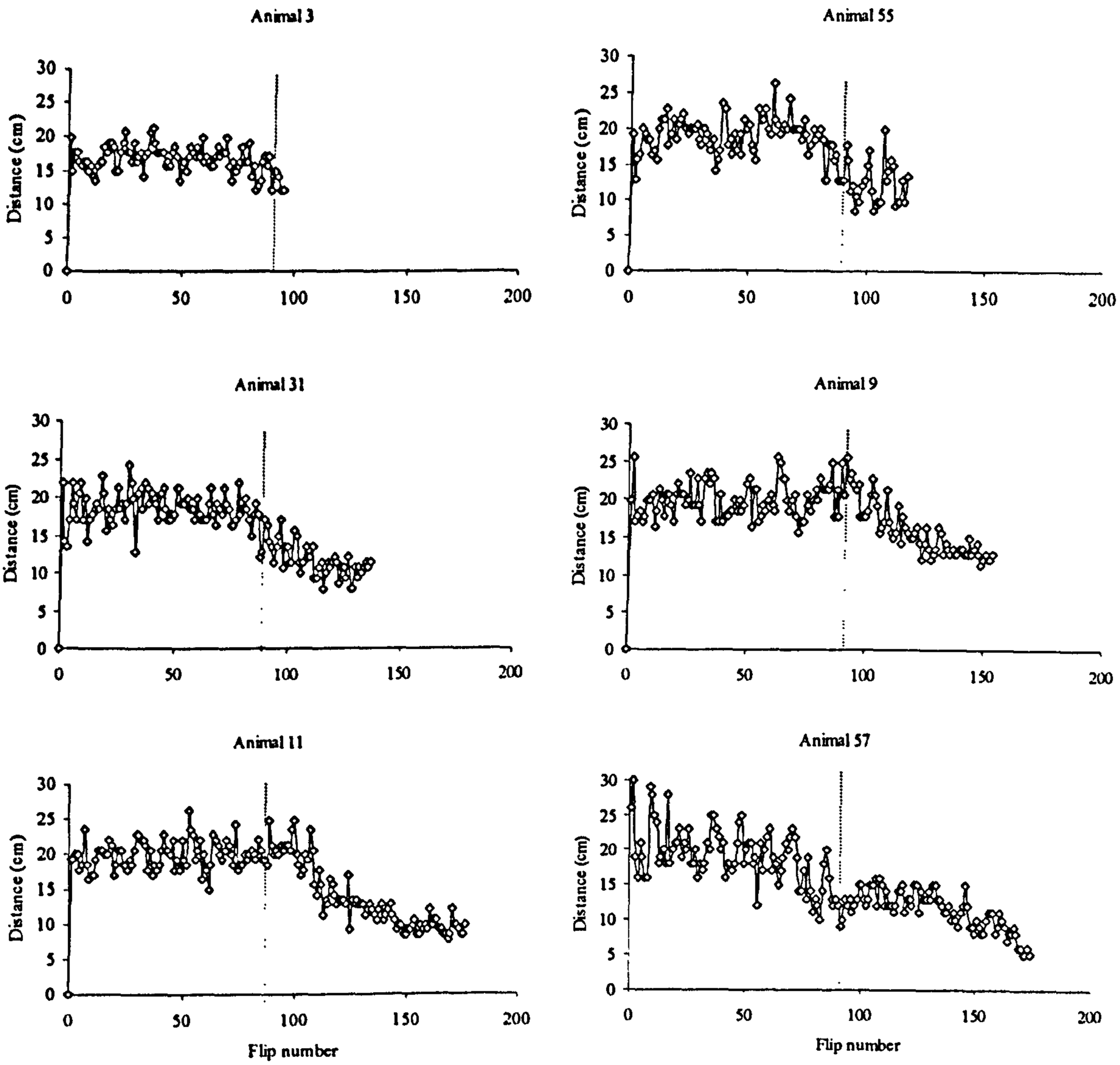


Fig. 2a-d. Distance travelled by individual tail flips, up to cessation of swimming in typical uninfected (Stage 0) and *Hematodinium*-infected (Stages 1, 2 and 3-4) lobsters The suggested point of transition to the 'second phase' of swimming (see text) is represented by a dashed line.

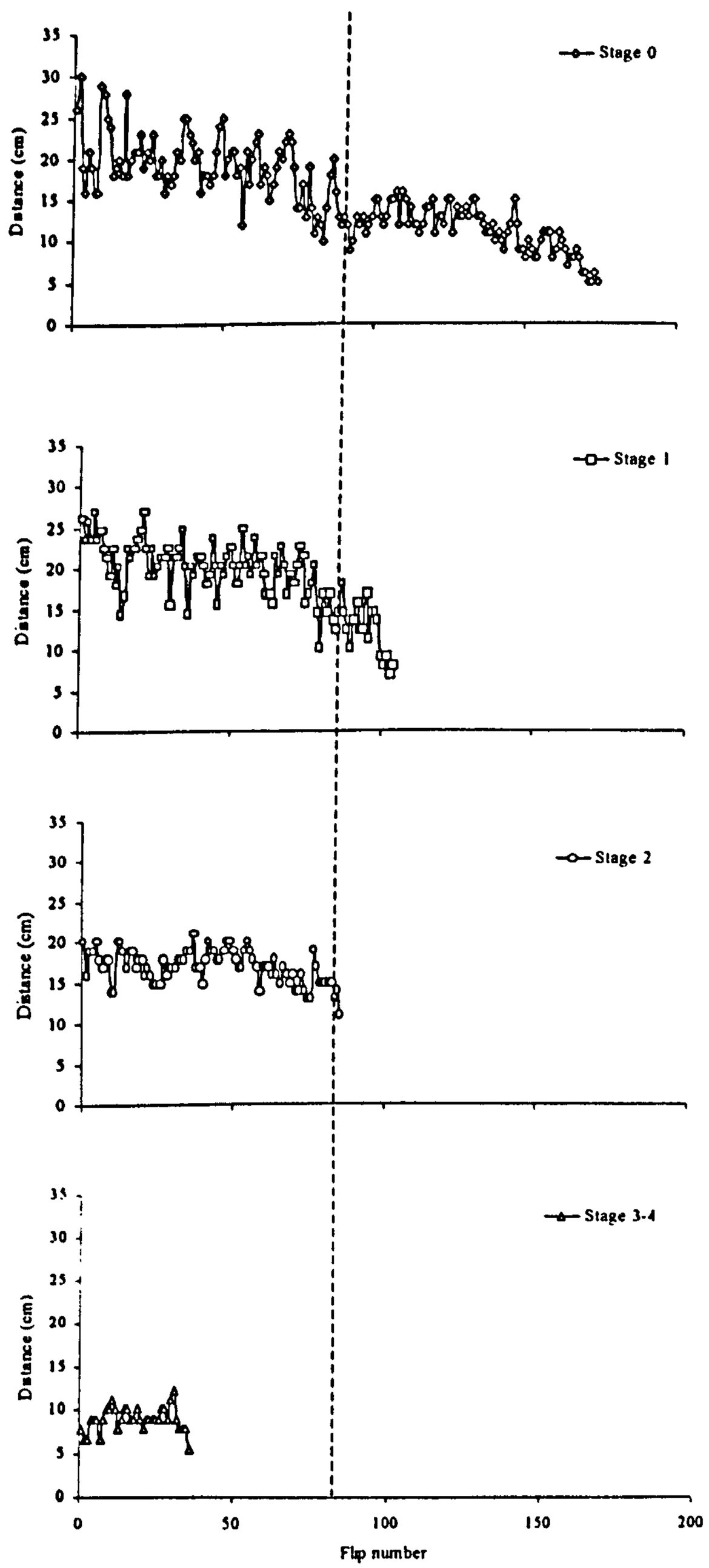


Fig. 3. Correlation plot of distance travelled against number of flips performed until cessation of swimming in uninfected (Stage 0) and *Hematodinium*-infected (Stages 1,2 and 3-4) *N. norvegicus*.

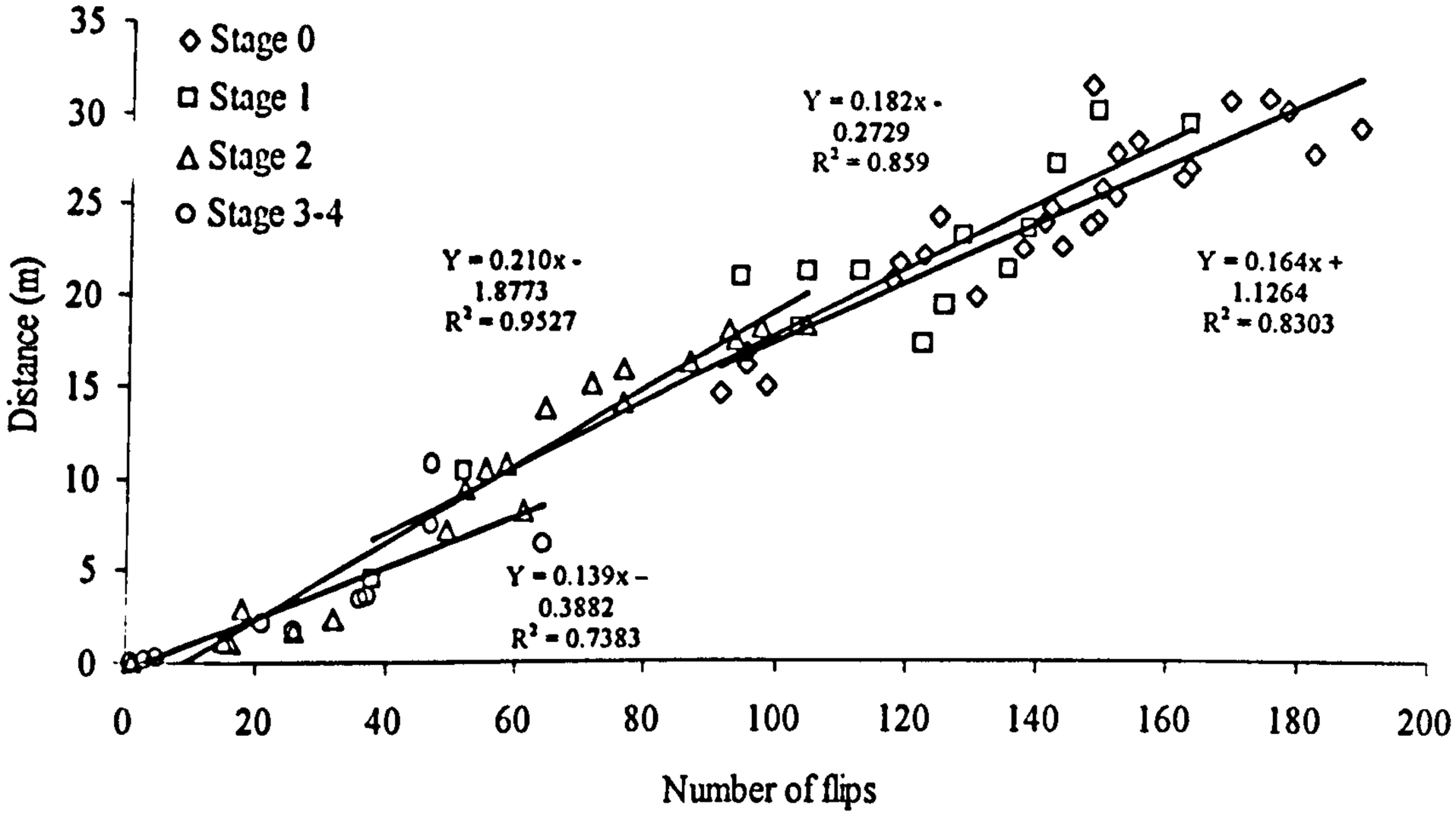


Fig. 4. Mean percentage recovery in the total number of tail flips performed by uninfected (Stage 0) and *Hematodinium*-infected (Stages 1,2 and 3-4) *N. norvegicus* in the 4 h run.

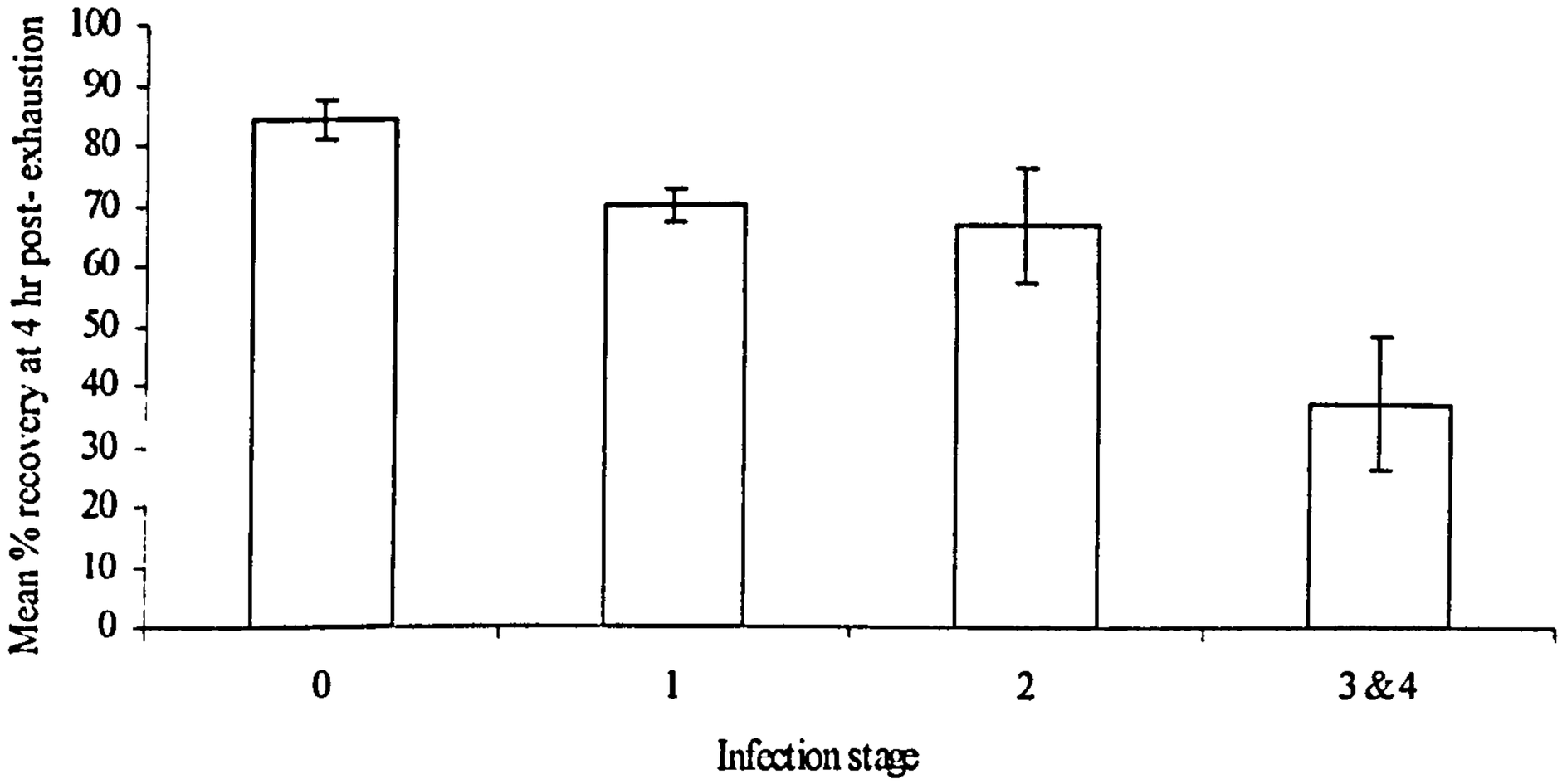


Fig. 5. Derived means of distance travelled and number of flips performed by uninfected (Stage 0) and *Hematodinium*-infected (Stages 1,2 and 3-4) *N. norvegicus*. The data for 0 h (solid symbols) and 4 h (open symbols) are plotted in relation to the lines of best fit for the 0 h data set (see Fig. 2).

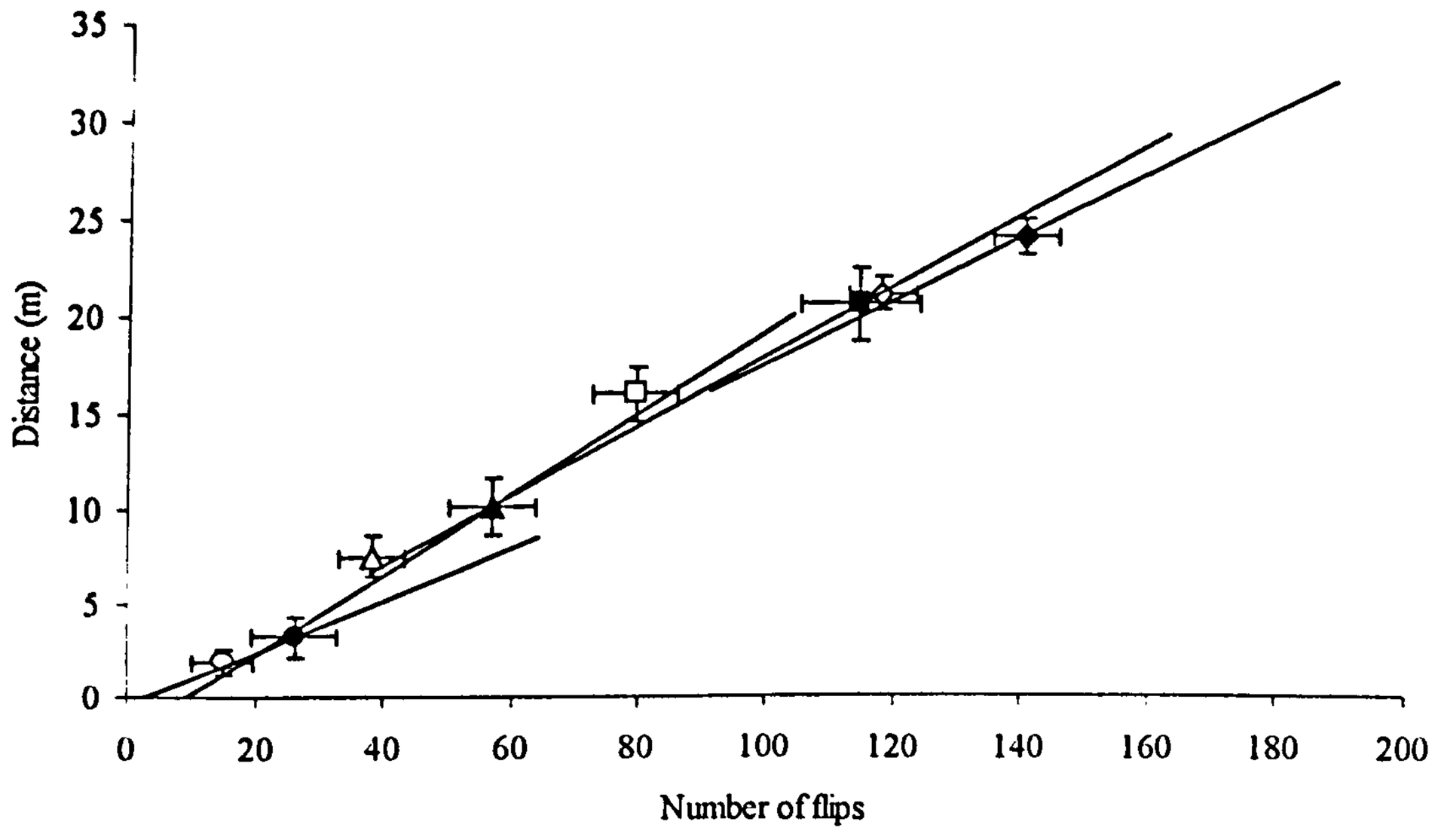
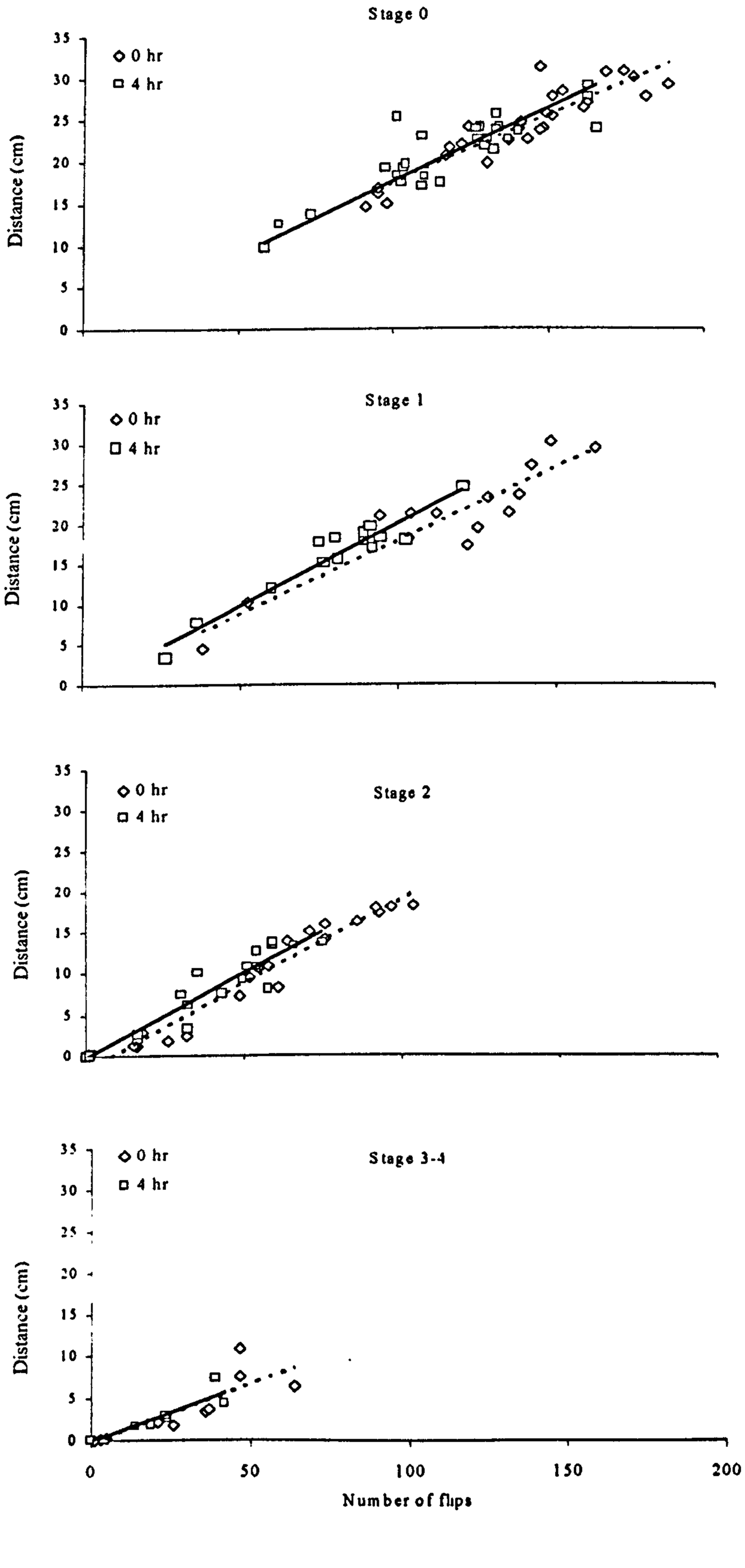


Fig. 6. Correlation plots of distance travelled against number of flips performed until cessation of swimming in uninfected (Stage 0) and *Hematodinium*-infected (Stages 1, 2 and 3-4) *N. norvegicus*. Lines of best fit are dashed (0 h run) or solid (4 h run).



Chapter 10

Alteration of burrow-related behaviour of the Norway lobster, *Nephrops norvegicus* (L.) during infection by the parasitic dinoflagellate *Hematodinium*.

Abstract

The current study has used time-lapse video recordings to analyze the burrow-related behaviour of *N. norvegicus* under a 12 h light : dark regime in aquarium conditions, and compared this to the behaviour of lobsters infected by the parasitic dinoflagellate *Hematodinium*. Uninfected lobsters performed more burrow departures, of longer duration, during the hours of darkness while infected lobsters performed a relatively constant number of similar-duration departures in the light and dark periods. However, the absolute number of departures performed by infected lobsters (70 day^{-1}) was more than double that of uninfected lobsters (30.1 day^{-1}), while the duration of burrow departures performed by infected lobsters (258.5 $s.departure^{-1}$) was more than six times greater than in uninfected lobsters (38.7 $s.departure^{-1}$). This led to a more than ten-times increase (from 1.7 to 19.4 %) in the percentage of the day spent out of the burrow by infected lobsters. The altered burrow-related behaviour could be due to the nutritional demands of the parasites on infected lobsters, causing an increased requirement to forage, or alternatively to a 'functional hypoxia', due to the additional respiratory demands of the parasite, causing the host to emerge from the burrow onto the sediment surface to seek oxygen. Implications for the increased time spent out of the burrow are discussed in relation to availability of infected lobsters to trawlers and predators, and to the use of infection prevalence data from trawl-caught samples in stock assessment models for *N. norvegicus*.

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Introduction.

Infection by parasites is known to have significant effects on the behaviour and life history of host organisms. Behavioural changes may bring the host to a more suitable site for the direct transmission of the parasite, or make intermediate hosts more susceptible to predation (Holmes & Bethel, 1972; Poulin, 1994). At the community level, parasites and pathogens are recognized as important factors in determining the density and long-term population dynamics of many animal populations (Dobson, 1988; Kuris & Lafferty, 1992). The discovery of a dinoflagellate parasitic infection in populations of the Norway lobster (*Nephrops norvegicus*) (Field *et al.*, 1992) provides an opportunity to study the effects of a parasitic infection on some well-studied aspects of the burrow-related behaviour of a scavenging crustacean, and to consider the practical consequences of such changes for the large fishery which exists for this commercially important shellfish species.

The Norway lobster (*Nephrops norvegicus*) is widely distributed on the continental shelf of the northeast Atlantic and in the Mediterranean sea, and inhabits burrows constructed in fine, cohesive mud substrata (Chapman, 1980). The general form of *N. norvegicus* burrows and the method of burrow construction have been well described (Rice & Chapman, 1971; Farmer, 1974a). The burrows provide protection against predatory attacks (Rice & Chapman, 1971) and are important for the incubation of eggs by females (Farmer, 1974b). Studies on the burrow-emergence behaviour of *N. norvegicus* have shown that its pattern is dependent on depth, with shallow water animals most active during darkness, deep water animals during the light and animals from intermediate depth showing a crepuscular activity pattern (Chapman, 1980). The basis for this emergence pattern may be associated with the reception of light by the eye and subsequent movement of the retinal shielding pigments (Aréchiga & Atkinson, 1975; Shelton & Gaten, 1986). As such, the relatively higher light intensity experienced in the middle of the day may inhibit out-of-burrow activity (Aréchiga & Atkinson, 1975). However, recent studies have shown that light-

induced eye damage has little effect on various aspects of *N. norvegicus* behaviour (including burrow-emergence) (see Chapman *et al.*, 2000), suggesting that activity rhythms may be entrained, at least in part, by light detection by other receptors, such as photosensitive neurones in the brain (Page & Larimer, 1976) and the caudal photoreceptor (Wilkens, 1988). In addition to the direct effects of light intensity, food availability and tidal strength may also be implicated in the observed burrow-emergence pattern (Atkinson & Naylor, 1976; Newland *et al.*, 1988), as may be some unknown endogenous determinant (Aréchiga and Rodriguez-Sosa, 1997).

N. norvegicus supports a major fishery in the United Kingdom, with the main method of capture being otter trawling, which relies upon lobsters being out of their burrows (Atkinson & Naylor, 1976; Newland & Chapman, 1989). It follows that any factor that increases the amount of time spent out of the burrow may be expected to lead to larger catches by trawling, and that such changes in behaviour provide the most likely explanation for the observed short term variation in catches (Atkinson & Naylor, 1976; Farmer, 1974c).

Stocks of *N. norvegicus* off the west coast of Scotland have been shown to harbour a seasonal infection by a parasitic dinoflagellate of the genus *Hematodinium* (Field *et al.*, 1992) which is responsible for a disease syndrome resembling the 'Bitter Crab disease' (BCD) reported in Alaskan tanner crabs (*Chionoecetes bairdi* and *C.opilio*) (Meyers *et al.*, 1987; Taylor & Khan, 1995) and also *Hematodinium* infections in other decapod hosts (Newman & Johnson, 1975; Wilhelm & Boulo, 1988; Latrouite *et al.*, 1988; Shields, 1992; Hudson *et al.*, 1993). A number of studies have established the basic characteristics of *Hematodinium* infection in *N. norvegicus* in terms of its progression, diagnosis and pathology (Field and Appleton, 1995, 1996), its effect on host physiology (Taylor *et al.*, 1996), on haemolymph and tissue biochemistry (Stentiford *et al.*, 1999 – Chapter 4, 2000a – Chapter 5) and on locomotion (Stentiford *et al.*, 2000b – Chapter 8).

Animals showing symptoms of patent infection (moribund, cloudy haemolymph, bright shell coloration) are seen in catches taken during the spring and early summer (Field *et al.*, 1992), and it was suggested by these authors that infected lobsters may be more prone to capture by trawlers than uninfected lobsters. Some evidence for a behavioural component to this susceptibility was obtained in a study which repeatedly sampled one fishing ground over a 24 h period (Field *et al.*, 1998), and found diurnal changes in the percentage of infected lobsters making up the catch. However, no systematic study has yet been performed to identify the behavioural changes induced by *Hematodinium* infection. The present study was therefore carried out to characterise any changes that may occur in the burrow-related behaviour of aquarium-held *N. norvegicus* infected by *Hematodinium*, and to consider these changes in relation to the catchability of infected lobsters.

Methods.

Collection and treatment of animals.

Norway lobsters were collected with an otter trawl from depths of approximately 80 m at a location south of Little Cumbrae in the Clyde Sea area, Scotland, UK (55.41°N, 4.56°W). Before being placed into experimental tanks, lobsters were maintained for approximately one week in a closed aquarium (9°C, 33 ppt salinity) at the University Marine Biological Station Millport, Isle of Cumbrae, Scotland. Animals were fed *ad libitum* upon capture on squid (*Loligo* spp.) and mussels (*Mytilus* sp.) but were not fed thereafter. Only male *N. norvegicus*, which are known to have the same endogenous emergence rhythms year round (Atkinson & Naylor, 1976) were used in experimental runs. Individuals with a carapace length of around 30 mm were chosen, as *Hematodinium* infection is most common in animals of this size (Stentiford *et al.*, 2000a – Chapter 5). All animals were in the intermoult state as defined by Aiken (1980). Since animals were to be held in the experimental tanks for five days, and patent infection would progress over this time, assessment of *Hematodinium* infection was performed at the end of

each trial. For this, the pleopod staging method of Field & Appleton (1995) was used, according to which Stage 0 denotes an apparently uninfected animal, with patent infection progressing from Stage 1 through to Stage 4. All animals used were of Stage 2 or Stage 3 infection (medium to heavy parasite burden).

Experimental set-up.

Experiments designed to examine the effect of *Hematodinium* infection on the burrow-related activity of *N. norvegicus* were carried out in a circular tank (∅ 1.5 m, depth 0.75 m – see Fig. 1A-F) containing well-settled sediment (0.35 m depth) obtained from the Clyde Sea area. The tank was placed in a light-controlled room and exposed to a 12 h : 12 h light-dark cycle (illumination from 0700 h to 1900 h). During the illuminated period, diffuse green lighting was used (c.1.0 $\mu E.\ s^{-1}\ m^{-2}$), while red lamps provided sufficient light for video recording for the whole light-dark period. A video camera (Panasonic WV-CL350) fitted with a 6.5mm wide-angle lens was mounted above the tank, providing a view of the whole sediment surface. The signal from the camera was fed to a time lapse video recorder (Panasonic AG-6730) set to a framing rate of 1 per second. Animals were placed individually in the tank, and their behaviour was recorded for a period of 5 days. In total 10 uninfected and 10 *Hematodinium*-infected lobsters were monitored in this way.

Video Analysis.

The video recordings were analyzed using a Panasonic AG-5700 VCR linked to a Panasonic AG-570 single-frame editing facility controller and a Panasonic WV-CM1000 colour monitor. The whole recording was reviewed, in order to assess the behaviour performed, but the first 24 h of the video recording was not used for the quantitative analysis of burrow-related behaviour. Thereafter, measurements were made over the next 3

consecutive days of the total time spent within and outside of the burrow, the number of burrow departures and the duration of individual excursions from the burrow. Thus data for a total of 30 animal-days were obtained for each of the two experimental groups.

Lobsters were scored as 'in-burrow' when part or all of their bodies were within the confines of the burrow (Fig. 1A, B and F) and 'out-of-burrow' when the whole body (including telson and uropods) were outwith the confines of the burrow (Fig. 1C, D and E). Data obtained from the three days of filming was averaged for each hour and pooled with other animals from the group (uninfected or *Hematodinium*-infected) to allow assessment of the effect of parasitic infection on burrow-related behaviour.

Data Analysis.

Comparisons of behavioural data from uninfected and infected lobsters were performed either by one-way analysis of variance (ANOVA) or a Kruskal-Wallace test followed by between stage comparisons with a Tukey's pairwise analysis (for normal distributed data), or by a Mann-Whitney test (for non-normal distributions). Tests were considered significant if $p < 0.05$.

Results.

Observations on burrow-related behaviour.

When a lobster was placed into the experimental tank, it took up occupancy of an existing burrow, or in some cases excavated a new burrow. Thereafter it extended and repaired the burrow, and evidence of this was obtained even when the animal was inside the burrow from the appearance of sediment plumes emerging from one or more openings. The lobster often occupied the main burrow opening (Fig. 1B), with only its claws visible, and from this position it would periodically either retreat into the burrow (Fig. 1A), or emerge onto the surface for an excursion (Fig. 1C, D and E). Both uninfected and infected animals

showed these patterns of behaviour, although their duration and timing were different in the two groups (see below).

Another observation was made that was unique to the *Hematodinium*-infected lobsters. In two cases, in the video recording beyond the 3 day period of measurement, the animals produced a series of relatively uncoordinated tail flexions ('spasms'), and between these spasms they lay in a quiescent state on the sediment surface. The frequency of these spasms increased over a 3-4 hour period, and after the final one of these the animals showed no further movement, and were found to be dead at the end of the trial.

Quantitative analysis of burrow-related behaviour.

Burrow departures. Measures of the number of burrow departures, expressed in terms of the mean hourly values over a 24 hour period, are shown in Figure 2. Uninfected lobsters performed a greater number of burrow departures during the hours of darkness than during the hours of light, the mean value for the complete periods of dark (1.72 departures h^{-1}) and light (0.79 departures h^{-1}) being significantly different ($p < 0.001$) (Fig. 2). In contrast, *Hematodinium*-infected lobsters displayed a pattern of burrow departures that was relatively constant over the 24 h period (Fig. 2), and the number of departures performed in the complete dark period (3.08 departures h^{-1}) and the complete light period (2.75 departures h^{-1}) (Fig. 3) are not significantly different ($p = 0.086$). However, both these values for infected lobsters are significantly greater than those for the uninfected lobster in their more active period, the dark ($p < 0.001$ in each case). Over the whole 24 hour period, the mean total number of burrow departures performed by infected lobsters (70 departures day^{-1}) was more than double the number performed by uninfected lobsters (30.1 departures day^{-1}) ($p < 0.01$) (Fig. 4).

Duration of excursions. Measures of the duration of individual excursions from the burrow onto the sediment surface, expressed in terms of the mean hourly values over a 24 hour period, are shown in Figure 5. This shows that uninfected lobsters made longer excursions from the burrow during the hours of darkness than during the hours of light, and the mean values calculated for the complete periods of dark and light (49.84 s departure^{-1} and 27.65 s departure^{-1} respectively) (Fig. 6) are significantly different ($p < 0.001$).

The excursion duration's of *Hematodinium*-infected lobsters also showed some variation throughout the 24 hour period, though the difference in the mean excursion duration between the light (212.9 s departure^{-1}) and the dark (304.2 s departure^{-1}) was not significant ($p = 0.061$). This was probably due to the large variation in the dark data set (see Fig. 5).

However, a more important feature of the excursions made by infected lobsters was their greatly increased duration, and values for both the dark and light periods are significantly greater than those for the uninfected lobster in their more active period, the dark ($p < 0.001$ in each case). Over the whole 24 hour period, therefore, the mean excursion duration for infected lobsters (258.5 s departure^{-1}) was more than six times the duration of the excursions performed by uninfected lobsters (38.7 s departure^{-1}) ($p < 0.001$) (Fig. 7).

Total time spent out of the burrow. By combining the data for the number of burrow departures and the duration of individual excursions, values were derived for the total time spent out of the burrow by uninfected and infected lobsters. Data expressed in terms of the mean hourly values over a 24 hour period are shown in Figure 8, and reflect the trends observed in the separate measures of burrow departures and excursion duration's. Thus uninfected lobsters spent significantly longer on the sediment surface during the period of darkness (mean for complete period 90.3 s h^{-1}) than during the period of light (mean for complete period 30.4 s h^{-1}) ($p < 0.001$) (Fig. 9).

Infected animals also spent a longer time out of the burrow during the period of darkness (mean for complete period 787.3 s h^{-1}) than during the period of light (mean for complete period 610.9 s h^{-1}) ($p < 0.05$). In this case, therefore, the combined measure reveals a significant difference between dark and light activity, despite the separate measures of burrow departures and excursion times not being significantly different (see Figs. 3 & 6).

However, the most important feature of the data is again the large and significant difference between the measures for the uninfected and infected animals (Figs 8, 9). Considered as the % time spent out of the burrow over the whole 24 hour period (Fig. 10), the value for infected lobsters (19.4 % of day out-of-the-burrow) is more than ten times greater than that for uninfected lobsters (1.7 % of day) ($p < 0.001$).

Discussion.

Burrow-related behaviour in uninfected lobsters.

Data presented for the burrow-related behaviour of male *N. norvegicus* show that under the conditions provided, lobsters were most active during the hours of darkness, with reduced out-of-burrow activity during illuminated periods. A prominent feature of emergence behaviour in uninfected male *N. norvegicus* was the relatively small proportion of the whole day spent on the sediment surface (mean 1.7%). This degree of activity fits well with field observations of emergence (Chapman & Rice, 1971; Chapman, 1980) and catch profiles for lobsters living at depths of 75-90 m (Atkinson & Naylor, 1976).

Uninfected male *N. norvegicus* make a greater number of burrow departures, of longer duration during the hours of darkness than during the hours of light. The burrows of *N. norvegicus* are principally for refuge from predators (Rice & Chapman, 1971) and emergence is probably governed by the availability of suitable food (Farmer, 1974c; Cristo, 1998) and the level of illumination (Atkinson & Naylor, 1976). The increased number and duration of departures made by uninfected lobsters during the hours of darkness probably

reflect a combination of a lowered perceived risk of predation and a physiological requirement to feed. Similar variations in diurnal activity are seen in crayfish: *Pacifastacus leniusculis* reduces the success of visual predators by remaining in shelter during the day (Blake & Hart, 1995), while *Procambarus clarkii* exhibits activity patterns which are strongly influenced by food availability (De Miguel & Aréchiga, 1994).

As the lobsters in this study were not captured in light-controlled conditions, there is likely to have been some damage to the retinal shielding system of the eyes (Aréchiga & Atkinson, 1975; Shelton & Gaten, 1986). However, the appearance of a light-dark rhythm, even in these visually compromised animals suggests that activity patterns in *N. norvegicus* from these depths may not be totally reliant on light reception by the eyes. This reinforces data presented by Atkinson & Naylor (1976) which showed that an endogenous activity rhythm occurs even when lobsters are held in constant darkness, and that this activity may be entrained by photosensitivity other than through the eye, possibly via the caudal photoreceptor (Wilkens, 1988; Simon & Edwards, 1990). Furthermore, Richardson (in Chapman *et al.*, 2000) has reported that light-induced damage has little effect on the burrow-emergence rhythm, feeding, agonistic behaviour or predator avoidance behaviour of *N. norvegicus*. The maximum absorbance of the retina of *N. norvegicus* is around 498 nm and the absorbency in the red end of the spectrum is known to be minimal (Loew, 1974). As constant red light was provided throughout the current experiments and reception of green light by the eyes during the illuminated period may have been compromised, the changes in burrow-emergence activity recorded in uninfected *N. norvegicus* may be a remnant of an endogenous activity rhythm (Atkinson & Naylor, 1976; Aréchiga and Rodriguez-Sosa, 1997). However, the current study was not designed to investigate the factors controlling burrow-related activity.

Burrow-related behaviour in Hematodinium-infected lobsters.

Male *Hematodinium*-infected lobsters were found to spend significantly more time on the sediment surface (mean 19.4 %) than their uninfected counterparts. This increase in the total time spent out of the burrow was due to the significantly increased number and duration of burrow departures. The lack of any significant difference between the number and duration of burrow departures performed in the light and dark periods suggests that infected male lobsters do not show the same degree of rhythmicity seen in uninfected male lobsters. However, when the number and duration of burrow departures were combined to give total time out of the burrow, the difference in activity between the light and dark periods was significant. This suggests that although the absolute level of activity is greatly increased in infected male lobsters, they may still retain some degree of emergence rhythmicity. Infected male lobsters were also significantly more active during the illuminated period than uninfected male lobsters were during darkness. This suggests that the motivation of infected lobsters to leave the relatively safe confines of the burrow exceeds the perceived risk of predation associated with emergence during the illuminated period.

Parasites are known to indirectly affect host behaviour through the depletion of the hosts energy reserves and by impinging upon their respiratory requirements (Barber *et al.*, 2000). The increase in foraging time necessary to provide the nutritional requirements of the host (and the parasite) appear to be made at the expense of predator avoidance behaviour (Millinski, 1985). In such cases, altered behaviour may make parasitized hosts more prone to predation by non-specific and definitive hosts for the parasite (Brassard *et al.*, 1982; Levri, 1999). The increased number and duration of burrow departures (especially during the illuminated period) observed in the current study is consistent with an increase of 'risk-prone' behaviour in male *Hematodinium*-infected lobsters. The basis for this increase in risk prone behaviour could be purely nutritional, as *Hematodinium*-infected *N. norvegicus*

are known to have severely depleted plasma and tissue carbohydrate reserves (Stentiford *et al.*, 2001, in manuscript – Chapter 6; Stentiford *et al.*, 2000a – Chapter 5, respectively), reduced concentrations of plasma protein (Taylor *et al.*, 1996) and a disrupted plasma and tissue amino acid profile (Stentiford *et al.*, 1999 – Chapter 4, 2000a – Chapter 5). It has therefore been suggested that *Hematodinium*-infected lobsters are undergoing 'physiological starvation' caused by the large burden of parasites in the haemolymph (Stentiford *et al.*, 2000a – Chapter 5). Such nutritional stresses have been related to increased foraging activity in parasitically infected hosts (Cunningham *et al.*, 1994).

The parasitic utilization of oxygen in the host plasma has also been implicated in the alteration of host behaviour during infection (Lester, 1971). During *Hematodinium* infection of *N. norvegicus*, a reduction in haemocyanin concentration leads to a deficit in the oxygen carrying capacity of the haemolymph. This coincides with an elevated oxygen demand from the developing parasites, leading to severe respiratory stress in the lobster (Taylor *et al.*, 1996). During conditions of moderate environmental hypoxia, *N. norvegicus* is known to exit the burrow, which is relatively more hypoxic than the water, in favour of the sediment surface (Baden *et al.*, 1990). The increased out of burrow activity observed in male *Hematodinium*-infected lobsters may thus represent the expression of such a respiratory stress response.

Is Hematodinium actively manipulating its host?

A well-known paradigm in host-parasite interactions is the ability of parasites to actively manipulate the host, in order to facilitate their own transmission (Levri, 1999). Thus alterations of host behaviour during parasitism may not simply be non-adaptive by-products of infection, but may represent a manipulation of host behavioral responses by the parasite (Poulin, 1994). However it is difficult to show unequivocally that manipulation of host behavioral responses by the parasite is occurring (Poulin, 2000), as this requires

evidence for a fitness gain by either the parasite or the host (Poulin, 1995). In the case of *Hematodinium* infection of *N. norvegicus*, incomplete knowledge of the life cycle of the parasite and the mode of transmission between hosts (Appleton & Vickerman, 1998) preclude conclusions about the 'motivational' basis for the altered behaviour observed in the present study. The component of behaviour in infected lobsters that is the strongest candidate for active manipulation by the parasite is the spasmodic tail flipping that was observed in two infected lobsters in the last hours before death. It certainly conforms to the precept that an 'active manipulation' by a parasite is most likely to occur when the intensity of infection is maximal Poulin (1994). Previous aquarium observations of *Hematodinium*-infected *N. norvegicus* have identified a terminal stage of infection (parasite sporolation), where swarming stages of the parasite are extruded via the integumental membranes, gills and the mouth, leaving the host moribund (Field and Appleton, 1995; Appleton and Vickerman, 1998). It is possible that spasmodic tail flipping is a behavioral manifestation of this sporolation event, causing rupture of integumental membranes, and allowing the motile dinospores to emerge. This behaviour will also bring the infected lobster to the sediment surface where broadcasting of the spores would be more effective.

Implications of altered behaviour for the N. norvegicus population and fishery.

Previous studies on *Hematodinium*-infected *N. norvegicus* have shown that infected lobsters exhibit severely reduced tail-flip swimming performance, which is likely to increase their likelihood of capture by predators and trawl nets (Stentiford *et al.*, 2000b – Chapter 8). Longer periods spent out of the burrow will exacerbate this effect by making the animals more susceptible to predation. However, increased predation is only likely to sustain infection in a population if infected hosts are consumed by other hosts for the parasite (Barber *et al.*, 2000). *Hematodinium* infection has been discovered in the hermit crab (*Pagurus bernhardus*) captured from the *N. norvegicus* fishing grounds in the Firth of

Clyde, Scotland, and it is possible that this species acts as an alternative host for this parasite on these grounds. Scavenging on moribund or recently dead *Hematodinium*-infected *N. norvegicus* by *P. bernhardus* may perpetuate the infection from one season to another. Further studies on the epidemiology of *Hematodinium* infection in *P. bernhardus* are necessary to resolve this issue.

Changes in the behaviour and locomotion of *Hematodinium*-infected *N. norvegicus* should also be considered when calculating infection prevalence from trawl-caught samples (see Stentiford *et al.*, 2000b – Chapter 8). Increased time spent out of the burrow when coupled with a reduced swimming performance, should increase the likelihood of infected lobsters being captured by trawl nets, thus leading to an overestimation of the true level of prevalence in the population sampled. Previous attempts to incorporate the observed high prevalence of *Hematodinium* infection into analytical stock assessments of the Clyde Sea area have modeled the effect of infection as an additional loading on the natural mortality rate (since animals showing symptoms of patent infection usually die) (ICES, 1997). However, this has led to unrealistic estimates of recruits entering the fishery, due in part to the fact that the infection may not necessarily add to overall natural mortality, but rather replace a proportion of it, since infected animals will also be more susceptible to predation through a reduced ability to escape. As such, it is suggested that the incorporation of infection prevalence data into stock assessment models for populations of *N. norvegicus* exhibiting significant infection prevalence, should take into account the effect of behavioural and locomotory modifications in the alteration of catchability. Also, changes in behaviour have to be considered in relation to lobsters at different stages of infection, and to the more complex burrow-related behavioural pattern of female lobsters.

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Fig. 1A-F. Still images from video recordings of *N. norvegicus* burrow-oriented activity; (A) completely within burrow (burrow entrances shown by arrows), (B) guarding main burrow entrance, (C) emerging for surface excursion, (D, E) on the surface of the sediment during an excursion and (F), guarding second burrow entrance. Scale bar = 50 cm.

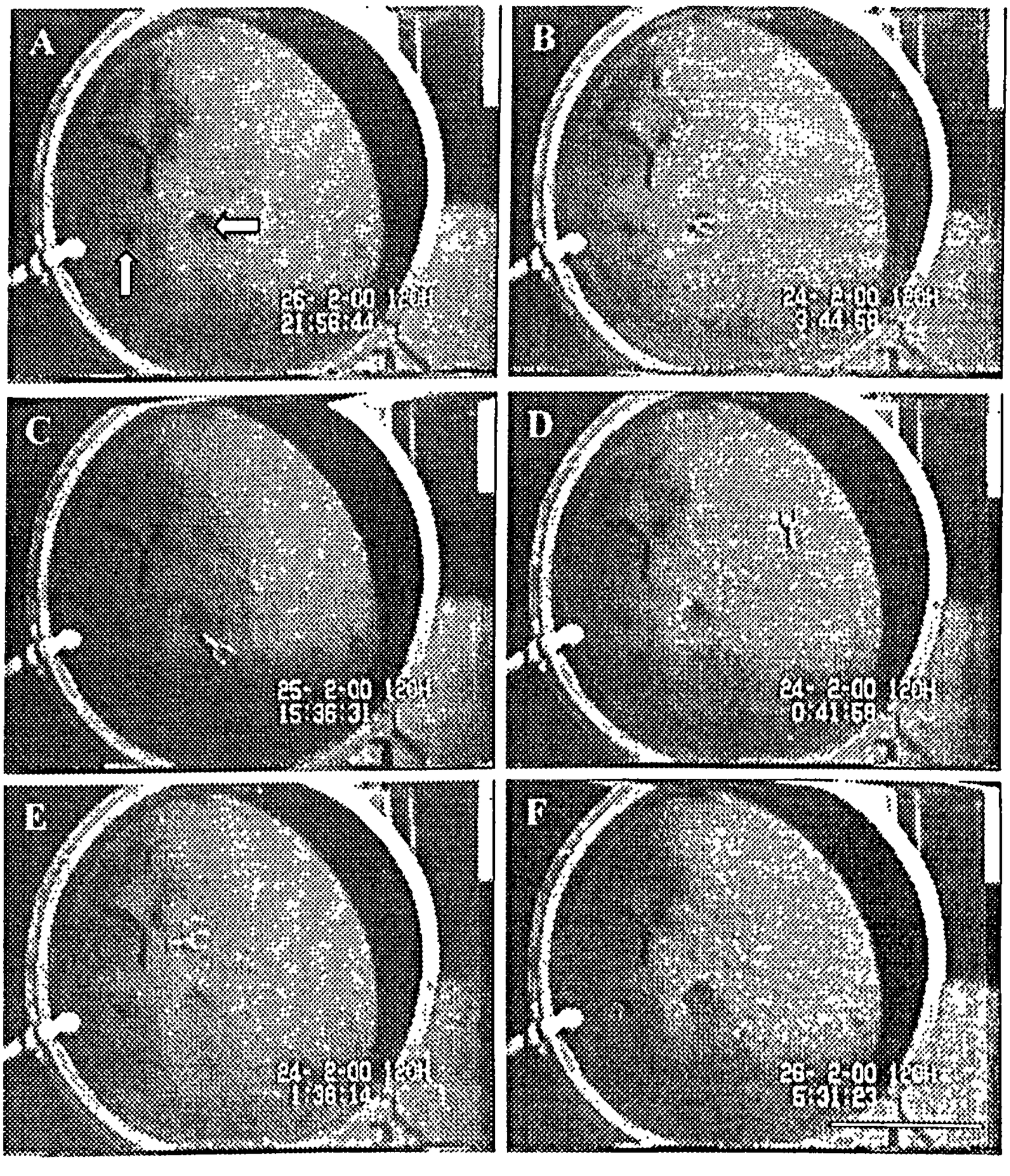


Fig. 2. Number of burrow departures by hour made by uninfected and *Hematodinium*-infected lobsters. Header bar indicates the illuminated and dark periods.

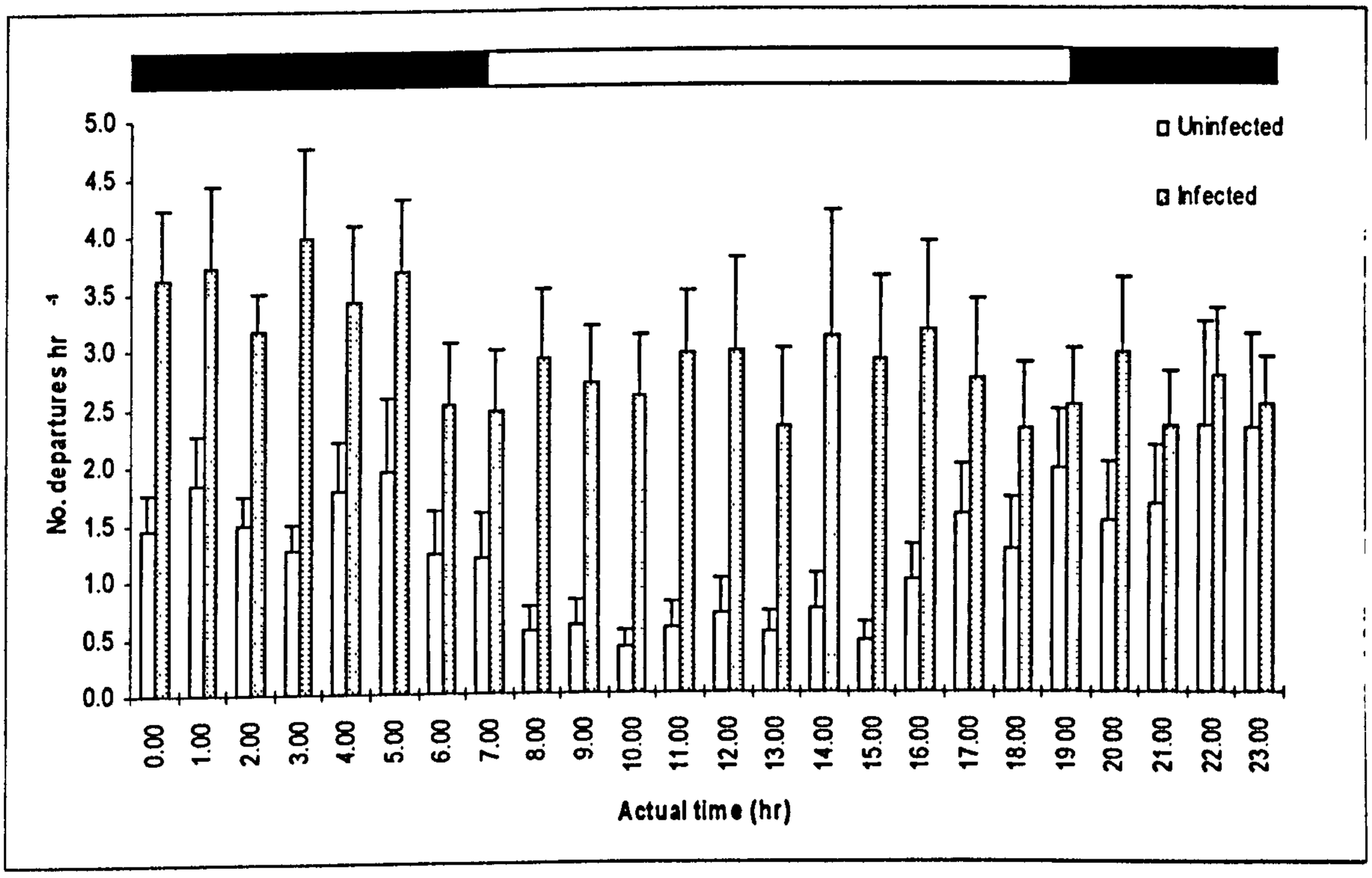


Fig. 3. Mean number of burrow departures per hour made by uninfected and *Hematodinium*-infected lobsters in the light and dark periods.

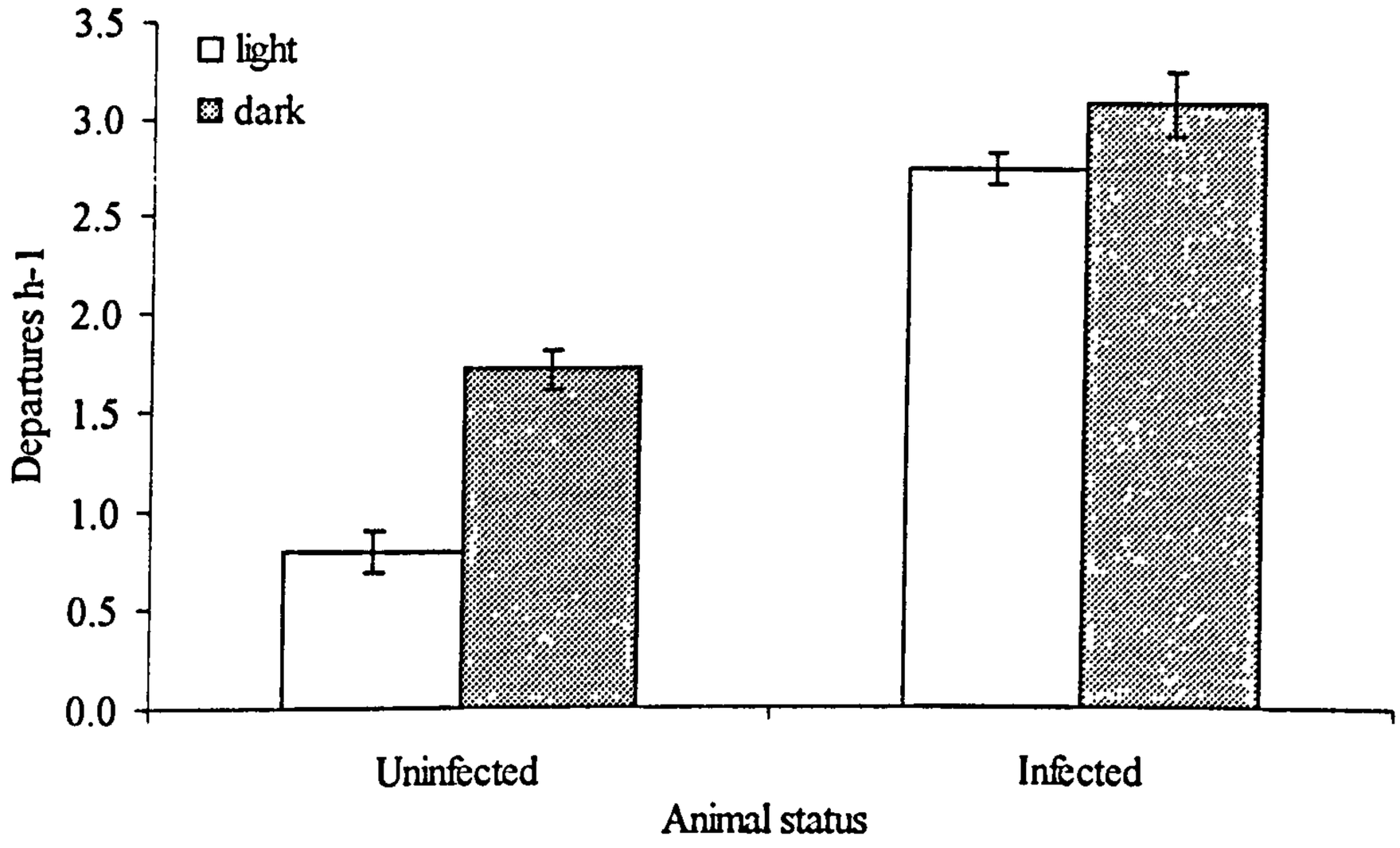


Fig. 4. Mean number of burrow departures per day by uninfected and *Hematodinium*-infected lobsters.

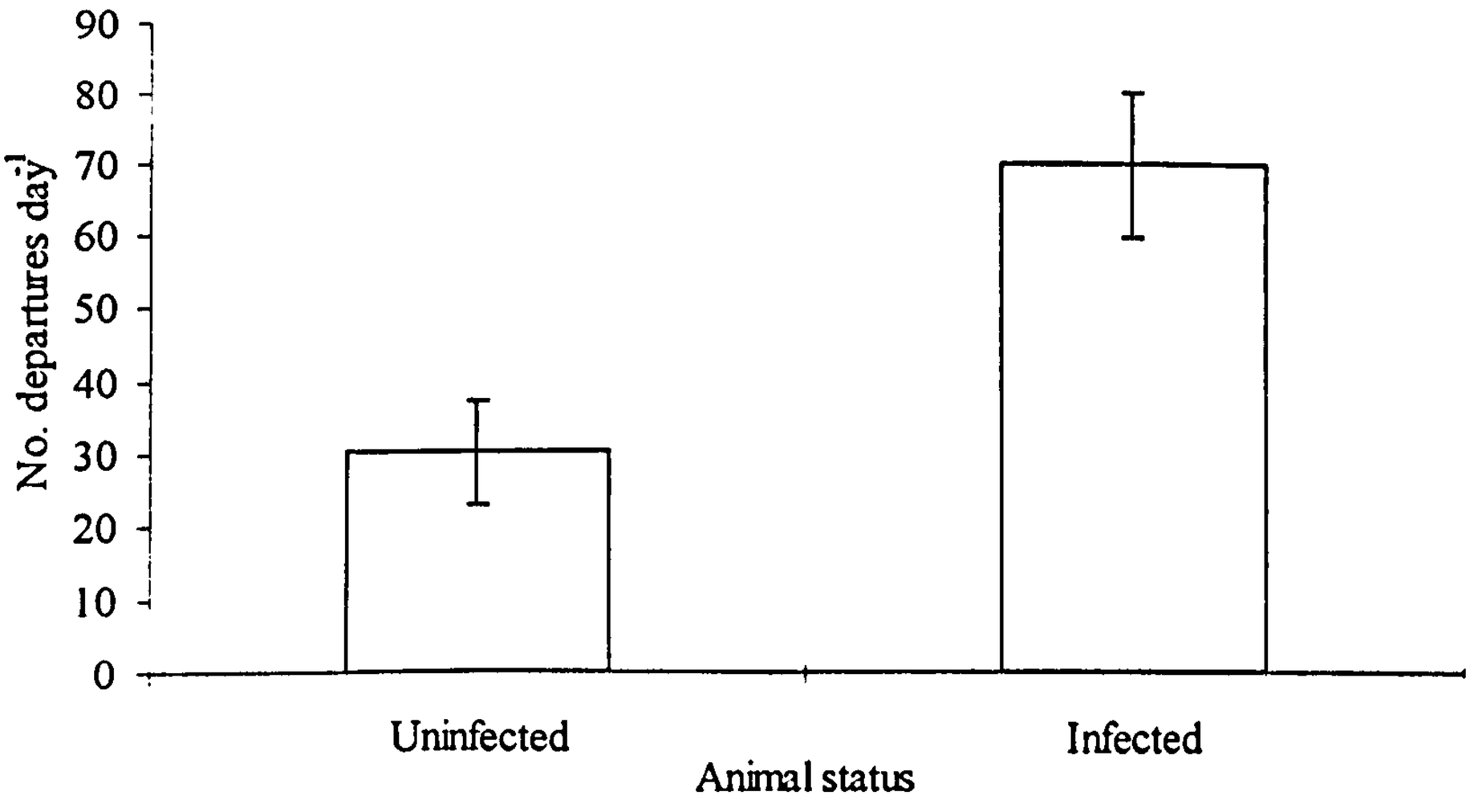


Fig. 5. Burrow excursion duration by hour for uninfected and *Hematodinium*-infected lobsters. Header bar indicates the illuminated and dark periods.

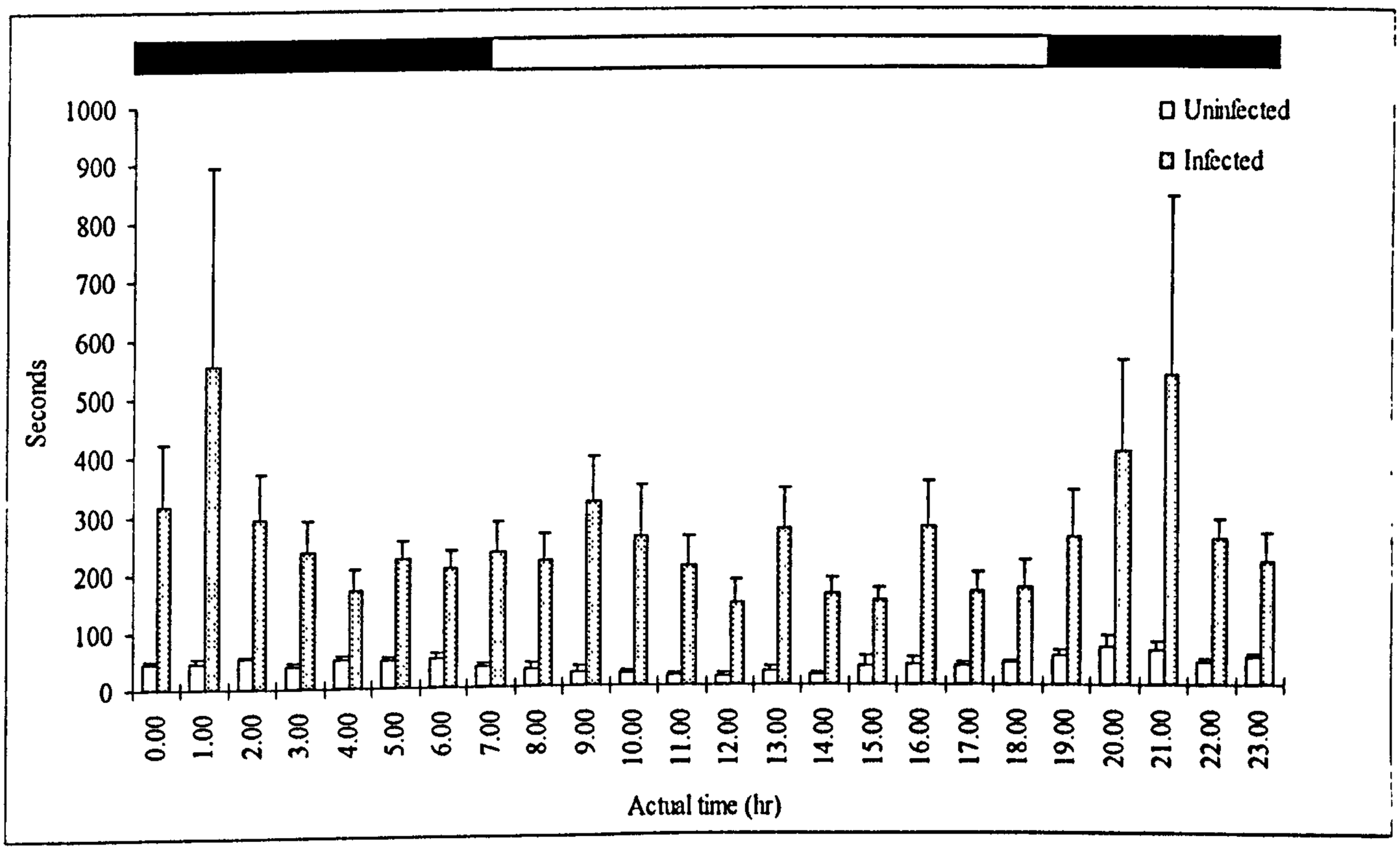


Fig. 6. Mean duration of burrow excursions made by uninfected and *Hematodinium*-infected lobsters in the light and dark periods.

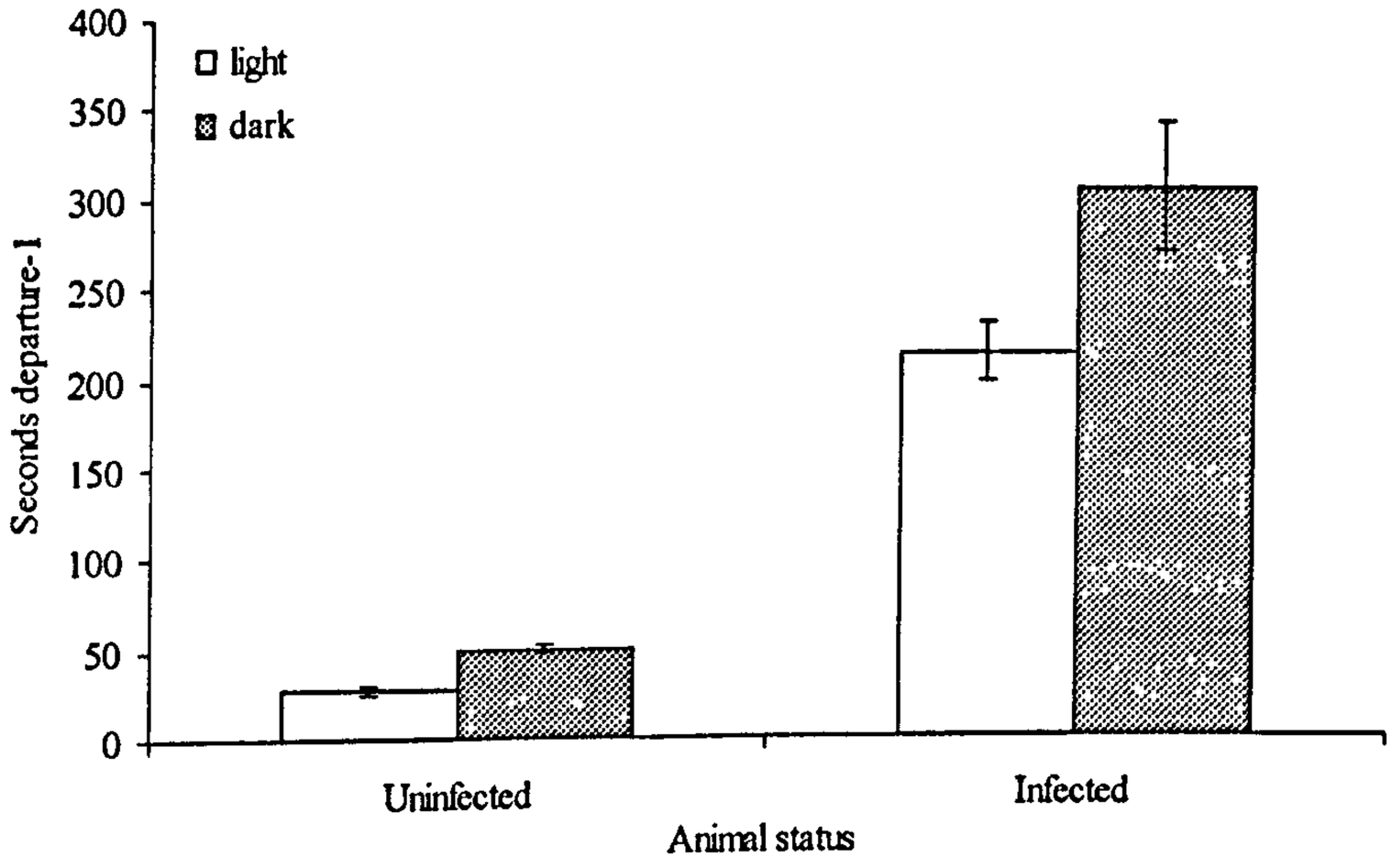


Fig. 7. Mean duration of burrow excursions over the whole day made by uninfected and *Hematodinium*-infected lobsters.

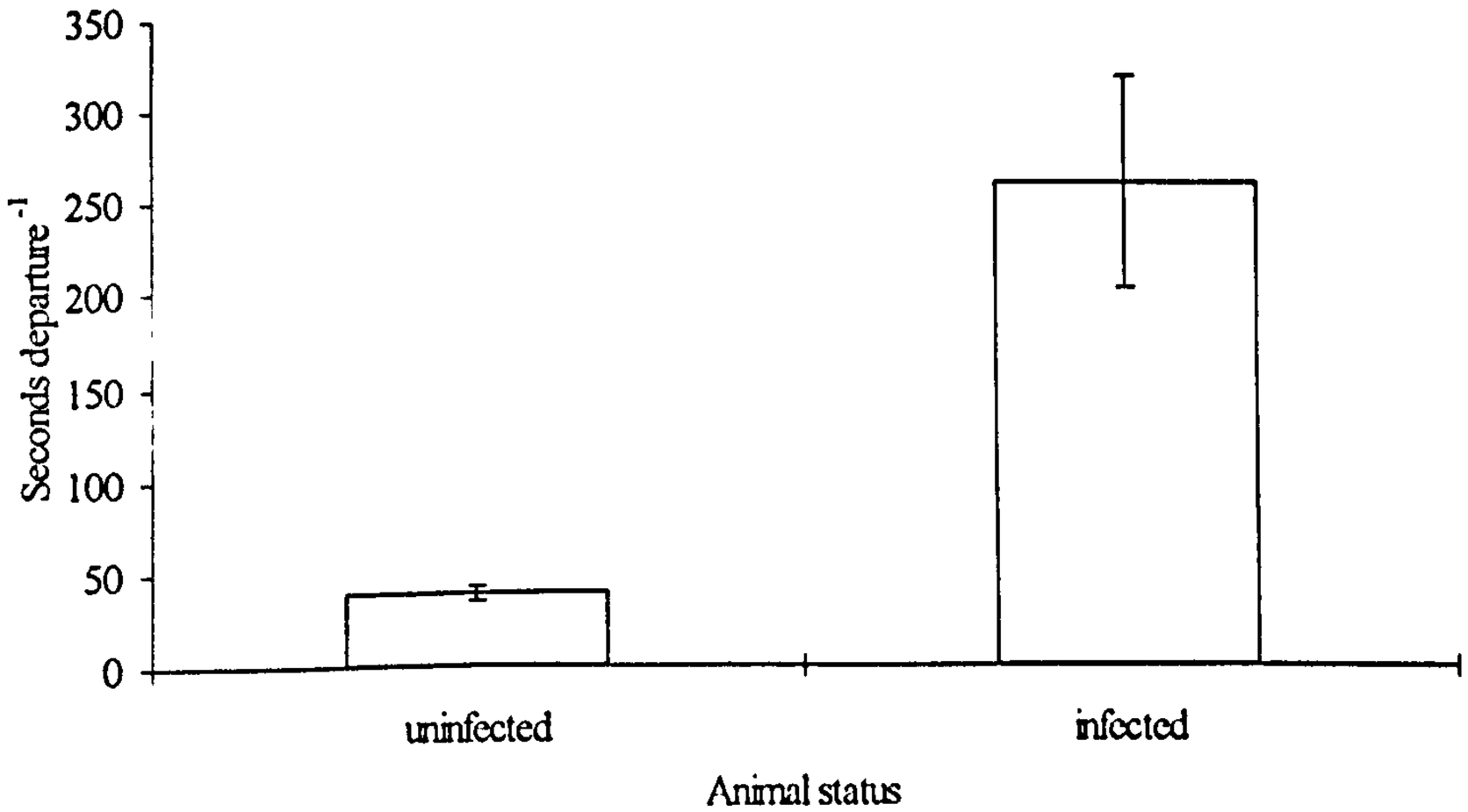


Fig. 8. Number of seconds per hour spent out of the burrow, by hour, for uninfected and *Hematodinium*-infected lobsters. Header bar indicates the illuminated and dark periods.

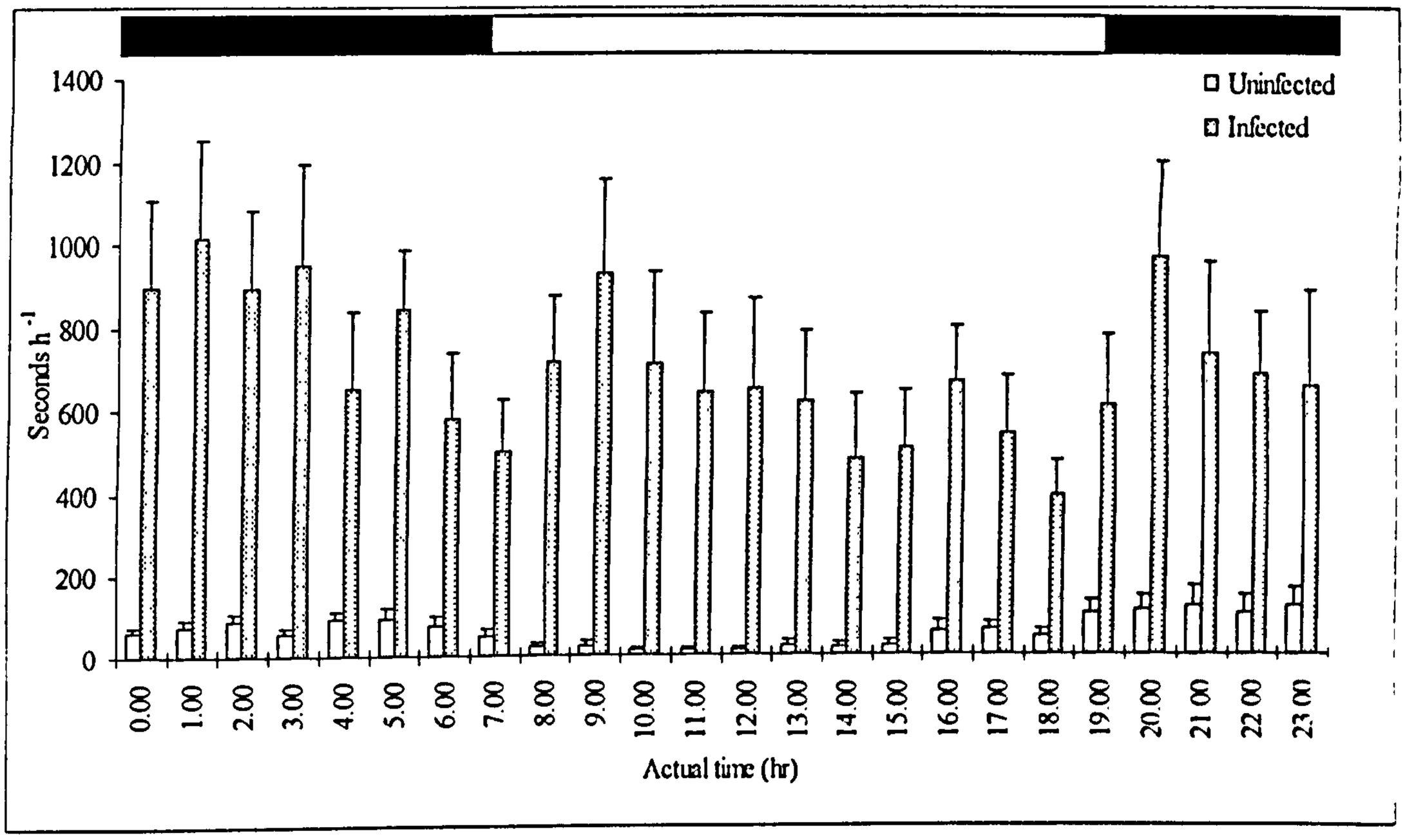


Fig. 9. Mean time spent out of the burrow per hour by uninfected and *Hematodinium*-infected lobsters in the light and dark periods.

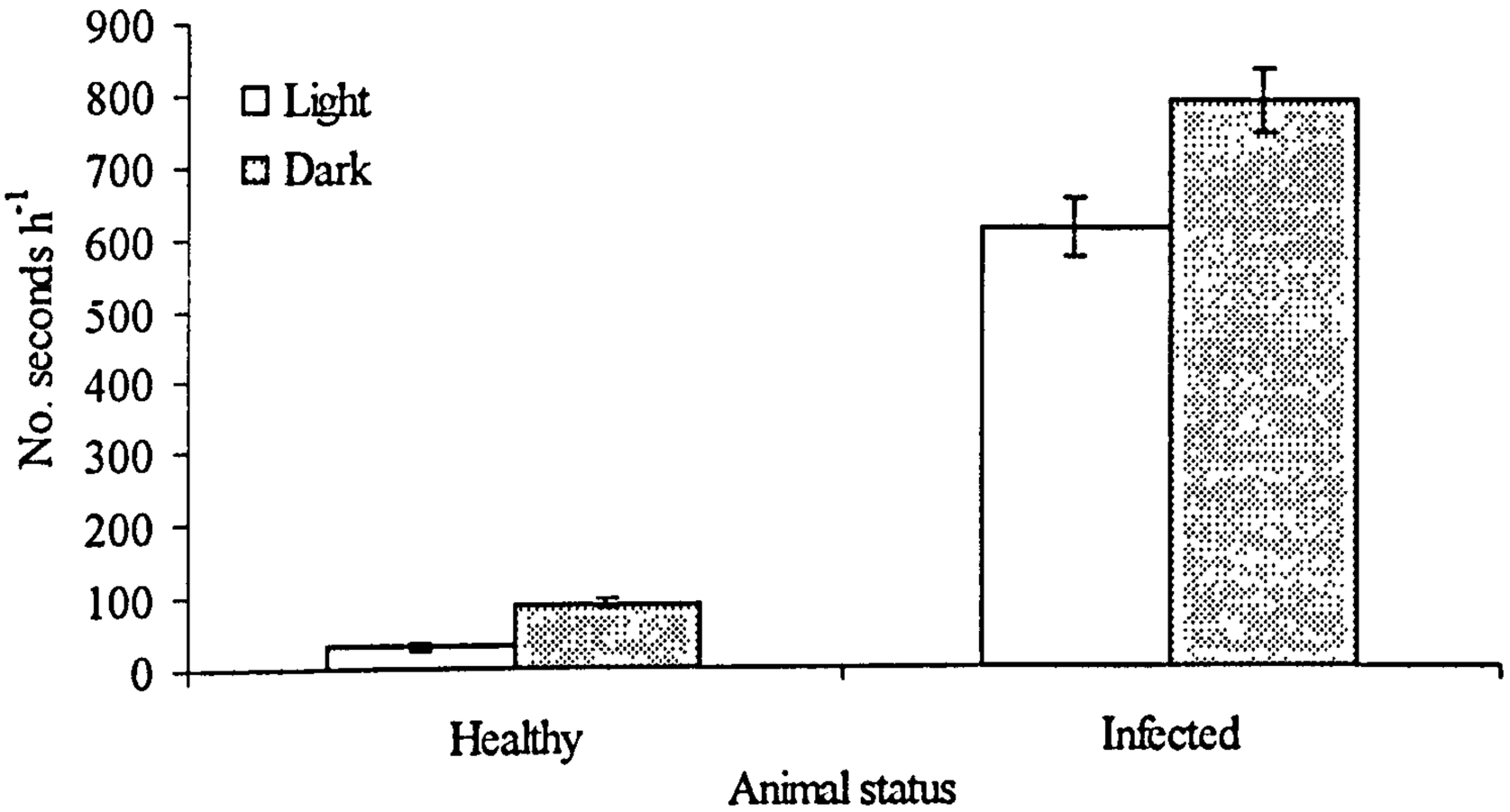
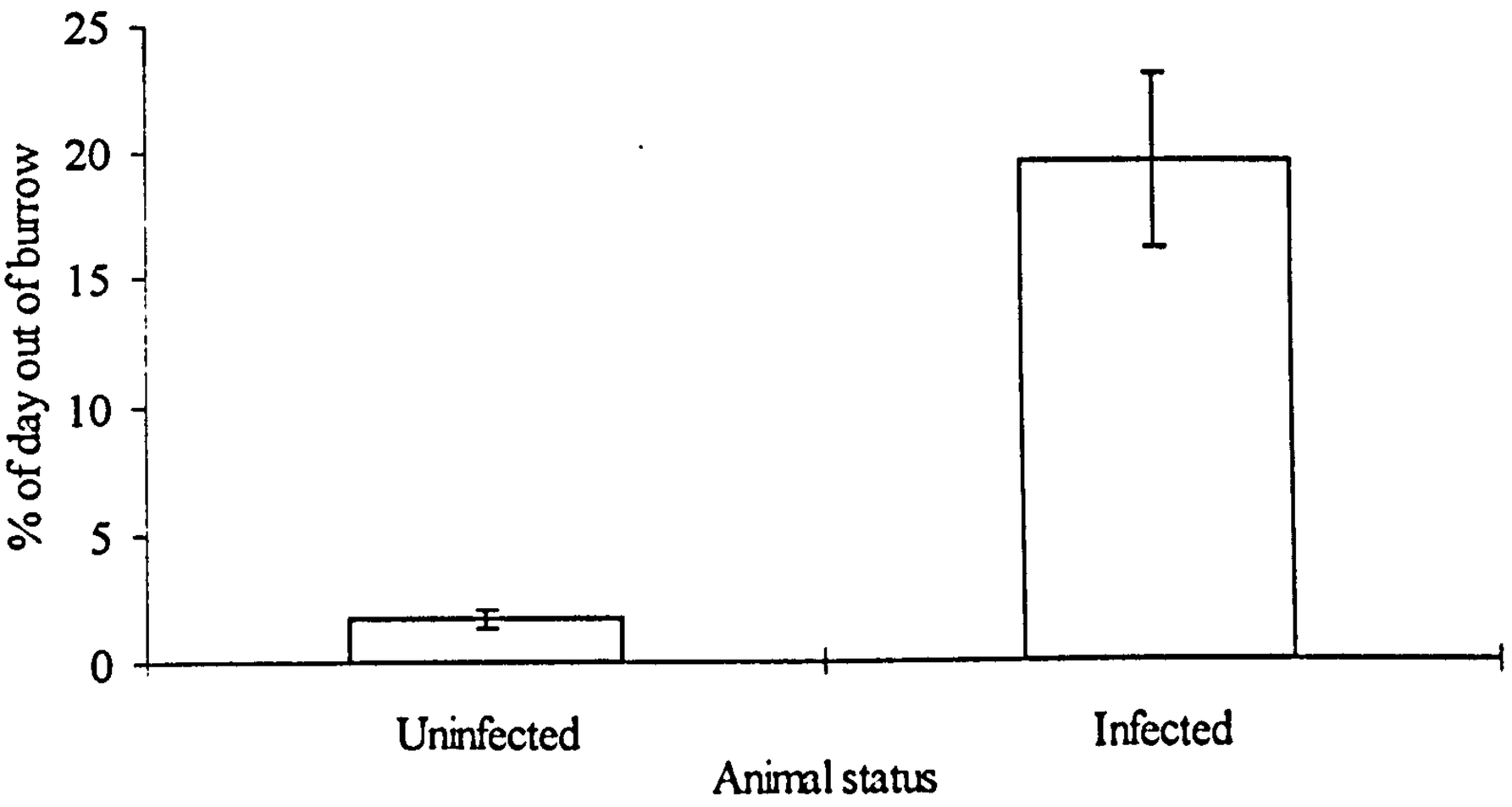


Fig. 10. Mean percentage of the whole day spent out of the burrow by uninfected and *Hematodinium*-infected lobsters.



Chapter 11

Conclusions and Prospects.

This study has furthered our understanding of the complex relationship between the protozoan parasite *Hematodinium* and its host, the Norway lobster, *Nephrops norvegicus*. A diverse range of experimental approaches and analytical techniques have been used to investigate the epidemiology, etiology and pathology of the infection, and its effect on the biochemistry, physiology and behaviour of the host. In the previous chapters the significance of these various findings has been considered in relation to the literature relevant to the particular aspects. In this final chapter, therefore, consideration is restricted to the wider relationships between these findings, and emphasis is placed on unresolved issues, and on suggestions for further studies.

Description of the parasite infecting N. norvegicus.

While still not described to species level, the dinoflagellate parasite found in *N. norvegicus* is now accepted to be a species of the genus *Hematodinium* (Appleton & Vickerman, 1998), and recent molecular studies have shown that the *N. norvegicus* isolate of *Hematodinium* is probably distinct from those infecting the other crustacean species that have been studied (Hudson & Adlard, 1996; Appleton & Vickerman, 1998; Coats 1999). However, attainment of species status for the *N. norvegicus* isolate of *Hematodinium* is an important and urgent aim for future taxonomic studies.

Field epidemiology.

Data describing the diagnosis and epidemiology of *Hematodinium* infection in populations of *N. norvegicus* from the Clyde Sea area, Scotland (Chapters 2 and 3) have provided a new appreciation of the cycling of the parasite in the field in relation to the

complex life history of the host, and add considerably to the work already reported by Field *et al.* (1992, 1998). Chapter 2 described how a simple morphometric diagnostic method (the pleopod method) could be used to study the epidemiology of *Hematodinium* infection in the field. It was shown that mean carapace length (the standard measure of animal size) of the population appears to be important in determining the absolute level of infection prevalence within a given year. Since an increased fishing effort has been implicated in the reduction of mean carapace length of *N. norvegicus* on certain fishing grounds (Sardá, 1998), studies are now required to determine whether increased fishing effort leads to the formation of a population that is particularly susceptible to disease epizootics.

Chapter 2 also showed how the synchrony of the moulting seasons of male and female lobsters may play an important role in the pattern of infection seen within a particular year. In some years, a sharp peak of infection is seen, while in others a plateau of infection occurs (see below). As such, the length of the infection season may be as important as the absolute infection prevalence in determining the natural mortality attributable to *Hematodinium* infection in any given year.

As a result of the finding that the pleopod method was ineffective in detecting sub-patent and latent *Hematodinium* infections (Field & Appleton, 1996), Chapter 3 described the further development and application of an diagnostic test for infection, based on detection of *Hematodinium* antigens in the haemolymph and tissues *of N. norvegicus* using immunoassay procedures. The data presented suggest that the pleopod method considerably under-diagnoses infection prevalence in trawl-caught samples, and that detection of parasite antigens by an immunoassay test may provide a more sensitive method for infection diagnosis. Further development of the immunoassay into a multi-sample, enzyme-linked immunosorbant assay (ELISA) is now being pursued. Such methods should allow for the routine assessment of *Hematodinium* infection prevalence in geographically-distinct *N. norvegicus* populations. In addition to antibody-antigen based

detection methods, diagnosis of *Hematodinium* infection has also been achieved using molecular techniques based on the polymerase chain reaction (PCR) (Hudson & Adlard, 1994, 1996). This technique utilizes nucleotide sequences that are specific to the parasite genome in order to amplify extremely small quantities of parasite DNA present in host tissue. The method is highly sensitive and is likely to be invaluable in future studies of *Hematodinium* parasite latency in *N. norvegicus*. Development of nucleotide primers specific to the *N. norvegicus* isolate of *Hematodinium* is being pursued.

Linking pathology to ecology.

Due to the severe pathological effects associated with advanced *Hematodinium* infections (Meyers *et al.*, 1987; Hudson & Shields, 1994; Field & Appleton, 1995; Wilhelm & Mialhe, 1996 and Chapters 4, 5 and 6 of this thesis), the survival of infected lobsters under aquarium conditions (Field *et al.*, 1992, Field & Appleton, 1995) and the sporolation response which leads to the death of the host lobster (Appleton & Vickerman, 1998), it is unlikely that recovery from patent infection is possible. This would explain why seasons of high infection prevalence have been associated with lower burrow densities on the fishing grounds, and with reductions in landings per unit effort from trawling (Field *et al.*, 1998). However, infection with *Hematodinium* may also contribute to determining the availability of *N. norvegicus* for capture, and an important aim of the present study was to identify the extent to which changes in the physiology, biochemistry and behaviour of the host upon infection determine the catchability of the infected lobsters.

Chapter 6 reported the reduction in hepatopancreatic glycogen and plasma glucose reserves during patent infection. This reinforced the data presented in Chapter 5 which showed that muscle glycogen was also greatly depleted during infection. Taken together with changes measured in the concentration of the crustacean hyperglycaemic hormone (CHH) in the plasma, these findings suggest that *Hematodinium* parasites act as a

'carbohydrate sink' in the haemolymph of *N. norvegicus*, by consuming free glucose and preventing repletion of tissue glycogen. It is thus envisaged that infected lobsters are in a state of 'physiological starvation' – a condition that may be compounded by 'functional hypoxia', due to the parasites consuming oxygen in the blood (Taylor *et al.*, 1996).

Schmidt and Platzer (1980) note that the condition of the host may determine the degree to which a parasitic infection develops, with hosts in the best physiological condition harbouring the largest parasite burdens. The prevalence of *Hematodinium* infection was seen to be highest in *N. norvegicus* during the spring, and in females (Chapter 2 and 3). Female crustaceans have relatively larger amounts of hepatopancreatic tissue than males as a means of supplying the nutritional requirements for egg rearing and spawning (Farmer, 1974). The higher incidence of *Hematodinium* infection in female lobsters may reflect some relative advantage of the female host to the parasite. Coincidence of peak infection with the onset of the main moulting period of *N. norvegicus* (Chapter 2) may reflect the fact that crustaceans are in their best physiological condition during the late intermoult and the premoult (Johnson, 1980), allowing *Hematodinium* parasites to develop and multiply under optimal conditions in the haemolymph. Data presented in Chapters 4, 5 and 6 have suggested that simple carbohydrate reserves may provide the initial energy source for developing parasites. This is followed by progressive depletion of more complex carbohydrates (such as glycogen) and then consumption of amino acids and proteins (see Taylor *et al.*, 1996). The sporolation phase of the parasite, in which motile parasite forms emerge from the host lobster, may occur when all available host resources have been depleted. This may explain why sporolation of the parasite can even occur in lobsters displaying only Stage 1 or 2 infection (Appleton & Vickerman, 1998). Further studies on the nutritional requirements of the parasite during different stages of development may reveal the nature of these more complex nutritional relationships between *Hematodinium* and its host.

The concepts of physiological starvation and functional hypoxia are also relevant to the interpretation of data presented in Chapters 8 and 9 on the swimming performance of *Hematodinium*-infected lobsters. These chapters described the progressive reduction in swimming performance during patent infection, and associated this mainly with the reduced glycogen reserves reported in infected muscle (Chapter 5). However, it was also suggested that the increased haemolymph and tissue lactate concentrations previously reported to occur in *Hematodinium*-infected lobsters (Taylor *et al.*, 1996), when coupled with alterations in water content and damage to the sarcolemmal membranes of infected muscle (Chapter 5), may also play a role in reducing swimming performance. Furthermore, the two-phase swimming pattern reported in Chapter 9 has highlighted how different metabolic substrates may be utilized throughout the normal swimming sequence of *N. norvegicus*. Further studies of the disruptions to metabolic processes in muscle that may affect the swimming capabilities of infected lobsters are required.

Alterations in tissue (Chapter 5) and haemolymph (Chapter 4) biochemistry provide further evidence for the severe pathological disruption that occurs in *Hematodinium* infection of *N. norvegicus*. While FAAs in the plasma of crustaceans are generally present at very low concentrations, during *Hematodinium* infection the plasma concentration of several FAAs increased considerably. The increase of taurine was the most marked, increasing 12-fold. Taurine is known to mimic the inhibitory effects of λ-aminobutyric acid (GABA) in crustaceans (Zatta, 1987) and glutamate, which also increases considerably during infection, is known to have pharmacological activity at low concentrations (Lin & Cohen, 1973). During infection, the concentration of free glutamate in the plasma increased to values that exceed the threshold for stimulating crayfish muscle. Due to open nature of the crustacean circulatory system, these elevated concentrations of neuro-active compounds could be at least partly responsible for the reduced swimming ability of *Hematodinium*-infected lobsters reported in Chapters 8 and 9. Additionally, high plasma

concentrations of these compounds may be involved in the unusual tail flipping spasms seen in heavily-infected lobsters shortly before death (Chapter 10).

Taurine and other FAAs (such as glutamate) have been identified as potent chemo-attractants in the marine environment. Altered excretion of chemo-attractive compounds from the antennal glands of *N. norvegicus*, which are known to be damaged during *Hematodinium* infection (Field & Appleton, 1995), and the subsequent predation of infected lobsters by other hosts, may provide an interesting link to parasite transmission in the field. This interaction between *Hematodinium*-infected lobsters and predators may be compounded by the increased amounts of time spent out of the burrow (Chapter 10) and the reduced swimming performance (Chapters 8 and 9) noted in *Hematodinium*-infected *N. norvegicus*.

The changes in FAA concentrations in the deep abdominal flexor muscle of patently infected lobsters suggest that the normal biochemistry of the muscle cells may also be considerably disrupted. An up-regulation in the production of taurine in the deep abdominal flexor muscle, as shown in Chapter 5, may explain the increased plasma concentration of this compound reported in Chapter 4. Additionally, as this muscle provides the majority of the commercially useful meat of *N. norvegicus*, changes to its composition may impact upon the marketability or edibility of infected lobsters (see Shirai *et al.* 1996). Alterations in the taste of *Hematodinium*-infected meat have already been described in the aptly-named 'Bitter Crab disease' of Alaskan tanner crabs *Chionoecetes opilio* and *C. bairdi*, though in this case it was assumed to be due to some exudate from the parasite itself (Meyers *et al.*, 1987; Taylor & Khan, 1996). As FAAs and other low molecular weight compounds are important components of the 'taste profile' of crustacean meat, future studies on the biochemical composition of *Hematodinium*-infected lobster meat are recommended.

Hematodinium infection as a stressor.

Evidence presented in the current study has suggested that *Hematodinium* infection may cause biochemical and physiological changes in host lobsters which are similar to those occurring under a range of other stresses (see Paterson & Spanoghe, 1997). In particular, a reduction in tissue glycogen and an elevation of plasma CHH are both common responses to hypoxia (see Baden *et al.*, 1990 and Santos & Keller, 1993, respectively). Additionally, changes in the concentration of plasma and tissue taurine (especially in respect to its ratiometric association with the amino acid glycine) are also known to be sensitive indicators of acute and chronic stress in marine invertebrates (Livingstone, 1982).

An interesting link was made in Chapter 6 between the onset of infection patency and the moult (itself a period of considerable physiological stress). Here, it was shown that CHH, the plasma concentration of which changes when animals are exposed to a number of different stressors (see Chung *et al.*, 1999), is significantly increased in sub-patently infected lobsters. Other recent studies have shown that the concentration of CHH is also increased significantly in the hours preceding ecdysis. This presumably leads to mobilization of the host's carbohydrate reserves, thereby providing the necessary energy for moulting (Chung *et al.*, 1999). The results of Chapter 6 suggest that the increased CHH titre in sub-patently infected lobsters may cause a similar response, producing conditions in the haemolymph that are suitable for the development of patent infection (i.e. colonization of the haemolymph by latent tissue forms). Elevated plasma CHH concentrations have also been implicated in the uptake of water by crustaceans immediately before the moult (Chung *et al.* 1999). The high concentration of CHH observed during the later stages of *Hematodinium* infection may therefore be responsible for the bloated appearance of heavily infected lobsters. This may eventually cause integumental membranes to break, allowing extrusion of the motile dinospores. Such swarming stages have been noted on several occasions under aquarium conditions (Appleton & Vickerman, 1998). As such, Chapter 6

provides the first description of parasite-induced hormonal disruption in a crustacean host. Furthermore, it suggests that *Hematodinium* infection in *N. norvegicus* may provide an excellent model for studying general stress responses in marine invertebrates, especially those involving hormonal and immunological mechanisms.

Effects of other stressors.

Although not an initial objective of the current study, in the course of routine examination of catches, an unusual pathology was noted in the abdominal muscle of trawler and creel-caught *N. norvegicus*. Chapter 7 provides the first description of this pathology, and showed that the condition is similar to the idiopathic or spontaneous muscle necrosis previously reported to occur in several other crustacean species (see Evans *et al.* 1999). The pathology described in *N. norvegicus* involves a rapid degeneration of the muscles of the abdomen, which renders the lobster immobile and then dead within 3-4 days. The economic impact of this condition has already been felt by wholesalers on the west coast of Scotland. This resulted from the mass mortality of *N. norvegicus* during vivier transport to the continent in the summer and autumn of 1999. The data presented in Chapter 7 for the prevalence of muscle necrosis immediately after trawl capture, and 4 hours later, reinforce the theory that the holding conditions of the animals in the period immediately following capture are crucial in determining whether the necrotic condition develops or regresses. This has important consequences both for the transport of live *N. norvegicus*, and for the quality of the meat of lobsters 'tailed' at sea. As sorting of undersized lobsters may take up to 4 h (see Chapman *et al.*, 2000), the pathology may also contribute to the high mortality of discarded *N. norvegicus* which are returned to the sea after trawl capture and several hours of emersion (Ulmestrand *et al.*, 1998). The pathology of muscle necrosis described in Chapter 7 was shown to be different from that occurring during *Hematodinium* infection (Chapter 5). As the protein composition of the deep

abdominal flexor muscles of the abdomen were largely unchanged during *Hematodinium* infection, it could be suggested that anecdotal reports of 'watery' and unstructured meat from the tails of apparently *Hematodinium*-infected lobsters may have in fact been describing the tail pathology noted in Chapter 7. Further studies on the conditions which lead to expression of this pathology and to the structure, biochemical composition and taste of cooked tail muscle from lobsters showing symptoms of necrosis would allow such issues to be addressed.

Hematodinium and the catchability of infected lobsters.

In terms of burrow-related behaviour, infected male *N. norvegicus* were shown to spend significantly longer periods out of the burrow, and appeared not to show the diel emergence rhythm seen in uninfected lobsters (Chapter 10). These changes in behaviour may be driven by the increased nutritional and/or respiratory requirements of *Hematodinium*-infected lobsters as mentioned above. As such, changes in burrow-related behaviour are likely to be implicated in an increased catchability of infected lobsters by trawl nets, especially when coupled with the reduced swimming performance noted in Chapters 8 and 9. Increased catchability may lead to overestimation of infection prevalence in the field, and may impact upon the accurate assessment of natural mortality attributable to *Hematodinium* infection (Anon, 1997). These findings suggest why previous attempts to incorporate the high observed prevalence of *Hematodinium* infection into analytical stock assessments of the Clyde Sea area, which modelled the effect as an additional loading on the natural mortality rate (since animals showing symptoms of patent infection usually die), led to unrealistic estimates of the number of recruits entering the fishery (Anon, 1997). Infected animals, while being more prone to capture by trawlers, may also be more susceptible to predation through both a reduced ability to escape and an increase in 'risk prone' behaviour (Chapter 10).

Current fisheries independent methods for *N. norvegicus* stock assessment are based on the estimation of larval production, the use of underwater television for making burrow density counts, and the use of trawl surveys. Comparisons between the number of lobsters present on the sediment surface and the burrow density suggest that only around 10 % of lobsters are out of their burrows at any given time (Tuck *et al.*, 1997). At higher burrow densities, proportional emergence may be greater than this, as individuals in such populations may have to spend longer foraging for food. However, while analytical models assume equal capture availability throughout the distribution of the stock (Tuck *et al.*, 1997), stocks exhibiting significant *Hematodinium* infection prevalence may also be showing greater proportional emergence than stocks with lower infection prevalence. As such, estimates of *N. norvegicus* abundance from trawler capture may overestimate true infection prevalence on these grounds. Further studies on the infection prevalence of trawl-caught populations in relation to the density of burrows and the proportional emergence of lobsters on the same grounds would represent a field test of the theories of increased burrow emergence presented in Chapter 10.

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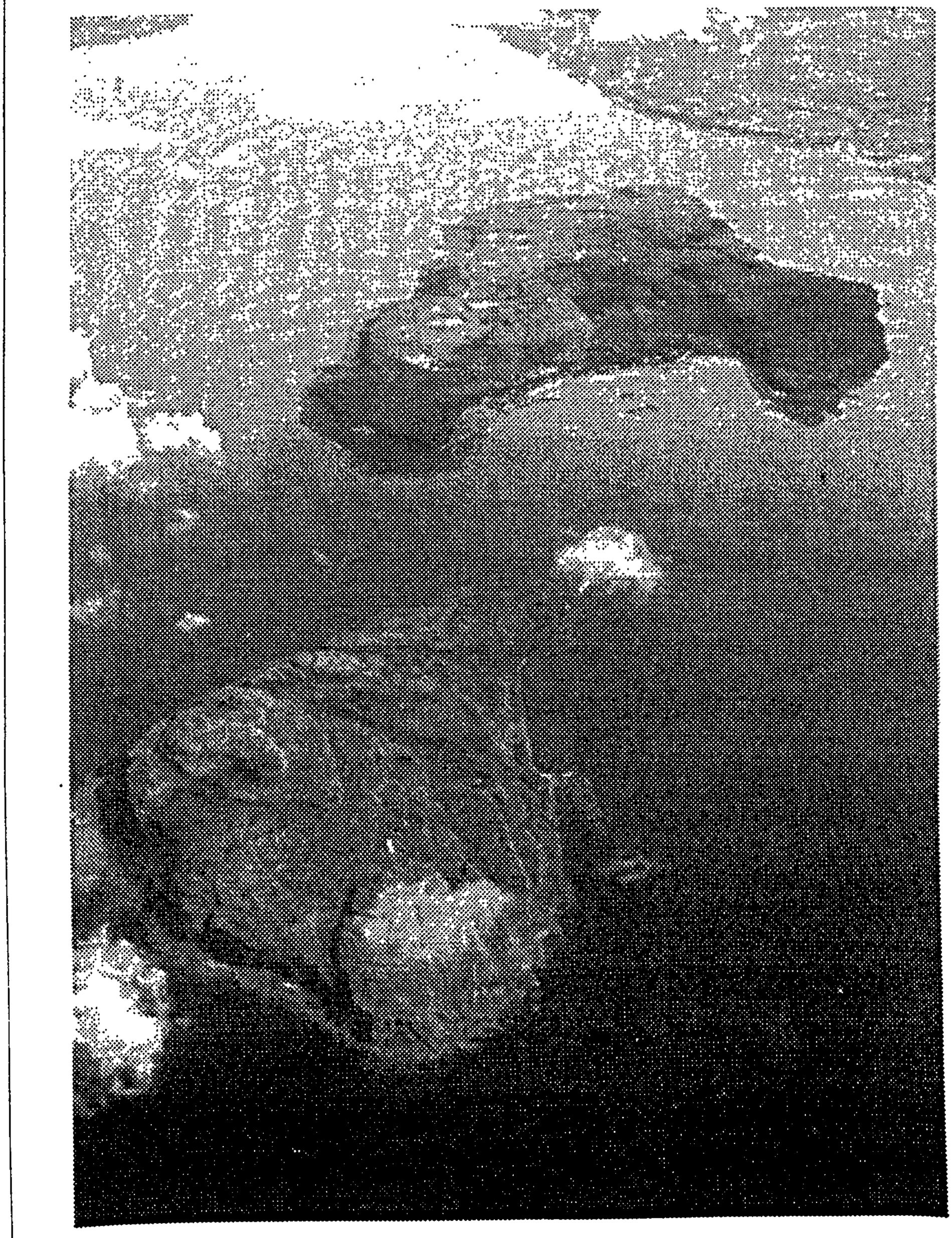

The Cumbraes. Firth of Clyde (G.D. Stentiford)

It is not storm or calm, but yesterday
The wild winds leapt in sudden thunder down;
Shook the dark waters into a starry spray,
And thrilled the soul of many a seaside town.

'The Sorrow of the Sea', John Hogben.