Infections of the Norway lobster, *Nephrops norvegicus* (L.) by dinoflagellate and ciliate parasites

Hamish J. Small

Division of Environmental and Evolutionary Biology
Institute of Biomedical and Life Sciences
University of Glasgow
Glasgow
G12 8QQ

This thesis is presented in submission for the degree of Doctor of Philosophy, to the Institute of Biomedical and Life Sciences, University of Glasgow.

June 2004

Abstract

Parasitic dinoflagellates of the genus *Hematodinium* have been reported from a number of commercially important crustacean hosts, including the Norway lobster, *Nephrops norvegicus*, from the coastal waters of Scotland. Several methods for detection of the parasite have been developed but each has associated drawbacks. As part of this study, an enzyme linked immunosorbent assay (ELISA) has been developed for the detection of the parasite in the haemolymph of *N. norvegicus* and other crustaceans. The ELISA is a simple, sensitive, and reproducible assay, with a detection limit of 5×10^4 parasites ml^{-1} haemolymph.

To further investigate low-level *Hematodinium* infection in *N. norvegicus* and other crustacean hosts, a set of *Hematodinium*-specific polymerase chain reaction (PCR) primers and DNA probes have been developed, based on *Hematodinium* ribosomal DNA (rDNA) regions. In the PCR assay, a diagnostic band of 380 bp is produced in the presence of parasite DNA. The limit of detection of the assay was found to be 1 ng DNA, which is equivalent to 6 parasites. The DNA probes detected *Hematodinium* cells in a range of tissues from *N. norvegicus* and from the crab *Carcinus maenas*.

The level of genetic similarity between nine isolates of *Hematodinium* originating from several species of Crustacea from the UK was examined. PCR was used to amplify and sequence the 3' of the small sub-unit (SSU) and the first internal

transcribed spacer (ITS1) of the parasite rDNA complex. Analysis of the SSU and ITS 1 sequences revealed that both the regions are highly conserved (92.2 % or greater) between isolates examined, and that there is no apparent geographical separation of isolates. The results suggest that the same species of *Hematodinium* infects a number of crustacean species from different geographical locations.

Hematodinium perezi, the parasitic dinoflagellate of the blue crab *Callinectes sapidus*, has been successfully isolated and cultured *in vitro*. Although the *in vivo* form of this parasite is morphologically and molecularly very different from that of the *Hematodinium* sp. infecting *N. norvegicus*, a number of similar life cycle stages were observed *in vitro*. These included syncytial networks, filamentous trophonts, and gorgonlock colonies. The isolation and *in vitro* culture of *H. perezi* and the *Hematodinium* sp. infecting *N. norvegicus* allowed the internal and external enzyme profiles of both species to be examined using the API ZYM system and biochemical assays, leading to the identification of several enzymes that may have pathogenic importance during *Hematodinium* infections. Differences in the secretion of acid phosphatase and leucine arylamidase by the two *Hematodinium* sp. studied may account for their different levels of virulence and infectivity.

A ciliate infection of wild and laboratory-held *N. norvegicus* was discovered during the course of this project. Extensive damage to heart muscle tissue was observed in affected lobsters. The ciliate was identified as belonging to the genus

Mesanophrys, based on silver carbonate impregnation of oral structures. However, molecular sequence data (ITS1 and ITS2) indicated that the ciliate sequences have 100 % identity with rDNA sequences from *Orchitophrya stellarum*, a ciliate parasite of sea stars. Since the morphology of *O. stellarum* differs from that of *Mesanophrys*, the possibility arises that the previous studies have misreported the molecular data. Otherwise, it must be concluded that morphological features cannot be used to discriminate between closely related ciliate species.

The initiation of *in vitro* cultures of the ciliate isolated from *N. norvegicus* allowed the investigation of proteolytic factors that may be involved in the initiation and progression of its infection. The ciliate was found to secrete a number of proteases into the culture medium, and these are exclusively of the metallo type. They have gelatinolytic and azocaseinolytic activities and are active at the physiological temperature and haemolymph pH of the host. Secreted proteases were selective in the degradation of several host proteins, including the myosin heavy chain, which is a common structural component of all lobster muscle tissues. Consequently, these proteases may have multiple roles in the invasion and progression of this ciliate infection, or in assisting nutrient uptake by the ciliate.

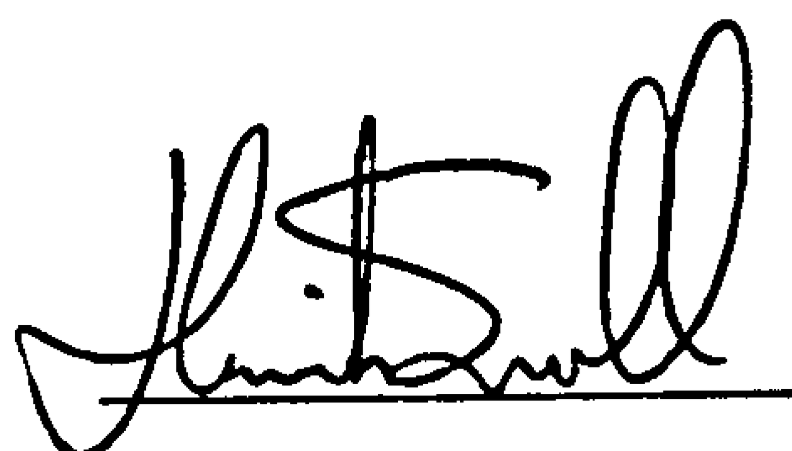
The results of these studies are discussed in terms of the technical development of diagnostic assays for *Hematodinium*, their potential application in examining the prevalence and transmission of this parasite in *N. norvegicus* and other

crustaceans, and the potential pathogenic mechanisms involved in parasitic infections of *N. norvegicus*.

Candidate Declaration

The results presented in this thesis are my own work, unless otherwise stated and that it is of my own composition.

Results from Chapters 2 and 3 have been published and are included in Appendix 1.



Hamish J. Small

June 2004

Acknowledgements

I wish to thank my supervisors Professors Graham Coombs and Alan Taylor, and especially Dr Douglas Neil for their help, ideas and encouragement throughout my PhD. Special thanks also go to Professor Keith Vickerman for providing initial parasite cultures and endless advice and enthusiasm for the study of parasites, to Professor Jim Atkinson (University Marine Biological Station Millport) for technical assistance with sample collection methods, and to Dr Grant Stentiford (The Centre for Environment Fisheries and Aquaculture Science (CEFAS) Weymouth Laboratory) for biological samples, discussions on *Hematodinium* and his uncanny ability to restore my motivation.

A special thanks to Mr Mick Parker and the crew of RV *Aora*, and to Mr Howard McKrindle and the crew of RV *Aplysia* (all from the University Marine Biological Station Millport) for their assistance with collection of animals.

A special thanks go to Fiona Seow, Dr Richard Burchmore, Dr Sanya Sanderson, Dr Gareth Westrop, Dr Rod Williams and all the rest of the staff and students based in the Laboratory for Biochemical Parasitology within the Division of Infection and Immunity, where I carried out the majority of my bench work for this study. My endless technical and theoretical questions were never left unanswered.

I would like to thank Dr Genoveva Esteban and Dr Bland Finlay (The Natural Environment Research Council Centre for Ecology and Hydrology, Windermere) for instruction and demonstration of the intricacies of silver impregnation techniques for ciliates. Thanks to Dr Ian Montgomery (Histotechnology, Glasgow University) for assistance with sample preparation, histology and microscopy. Thanks also to Dr Liz Smith (FRS Marine Laboratory, Aberdeen) for providing samples of free-living dinoflagellates, to Dr Dave Taylor (Fisheries and Oceans Canada) for providing biological samples, to Kelly Bateman (CEFAS, Weymouth lab) for EM sample preparation and images, and to Dr Beth Mouat (Scottish Natural Heritage), Michael McAliskey (Department of Agriculture and Rural Development, Northern Ireland) and Karin Frohlund (Institute of Marine Research, Lysekil, Sweden) for Norway lobster samples from Scotland, Ireland and Sweden (respectively).

I would also like to thank Dr Jeff Shields of the Virginia Institute of Marine Science (VIMS) in the US for allowing me to work in his laboratory for 4 weeks in November 2002 and for providing biological samples.

Thanks to my office mates Dr Dominic Lewis, Dr Aaron Johnston, Dr Beth Mouat and Iain Ridgway for hours of entertainment over the last three and a half years.

Thanks go to the Natural Environment Research Council (NERC) for funding the current project, and for a Mac Robertson Travelling Scholarship (jointly between the Universities of Glasgow and Strathclyde) that allowed me to visit Dr Jeff Shields at VIMS in November 2002.

And finally, a very special thanks goes to my family, and to Leeanne, Heather and Nadia for being there when I needed them the most.

Contents Page

Title page

Abstract i

Declaration v

Acknowledgements vi

Contents ix

List of Abbreviations xv

Chapter 1

General Introduction 1

1.1	The Norway lobster *Nephrops norvegicus* (L.)	1
1.2	Crustacean diseases	3
1.2.1	Non-infectious diseases of crustaceans	3
1.2.2	Bacterial infections of crustaceans	4
1.2.3	Viral diseases of crustaceans	7
1.2.4	Fungal diseases of crustaceans	8
1.2.5	Protozoan parasites of crustaceans	10
1.2.6	Dinoflagellate parasites of crustaceans	14
1.2.7	*Hematodinium* species infecting crustaceans	16
1.3	Hematodinium infection of the Norway lobster	18
1.4	Aims of this project	25

Chapter 2

Development and application of an ELISA for the diagnosis of *Hematodinium* infection in *N. norvegicus* and other crustaceans. 28

2.1	Introduction	28
2.2	Materials and Methods	30
2.2.1	Collection and maintenance of experimental lobsters	30
2.2.2	Haemolymph preparation and development of assay	30
2.2.3	Sensitivity and comparison of assay with other methods	31
2.2.4	Specificity of antiserum	32
2.2.5	Application of assay	33
2.3	Results	33
2.3.1	Evaluation, sensitivity and specificity of assay	33
2.3.2	Comparison of diagnostic methods	34
2.3.3	Seasonality of Infection	35
2.4	Discussion	35

Chapter 3

Development and application of a PCR based assay and DNA probes for the diagnosis of *Hematodinium* infection in *N. norvegicus* and in other crustaceans. 41

3.1	Introduction	41
3.2	Materials and Methods	43
3.2.1	Specimen preparation	43
3.2.2	DNA extraction	44
3.2.3	Primary amplification, cloning and sequencing	45
3.2.4	Primer and probe design	46
3.2.5	PCR sensitivity and specificity	47
3.2.6	Verify PCR assay and secondary host investigation	48
3.2.7	Labelling of DNA probes	48
3.2.8	DIG-labelled probe specificity	49
3.2.9	*In situ* hybridisation	51
3.3	Results	52

3.3.1 *Hematodinium* rDNA sequences 52
3.3.2 PCR primer design, sensitivity and specificity 53
3.3.3 Verify PCR assay for *Hematodinium* 54
3.3.4 *Hematodinium* infection in *O. nanus* 55
3.3.5 DNA probe 1 specificity 55
3.3.6 *In situ* hybridisation 55
3.4 Discussion 56

Chapter 4

Genetic variation of *Hematodinium* species infecting crustaceans in the UK. 70

4.1 Introduction 70
4.2 Materials and Methods 72
4.2.1 Isolates 72
4.2.2 DNA extraction, and amplification 72
4.2.3 Cloning and sequencing 73
4.3 Results 74
4.3.1 Sequence alignment 74
4.3.2 Analysis of the ribosomal SSU region 74
4.3.3 Analysis of the ribosomal ITS1 region 75
4.4 Discussion 76

Chapter 5

Isolation and *in vitro* culture of *Hematodinium perezi* from the blue crab *Callinectes sapidus*. 87

5.1 Introduction 87
5.2 Materials and Methods 89

5.2.1 Crab collection 89
5.2.2 Diagnosis of infection 89
5.2.3 Parasite isolation 90
5.2.4 Parasite culture 91
5.3 Results 91
5.3.1 Initial isolation of *H. perezi* 91
5.3.2 Parasite forms in early *in vitro* cultures 92
5.4 Discussion 94

Chapter 6
Possible virulence factors produced by *Hematodinium* species. 103

6.1 Introduction 103
6.2 Materials and Methods 105
6.2.1 Collection and maintenance of experimental animals 105
6.2.2 Diagnosis of *Hematodinium* infection and parasite isolation 106
6.2.3 Preparation of parasite lysates 107
6.2.4 Parasite culture 108
6.2.5 API ZYM enzyme analysis 109
6.2.6 AP assay 109
6.2.7 AP histochemistry 110
6.3 Results 111
6.3.1 *Hematodinium* cell culture 111
6.3.2 API ZYM enzyme profiles of *Hematodinium* cell lysates and cell culture media 111
6.3.3 AP activity in *Hematodinium* cell lysates 113
6.3.4 AP activity in *Hematodinium* cell culture medium 113
6.3.5 AP activity in *Hematodinium*-infected *N. norvegicus* serum 114
6.3.6 Localisation of AP activity in *Hematodinium* cells 114
6.4 Discussion 114

Chapter 7

Identification of a parasitic ciliate infecting the Norway lobster (Nephrops norvegicus). 125

7.1	Introduction	125
7.2	Materials and Methods	126
7.2.1	Sample collection and infection monitoring	126
7.2.2	Histology	127
7.2.3	Ciliate culture	127
7.2.4	Pyridine silver carbonate staining	128
7.2.5	Electron microscopy	129
7.2.6	rDNA amplification and sequencing	130
7.3	Results	132
7.3.1	Prevalence of ciliates in *N. norvegicus*	132
7.3.2	Histopathology	132
7.3.3	Morphology and cell structure of the ciliate	133
7.3.4	rDNA sequence comparison	134
7.4	Discussion	135

Chapter 8

Characterisation of proteases secreted by a ciliate parasite of the Norway lobster. 149

8.1	Introduction	149
8.2	Materials and Methods	151
8.2.1	Isolation and culture of parasitic ciliate	151
8.2.2	Preparation of ciliate lysates	152
8.2.3	Spectrophotometric assay for protease activity	153
8.2.4	Substrate–impregnated SDS-PAGE	153
8.2.5	Effect of inhibitors on protease activity	154

8.2.6 Effect of temperature and pH on protease activity 156
8.2.7 Effect of proteases on host muscle proteins 157
8.3 Results 157
8.3.1 Protease activity and ciliate growth *in vitro* 157
8.3.2 Detection of proteases in ciliate culture medium and lysates by gelatin SDS-PAGE 158
8.3.3 Effect of inhibitors on proteases present in ciliate culture medium 158
8.3.4 Effect of temperature and pH on protease activity in ciliate culture medium 159
8.3.5 Host protein degradation by proteases present in the ciliate culture medium 160
8.4 Discussion 160

Chapter 9
General Discussion 169

References 181

Appendix 1 216

List of Abbreviations

AFLP	Amplified fragment length polymorphism
AP	Acid phosphatase
BLAST	Basic local alignment search tool
BMD	Black matt disease
BSA	Bovine serum albumin
CCAP	Culture collection of algae and protozoa
CHH	Crustacean hyperglycaemic hormone
DIG	Digoxygenin
DNA	Deoxy ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
FCS	Foetal calf serum
Ft	Filamentous trophont
Gc	Gorgonlock colony
Hc	Host haemocyte
H & E	Haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFAT	Indirect fluorescent antibody technique

IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITS	Internal transcribed spacer
LB	Luigi-bertani
MAM	Modified appleton medium
NCBI	National center for biotechnology information
OD	Optical density
ODK	Oral dikinetid
OPK	Oral polykinetid
PBS	Phosphate buffered saline
PCD	Pink crab disease
PCR	Polymerase chain reaction
PNP	P-nitrophenol
PNPP	P-nitrophenylphosphate
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
SSC	Saline citrate solution
SSU	Small sub unit
TCA	Trichloroacetic acid
TEM	Transmission electron microscope
UK	United Kingdom

USA	United States of America
UV	Ultraviolet
VIMS	Virginia institute of marine science
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Chapter 1

General Introduction.

1.1 The Norway lobster *Nephrops norvegicus* (L.)

The sub phylum Crustacea (phylum Arthropoda) contains over 26,000 species dwelling in a wide range of habitats. The Norway lobster *Nephrops norvegicus* (L.) belongs to the infraorder Astacidea and family Nephropidae. The Norway lobster is a bottom-dwelling, burrowing decapod crustacean, which is common in European waters. It has been recorded from depths of around 10 to almost 900 metres, though it is more often found in waters of 30 to 200 metres (Farmer, 1975; Chapman, 1980; Atkinson, 1987; Abello *et al.*, 1988). It appears to have a wide adaptability to temperature and salinity and is present in NE Atlantic waters from southern Greenland to off the NW African coast, and in the Mediterranean (Farmer, 1975; Merella *et al.*, 1998). However, its distribution is restricted by its requirement for soft sediments in which to construct burrows (Chapman, 1980). Due to its commercial importance, large numbers and relative ease of capture throughout the year, it has become the model species for research into a number of important biological processes such as feeding (Loo *et al.*, 1993), reproduction (Farmer, 1974a) and behaviour (Atkinson and Naylor, 1976).

Up until the early 1950s *N. norvegicus* was considered to be a nuisance species and was discarded by fishermen working in the Firth of Clyde (Bailey *et al.*, 1986) and adjacent waters (Briggs, 1997). However in recent years, *N. norvegicus* has become one of the most important shellfish stocks fished in the Northeast Atlantic. UK landings for this species are currently worth around £68.5 M per annum at first sale (Sea Fish Industry Authority, 2001), making it one of the most valuable lobster resources in the world. The main fishing grounds for *N. norvegicus* in Scotland consist of the Firth of Clyde (W. Scotland), the Firth of Forth (E. Scotland), the North and South Minches (N. W. Scotland), the Noup (N. Scotland), the Fladen (N. E. Scotland), and the Farn Deeps (S. E. Scotland) (Anon. 2001). The majority of Scottish landings are from trawler capture, with the smaller sized lobsters being processed at sea and sold on return to land as "scampi tails". However, in some areas trawling is not practical or possible. Larger lobsters are usually caught by baited creels set within inshore sheltered waters (where there may sometimes be conflicts between trawling and creeling interests). These lobsters are landed live and supply a lucrative export market to continental Europe.

The reproductive cycle of the Norway lobster greatly influences its availability for capture. Mating occurs just after moulting by the female, which takes place from May through to August (Farmer, 1974a). The female stores the sperm packet transferred from the male until egg laying occurs in August and September (Farmer, 1974a). Subsequently, there is an incubation period during which the

fertilized eggs are carried on the pleopods of the female lobster. This incubation period lasts until the following April/May when the planktonic larvae are released. During this incubation period, females reside in their burrows and are unavailable for capture, which causes a strong bias in the sex ratio of catches (Farmer, 1974b; Chapman, 1980).

1.2 Crustacean diseases

1.2.1 Non-infectious diseases of crustaceans

An example of a non-infectious disease is that of idiopathic muscle necrosis, a condition for which the cause is unknown since infecting microorganisms have never been observed in the necrotic foci. It occurs in several marine and freshwater crustaceans when experiencing sub-optimal environmental conditions, such as hypoxia, hypo- and hypersalinity, hypo- and hyperthermia, hyperactivity, overcrowding and exposure to air and stress associated with handling. Necrotic lesions occur in the abdominal muscles of affected individuals, with associated infiltration of haemocytes. Lesions show disorganization of myofibrils, loss of sarcomeres and disintegration of interfibrillar mitochondria. The condition has been reported in the freshwater crayfish *Cherax tenuimanus*, in which it is thought to be due to air exposure (Evans *et al.*, 1999); in the brown shrimp *Penaeus aztecus*, due to host metabolic reactions caused by temperature and anoxia-induced stress (Rigdon and Baxter, 1970); in kuruma shrimp, *Penaeus japonicus*,

due to overcrowding in harvest nets (Momoyama and Matsuzato, 1987); and in the freshwater prawn *Macrobrachium rosenbergii*, due to hyperactivity and hypoxic water conditions (Akiyama *et al.*, 1982). Recently, Stentiford and Neil (2000) reported a post-capture idiopathic muscle necrosis of Norway lobsters from the West coast of Scotland. In this description, Scottish lobster wholesalers encountered economic losses due to mortality of lobsters during live storage and transport. Necrosis of abdominal muscle tissues was apparent within hours of capture and progressed to complete opacity of the abdominal musculature within a number of days. Affected tissues displayed a progressive disruption of sarcomere organization, loss of z-line material and infiltration of necrotic region by granulocytes. In addition, there was a reduction in the major contractile proteins of the affected abdominal muscle tissues.

The majority of diseases in crustaceans, however, are caused by pathogens of bacterial, viral, fungal and protozoan origin, largely in intensive rearing conditions.

1.2.2 Bacterial infections of crustaceans

Low levels of bacterial epibionts are present and occur naturally on the outer surfaces of all crustaceans, most having no detrimental effect to the host. However, colonisation of the body surfaces by filamentous bacterial species such as *Leucothrix mucor*, which is commonly observed on both natural and cultured

marine crustaceans, can be a serious problem by interfering with gas exchange across gill and egg membranes (Stewart, 1993). Incidents of chitinolytic shell disease (also known as rust disease, black spot and brown spot) have been reported among crustaceans living in degraded environmental conditions such as aquaculture settings (Prince *et al.*, 1993) or polluted environments (Young and Pearce, 1975; Sawyer, 1991). Loss of the epicuticle outer layer of the crustacean exoskeleton occurs as a result of proteolytic bacterial activity, predatory or cannibalistic attack, chemical attack, and the abrasive action of the host's interaction with the sediment. The underlying chitin-containing procuticle is then exposed and shell degeneration occurs. Bacteria belonging to the genera *Vibrio, Pseudomonas, Alteromonas, Aeromonas* and *Pasteurella* have all been reported to be involved in the disease syndrome (Getchell, 1989). The disease is not thought to be fatal in its initial stages, but mortality results in the later stages due to an inability of the animal to moult and also to septicaemia when pathogenic bacteria gain entry through lesion sites. Incidents of shell diseases are common in wild populations of crustaceans: Baross *et al.* (1978) reported a 76 % incidence in female tanner crabs, *Chionoecetes bairdi*, off the Oregon coast, and Vogan *et al.* (1999) reported a high prevalence in the edible crab, *Cancer pagurus*, from Swansea bay in South Wales.

Bacteria have been isolated from the haemolymph of apparently healthy crustaceans (Colwell *et al.*, 1975; Lightner, 1977) indicating that haemolymph is not a sterile environment and that the host immune system is able to control low-

level bacterial infection (Brock and Lightner, 1990). In contrast, Bang (1970) and Johnson (1976) reported that bacteria are not present in the haemolymph of crustaceans. However, what is clear is that stress plays an important role in bacterial disease initiation and progression. Most internal bacterial infections are caused by invasion of the haemolymph by gram-negative *Vibrio* species (Lightner and Lewis, 1975). Symptoms and pathology of infection include lethargy, black or brown cuticular lesions, tissue necrosis and melanization of appendages (Aguirre Guzmán and Ascencio Valle, 2000). Large numbers of bacteria can be observed in the haemolymph of severely affected individuals. Many cultured shrimp species (*Penaeus monodon, P. merguiensis, P. japonicus*) suffer major outbreaks of vibriosis attributed to poor water quality and aquaculture management (Lightner, 1996). Vibriosis has also been reported in *Homarus americanus* (Bowser *et al.*, 1981) and *Callinectes sapidus* (Johnson, 1976).

Gaffkemia is the most important disease caused by gram-positive bacteria. It is a fatal condition of Canadian origin, where it is endemic in stocks of *H. americanus* (Stewart *et al.*, 1966). However, outbreaks of infection in *H. gammarus* have occurred regularly in European waters in recent years (Alderman, 1996). The disease is caused by the proliferation of *Aerococcus viridans* var. *homari* in the haemocoel and haemolymph, resulting in depletion of circulating haemocytes (Johnson *et al.*, 1981). The bacterium is resistant to destruction within haemocyte phagosomes, possibly due to its acidic polysaccharide capsule (Johnson *et al.*, 1981). The bacterium is also resistant to agglutinins, and its growth is stimulated

by haemolymph serum (Cornick and Stewart, 1968). The clotting ability of the haemolymph is also lost in infected lobsters. Time to death appears to be temperature-dependent, as infected lobsters held at low temperatures (4 °C) survive longer than those held at higher temperatures. Several decapod crustacean species can become infected by *A. viridans*, and in some it has only a low virulence, so they may act as a reservoir for the pathogen (Stewart and Rabin, 1970).

1.2.3 Viral diseases of crustaceans

The first viral disease of a marine crustacean was reported by Vago (1966), for the swimming crab *Liocarcinus* (as *Macropipus*) *depurator*, and since then over 30 species of virus have been identified infecting crustaceans in both wild and culture conditions (Brock and Lightner, 1990). Viruses have been associated with disease in penaeid shrimp, the blue crab *C. sapidus*, the swimming crab *L. depurator*, and members of the crab genera *Carcinus* and *Paralithodes* (Johnson, 1984; Brock and Lightner, 1990; Sindermann, 1990). Viral disease has emerged as an extremely serious economic problem for shrimp farming around the world (Lightner and Redman, 1998). Many viruses have been found to have deleterious effects on aquacultured shrimps, while they have no effect on natural populations. The occurrence of a virus in a crustacean does not necessarily mean that a disease will develop since many viruses are latent throughout large parts of the crustacean life cycle, and indeed a small number have never been associated with any

pathological condition. Crustacean viral infection is usually associated with stress from high temperature, overcrowding and pollutants. Recently, the first viral disease of a lobster host has been reported by Behringer *et al.* (2001) as a herpes-like DNA virus infecting haemocytes of wild Caribbean spiny lobsters *Panulirus argus*, in the Florida Keys. It was suggested that the social grouping behaviour of this lobster may facilitate disease transmission by water-borne particles, direct contact or ingestion of infected tissues.

1.2.4 Fungal diseases of crustaceans

Fungal infections occur frequently in marine crustaceans, usually resulting from invasion of injured or stressed hosts, with eggs and larvae being particularly vulnerable. Fungal infections are also commonly associated with shell disease outbreaks, either alone or in conjunction with bacterial-induced lesions. An example of this is burned spot disease of the European crayfish. In this, fungal hyphae grow within the cuticle causing breaching and dark lesions, which permit secondary bacterial invasion to occur. The tanner crab, *Chionoecetes bairdi*, is subject to a condition described as black matt disease (BMD), caused by the ascomycete, *Trichomaris invadens*. Initially this was reported as a fungal infection of external surfaces only. However Sparks (1982) revealed that the infection was not only restricted to external surfaces, but was invasive, with fungal hyphae penetrating the thick cuticle and proliferating within underlying epidermal and connective tissues surrounding internal organs. The disease seems to be genus-

specific (*Chionoecetes*), with both *C. opilio* and *C. tanneri* being infected to a lesser extent (Hibbits *et al.*, 1981). The blue crab, *Callinectes sapidus*, is affected by sponge disease caused by *Lagenidium callinectes*. Up to 25 % of the egg mass of affected crabs is destroyed, and on occasion larvae can also be affected (Sindermann, 1990). Infections by *L. callinectes* have also been reported for *Cancer magister* larvae (Armstrong *et al.*, 1976) and on the eggs of the estuarine shrimp *Palaemon macrodactylus* (Fisher, 1983). Fungal disease caused by *Fusarium* sp. is common in the aquaculture of prawn and shrimp species. They are considered as opportunistic fungal pathogens which cause fouling of the gills and cuticle. Probably the most serious example of a fungal disease of wild crustaceans is the European crayfish plague caused by the phycomycete *Aphanomyces astaci*. This condition was first reported from Italian crayfish *Astacus astacus* in 1865. The fungal condition then spread rapidly throughout European populations of *A. astacus*, also infecting *A. leptodactylus* and *Austropotamobius pallipes* by the late 20th century (Johnson, 1983). North American crayfish species show resistance to *A. astaci*, but have been found to act as carrier hosts.

Another important group of crustacean fungal pathogens are the Microsporidia, with over 140 species documented to infect all crustacean Orders (Brock and Lightner, 1990). These amitochondrial intracellular parasites were formerly within the kingdom Protozoa (Protista) but have now been placed within the kingdom Fungi, based on DNA sequence evidence that they are more related to

conventional fungi (Cavalier-Smith, 1998). They occur in shrimps, crabs and lobsters where they are obligately parasitic and frequently cause extensive mortalities. Common symptoms of infection include the whitening of body musculature, in particular the abdominal flexor muscles, which gives rise to the term "cotton tail" for this condition. The whitening is due to the invasion and necrosis of muscle fibre cells by the microsporidian after its initial entry to the host through the midgut epithelium. Infection is thought to be due to spore ingestion; spores then hatch and initially infect the gut wall, then the infection spreads to other tissues and organs. Once inside the target host cell, asexual schizogony takes place followed by sporogony, resulting in the production of sporonts that divide to form sporoblasts and ultimately to form spores, which can infect other host cells (Canning, 1977). Microsporidians have been observed to infect crustacean skeletal muscle (Dennis and Munday, 1994), the hepatopancreas (Kabre, 1992), the gut wall (Kelly, 1979) and reproductive tissues (Baticados and Enriquez, 1982). The identification of microsporidian infection is problematic however, as this group can resemble several other pathogens including protozoa.

1.2.5 Protozoan parasites of crustaceans

Sparks (1985) concluded that protozoan parasites are the most common cause of disease in invertebrates. Protozoan parasites are thought to exist in all but a few decapod crustaceans due to their abundance and versatility. The sarcomastigophoran parasite *Paramoeba perniciosa* is an example of an important

protozoan pathogen. It causes grey crab disease in the blue crab *C. sapidus*, resulting in a seasonal infection along the Eastern coast of the USA (Johnson, 1977). The parasite multiplies in connective tissues, haemal spaces and in the haemolymph, impacting a grey colouration to the ventral surface of infected individuals. The parasite causes commercially significant mortalities of crabs both in wild populations and when held in holding tanks (Sawyer *et al.*, 1970; Johnson, 1988).

Opportunistic free-living ciliates have been reported to parasitize several crustacean species around the world. The first report of ciliates infecting crustaceans was by Cattaneo (1888) in which *Anophrys maggii* was found in the haemolymph of a European shore crab (*Carcinus maenas*) from Italy. Several other ciliate species are known to be important crustacean parasites. The scuticociliate *Anophrys haemophila* causes "bumper car" disease in the American lobster *H. americanus* (Cawthorn *et al.*, 1996). The term "bumper car" refers to the rapid motility of the ciliates *in vitro* and *in vivo* at low temperatures. The ciliate was first observed as a pathogen of captive *H. americanus* held in a flow-through system (Aiken *et al.*, 1973). Epizootics of this disease have been documented to occur in the winter months in Maine and New Brunswick, with mortalities of up to 25 % (Shelburne and Bean, 1991; Loughlin *et al.*, 1994). Affected lobsters have reduced muscle mass, poor meat quality and an unsavoury flavour (Loughlin *et al.*, 1993). Recently, mass mortalities of krill (several euphausiid species) have been reported and attributed to parasitic ciliates of the

genus *Collinia* (Gómez-Gutiérrez *et al.*, 2003). This ciliate was observed to consume all the hosts' tissues and to rupture the cephalothorax, killing the host within 40 hours of infection.

The taxonomy of ciliates is confusing and often contentious, and has undergone many changes, especially recently in the light of genetic research. The description of several ciliates resembling *Anophrys maggii* (Cattaneo, 1888) following its initial discovery, are a good example of this problematic issue. The genus *Anophrys* was previously established for a free living scavenging ciliate *A. sarcophaga*, found in a marine aquarium (Cohn, 1866). Substantial confusion then followed, with incomplete or poor morphological descriptions of several ciliates of the genus *Anophrys* infecting crustaceans (Poisson, 1930; Bang, 1962; Bang *et al.*, 1972). These early descriptions were made without the use of silver impregnation methods that allow precise staining and identification of ciliary structures. Grolière and Leglise (1977) then described a ciliate from *Cancer pagurus* as a new species, *Paranophrys carcini*, without any reference to the previous descriptions of ciliates infecting crustaceans. Following this, De Puytorac and Grolière (1979) reported basic differences between *A. sarcophagi* and *A. maggii*, and grouped *P. carcini* with *A. maggii* as *Paranophrys maggii*, suggesting that all previously described crab haemolymph ciliate parasites from European waters should be designated as this. Armstrong *et al.* (1981) reported a *Paranophrys* infection in dungeness crabs (*Cancer magister*) from Oregon in the US, and speculated that the ciliate was probably *P. maggii* from Europe. Hibits

and Sparks (1983) also described a *Paranophrys* sp. infection, this time in the isopod *Gnorimosphaeroma oregonensis* from Alaska. Based on the morphology of protargol-stained ciliate oral structures, a new genus, *Mesanophrys,* was erected by Small and Lynn (1985). This was defined by the second oral polykinetid being roughly equal in length to the first, and the forward part of the oral dikinetid ending at the level of the third oral polykinetid. *A. maggii* and *P. carcini* were then placed in this new genus. Since the construction of this genus there have been several descriptions of crustacean ciliate infections of the genus *Mesanophrys*; Morado and Small (1994) reported *M. pugettensis* from dungeness crabs, and Messick and Small (1996) reported *M. chesapeakensis* from blue crabs. Both descriptions were attributed to separate species based on minor differences in protargol-stained oral structures, the host species and geographical location. However, Goggin and Murphy (2000) surprisingly found no differences in the highly variable first and second internal transcribed spacers (ITS1/ITS2) of the ribosomal DNA gene complex from *M. pugettensis* and *M. chesapeakensis,* suggesting either that the ITS sequences are highly conserved and cannot be used to discriminate between species, or that they are both the same species of *Mesanophrys*. In addition, Wiąchowski *et al.* (1999) reported a *Mesanophrys* sp. ciliate infecting the isopod *Saduria entomon*. They suggested that all nominal *Mesanophrys* species should be referred to as *M. maggii,* based on strongly overlapping morphometric characteristics between described species, unless new information on host specificity, life cycle or biochemical dissimilarity indicates a taxonomic difference.

The discovery of a ciliate in a new host raises issues of parasite host specificity and species diversity. This has occurred in the present study since a *Mesanophrys*-like ciliate was discovered in *N. norvegicus*. The opportunity was therefore taken to include studies of its identity and virulence within the scope of the project aims.

1.2.6 Dinoflagellate parasites of crustaceans

The dinoflagellates occur as essential components of phytoplankton, and can be both parasites and symbionts of marine invertebrates (Shields, 1992). Dinoflagellates are principally biflagellated with characteristic transverse and longitudinal flagella, cortical alveoli and tubular mitochondrial cristae. The dinoflagellates are composed of motile and non-motile forms, pigmented and non-pigmented types, solitary and colonial species, and armoured and naked forms. Of 2000 recognised species of dinoflagellate, over 140 species are parasitic (Drebes, 1984). Parasitic dinoflagellates are known to infect a wide range of marine organisms including fish, tunicates, molluscs, annelids, protozoa, algae and crustaceans (reviewed in Shields, 1994). The first report of a parasitic dinoflagellate was by Pouchet (1884), in which he described appendicularian tunicates parasitized by *Gymnodinium pulvisculus*. Little else was discovered about parasitic dinoflagellates until a substantial monograph was produced by Edouard Chatton in 1920 and reviewed in the 1930s (Chatton and Poisson, 1931). Jean and Monique Cachon (Chatton's students) continued his work, describing the life history and structure of many parasitic dinoflagellates (Cachon and Cachon,

1987). Parasitic species occur in around 35 genera, encompassing the four orders of dinoflagellates (Phytodiniales, Gymonodiniales, Blastodiniales, and Syndiniales).

The orders Blastodinia and Syndinia contain at least 25 species of dinoflagellate parasites of crustaceans. There are seven families forming the Blastodinia (Cachon and Cachon, 1987), but only two contain parasites of crustaceans. Members of the family Blastodiniae are extracellular marine parasites occurring in copepod guts. The mode of infection is not known but is believed to involve the ingestion of dinospores that develop into the trophont form and then divide through several vegetative stages into a sporocyst, eventually releasing dinospores (Chatton, 1920). Host castration is a common consequence of infection. The family Chytriodinidae contains four genera that parasitize crustaceans, which live as ectoparasites on the eggs of euphausiids, copepods and penaeid shrimps. The parasitic dinospore attacks, penetrates and consumes the host egg in a period of 1-2 hours, after which sporulation occurs. Consequently, the reproductive dynamics of infected species are greatly affected by this parasite (Wickham, 1986).

The Syndinidae are osmotrophic parasites of the cytoplasm and body cavity. Four genera of the Syndinidae parasitize crustaceans: *Actinodinium, Trypanodinium, Syndinium* and *Hematodinium.* The *Actinodinium* and *Trypanodinium* are not well documented. *Actinodinium apsteini* was found in the stomach wall of the copepod *Acarti clausi* (Chatton and Hovasse, 1938), while *Trypanodinium ovicola*

parasitizes the eggs of several species of copepods (Chatton, 1920). Three species of the genus *Syndinium* parasitize crustaceans and are found in the soft tissues and haemal spaces of hosts. Infection of copepods by the type species *S. turbo* (Chatton 1920) is thought to occur by ingestion of dinospores. The dinospore penetrates the gut wall and develops into a plasmodium, which grows and causes muscle degeneration and host castration leading to death (Shields, 1994).

1.2.7 *Hematodinium* species infecting crustaceans

Members of the genus *Hematodinium* are predominantly parasites of decapod crustaceans. There are two described species of *Hematodinium*, although several *Hematodinium*-like infections in decapods probably warrant new species descriptions based on rDNA sequence information (Hudson and Adlard, 1996). The type species, *Hematodinium perezi,* was originally described as infecting the portunid crabs *Carcinus maenas* and *Liocarcinus depurator* from France (Chatton and Poisson, 1931). It was found in only four out of 3570 individuals examined. A second species, *H. australis*, from the Australian sand crab, *Portunus pelagicus,* was described by Hudson and Shields (1994). *Hematodinium*-like infections have been reported for a wide range of crustacean species including *Callinectes sapidus* from the US East coast waters of Florida, Maryland, Virginia, and Georgia (Newman and Johnson, 1975; Messick, 1994; Messick and Shields, 2000; Lee, 2001). *Hematodinium* infections have also been found in *Cancer irroratus, C. borealis* and *Ovalipes ocellatus* from the New York Bight on the Eastern seaboard

of the US (MacLean and Ruddell, 1978); *Cancer pagurus* from France and the Channel Islands (Latrouite *et al.*, 1988; Stentiford *et al.*, 2002); *Necora puber* from the West coast of France (Wilhelm and Mialhe, 1996); *Scylla serrata* (Hudson and Lester, 1994) and *Trapezia aerolata* from the East coasts of Australia (Hudson *et al.*, 1993); *Chionoecetes bairdi* (Meyers *et al.*, 1987; Eaton *et al.*, 1991; Love *et al.*, 1993) and *Chionoecetes opilio* (Taylor and Khan, 1995) from the Baring Sea and South East Alaskan waters. Benthic amphipods infected by dinoflagellates similar in appearance to *Hematodinium* species have also been described (Johnson, 1986; Messick and Shields, 2000) suggesting their involvement as a secondary or intermediate host in the life cycle of *Hematodinium.*

Hematodinium infection is thought to be a gradual process, with early and moderate infections hard to diagnose because circulating trophonts resemble immature haemocytes. In advanced infections the haemolymph is milky white in colour due to the number of trophonts present in the circulating haemolymph. As the parasite cells proliferate there is marked degeneration of the hepatopancreas and muscle tissues, and a general congestion of the gill filaments and haemal sinuses with circulating parasites. There is also a marked reduction in the numbers of circulating host haemocytes, even though *Hematodinium* is not found intracellularly. Respiratory dysfunction indicated by reduced oxygen-carrying capacity of the haemolymph results in lethargy of the host crustacean (Taylor *et al.*, 1996).

1.3 *Hematodinium* infection of the Norway lobster

During the early 1980s, routine investigations into the biology of the Norway lobster *N. norvegicus* in the Firth of Clyde led to the discovery in the spring months of a number of lobsters in a moribund state with an opaque rather than the normal translucent appearance, and white coloured haemolymph. The condition was first thought to be moult-related as this takes place in both the female and male between March-April. By 1987 the poor appearance and condition of these lobsters had also evoked comment from fishermen and food processors, with at least one catch of lobsters refused at market. A sampling programme was therefore instigated to monitor the incidence and geographical extent of this condition around the West Coast of Scotland. This led to the discovery of non-motile protozoan parasites in the haemolymph of severely infected lobsters (Field *et al.*, 1992). The parasite was identified as a dinoflagellate resembling *Hematodinium perezi*. *Hematodinium* infection of Norway lobsters has since been identified in the Irish Sea (Briggs and McAliskey, 2002) and the Swedish Skagerrak (K. Frohlund, Havsfiskelaboratoriet Sweden, personal communication). At present these are the only descriptions of *Hematodinium* infection in a lobster host, and their genetic similarity to each other is not known.

Several studies have shown that there is a seasonal pattern of infection between February and July with peak numbers of infected animals occurring between March and May (Field *et al.*, 1992; Appleton *et al.*, 1997; Field *et al.*, 1998). In

the Clyde Sea Area, the proportion of infected lobsters was observed on occasions to be as high as 80 % of the total trawl catch (Field *et al.*, 1992), with female lobsters being found to have a higher infection prevalence than males (Field *et al.*, 1992; Stentiford *et al.*, 2001b).

Hematodinium infection in the Norway lobster results in a reduced swimming performance of the host (Stentiford *et al.*, 2000a), and to the depletion of glucose levels in the host haemolymph. This is thought to be due to the parasite cells circulating in the haemolymph affecting the carbohydrate dynamics of the tissues, thus causing the release of crustacean hyperglycaemic hormone (CHH) (Stentiford *et al.*, 2001a). Infection also changes the plasma free amino acid profiles, and causes alterations in muscle sarcolemmal structure and disruption of myofibrillar bundles (Stentiford *et al.*, 2000b). In contrast, little or no research has been carried out on how *Hematodinium* infects the lobster, or the mechanisms of host immune reaction evasion by the parasite. Because of the dearth of information, the possible mechanisms for host immune reaction evasion were investigated in this study.

The *Hematodinium* species infecting *N. norvegicus* has been continuously cultured for a number of years using a balanced salt solution (*Nephrops* saline), supplemented with 10% (v/v) foetal calf serum (Appleton, 1996; Appleton and Vickerman, 1998). A proposed life cycle consists of the development of filamentous trophonts, which aggregate into unusual colonies of plasmodia, called

"gorgonlocks", followed by continual cell differentiation into arachnoid trophonts, clump colonies, arachnoid sporonts, sporoblasts and finally dinospore release (Appleton, 1996; Appleton and Vickerman, 1998). Dinospore release has only rarely been observed and recorded *in vivo* with dinospores exiting the host at sites of cuticle damage, particularly in the gills (Appleton *et al.*, 1997).

In contrast to the above studies on the culture of the *Hematodinium* sp. from *N. norvegicus*, little is known of the life cycle stages of the type species *H. perezi* from *C. sapidus*, due to a lack of success with *in vitro* culture systems (J. Shields, VIMS, personal communication). There is however, considerable information on the virulence and effect of *H. perezi* on *C. sapidus* survival, as artificial infection can be achieved by injection of infected haemolymph (Shield and Squyars, 2000).

Artificial infection of naïve Norway lobsters by ingestion of infected tissues, and by inoculation of infected haemolymph and culture stages have all been attempted without success. Therefore the natural mode of *Hematodinium* infection of Norway lobsters is unknown. The lobster may ingest spores while suspension feeding, or dinospores may gain access during the host moulting period. Resting cyst stages may also be present in the sediments, infecting lobsters during ecdysis. Other possible routes of *Hematodinium* transmission include cannibalism and lobsters feeding on a secondary host of the parasite.

Methods used to diagnose *Hematodinium* infection in *N. norvegicus* have previously included visually recognizing infected lobsters based on the dull orange colouration of the carapace and appendages (Fig. 1.1 and 1.2). However, this method lacks sensitivity and only the heaviest infections can be identified. A more reliable and quantitative technique is based on pleopod examination (Field and Appleton, 1995), in which the pleopod is assessed under low power microscopy for the aggregation of parasites. The severity of infection is assigned a stage on a scale from I through IV (Field and Appleton, 1995). This has been used to show that *Hematodinium* infection progresses from stage I with low haemolymph burdens to stage IV, in which large numbers of spores outnumber host haemocytes. Pleopod examination has proved to be reliable as a field method for identifying advanced infections, but it is unable to detect low-level tissue and haemolymph infections, and also requires a degree of training, careful interpretation and standardization.

Because of these limitations of the inspection methods, an immunological approach to infection diagnosis was pursued (Field and Appleton, 1996). Such antibody-based diagnostic assays have been used in a wide range of applications to detect pathogens of marine inhabitants, including Taura syndrome in the penaeid shrimp (Poulos *et al.*, 1999), *Penaeus monodon*-type baculovirus in *Penaeus monodon* (Hsu *et al.*, 2000) and *Cryptocaryon irritans* in barramundi, *Lates calcarife* (Bryant *et al.*, 1999).

A polyclonal rabbit antibody was raised against a mixed *in vitro* culture of vegetative forms of the *Hematodinium* species infecting *N. norvegicus* (Field and Appleton, 1996). The antibody was first assessed to have good specificity for the parasite, and then employed as part of an indirect fluorescent antibody technique (IFAT). This demonstrated that apparently uninfected lobsters, as assessed by the pleopod method, harboured both low level haemolymph infection, and tissue-only infection, outside the main season (Field and Appleton, 1996). More recently, a Western immunoassay blotting technique was developed utilising the original polyclonal anti-*Hematodinium* antibody, and this has been used to detect low-level tissue-based infections in a given population (Stentiford *et al.*, 2001c). This study failed to detect infected animals in the summer months, which may indicate that infection is not carried over to the next season. Alternatively, the sensitivity of the Western blot method may not be sufficient to detect small numbers of parasite cells, or it may be the case that the parasite shows a preference for certain tissues during early infection. However, previous studies using the IFAT procedure did demonstrate tissue-based *Hematodinium* infection in lobsters outside the peak season (Field and Appleton, 1996). Both the IFAT and Western blot procedures offer greater sensitivity than previous methods but are relatively long and complex to perform. Consequently, it would be beneficial to develop an antibody-based assay that has the potential for processing large numbers of samples in a short time, and for detecting both low level and advanced infections. This was therefore one of the major aims of this study.

The recent expansion of molecular diagnostic methods, such as the polymerase chain reaction (PCR), which display levels of sensitivity and specificity, irrespective of life cycle stage, far beyond any existing technique, has permitted the recent identification of several marine pathogens. These include *Marteilia sydneyi* in the Sydney rock oyster (Kleeman and Adlard, 2000), *Perkinsus marinus* in the Eastern oyster (Reece *et al.*, 1997; Robledo *et al.*, 1999) and *Haplosporidium nelsoni* in oysters (Stokes and Burreson, 1995). PCR also provides a method for detection of a parasite, despite the presence of host DNA. In addition, very small amounts of DNA can be detected by exponential amplification of a particular region of the genome. DNA techniques can also be used to eliminate misidentification of infective agents. Thus Bower *et al.* (1993) reported a disease of spot prawns *Pandalus platyceros*, caused by bacteria and a dinoflagellate-like parasite. However, the parasite was later identified as belonging to the Haplosporidia, based on antibody binding affinities and DNA sequence analysis (Reece *et al.*, 2000).

A PCR-based assay for the detection of *Hematodinium* infection in decapod crustaceans was developed by Hudson and Adlard (1994), although primers were designed in conserved ribosomal gene regions and as such were not specific to *Hematodinium* species. Sequencing of ribosomal gene internally transcribed spacer regions (ITS) from several presumptive species of *Hematodinium* originating from different geographical locations and hosts (*N. norvegicus, Callinectes sapidus, Chionoecetes opilio* and *C. bairdi*), demonstrated that

substantial sequence variation exists among the isolates, and that *Hematodinium* species remain genetically and geographically distinct from each other (Hudson and Adlard, 1996). This assumption, however, was based on single *Hematodinium* samples from each host crustacean. Moreover, strain differentiation within the same host species, or differentiation in different host species in the same geographical location, was not analysed. More recently, a second PCR-based assay has been developed for *H. perezi* from blue crabs, again based on conserved ribosomal gene regions (Gruebl *et al.*, 2002). However, no DNA-based probes have yet been developed to identify and localise *Hematodinium* parasites within infected crustacean tissues.

Thus a major aim of this study was to develop a suite of molecular diagnostic tools that will facilitate investigation of low-level *Hematodinium* infection, secondary host identification and transmission issues, all of which are currently unresolved. In conjunction with the development of molecular diagnostics, the sequencing of parasite rDNA gene regions has facilitated investigation of the level of genetic diversity of *Hematodinium* sp. infecting *N. norvegicus* and other crustaceans in UK waters, and this represents another aim of this project.

1.4 Aims of this project

For the reasons identified in the sections above, the following specific aims of the project were formulated:

- To develop the Western blot method into a sensitive, multi-sample diagnostic ELISA for the detection of *Hematodinium* infection in *N. norvegicus* and other crustaceans (Chapter 2).

- To develop a set of PCR primers and a DNA probe for the diagnosis of *Hematodinium* infection in *N. norvegicus* and other crustaceans (Chapter 3).

- To investigate the level of genetic variation between isolates of *Hematodinium* from the UK (Chapter 4).

- To initiate *in vitro* cultures of *Hematodinium perezi* from the blue crab *Callinectes sapidus*, and to document the early culture life cycle stages (Chapter 5).

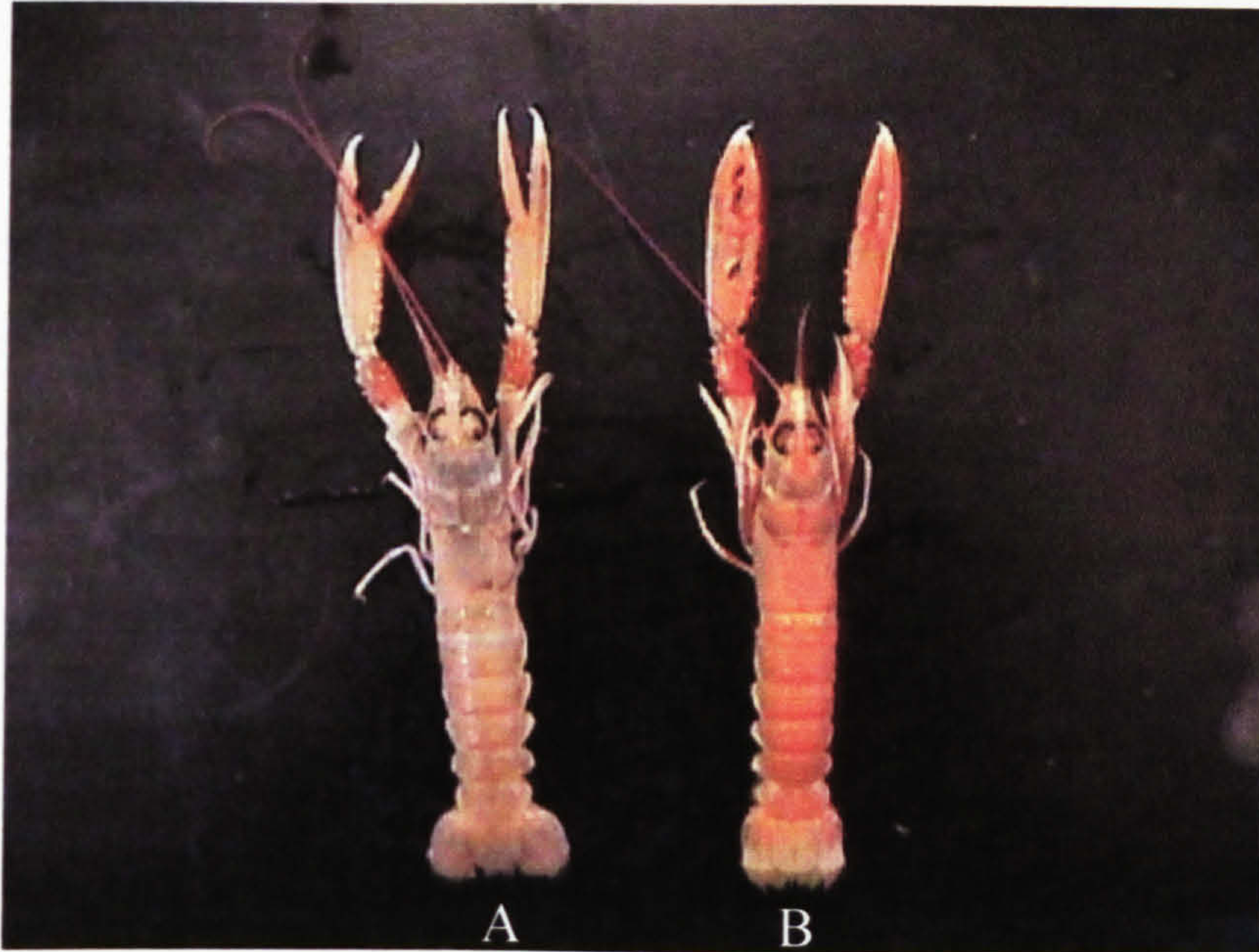
- To investigate possible virulence factors that *Hematodinium* parasites may possess (Chapter 6).

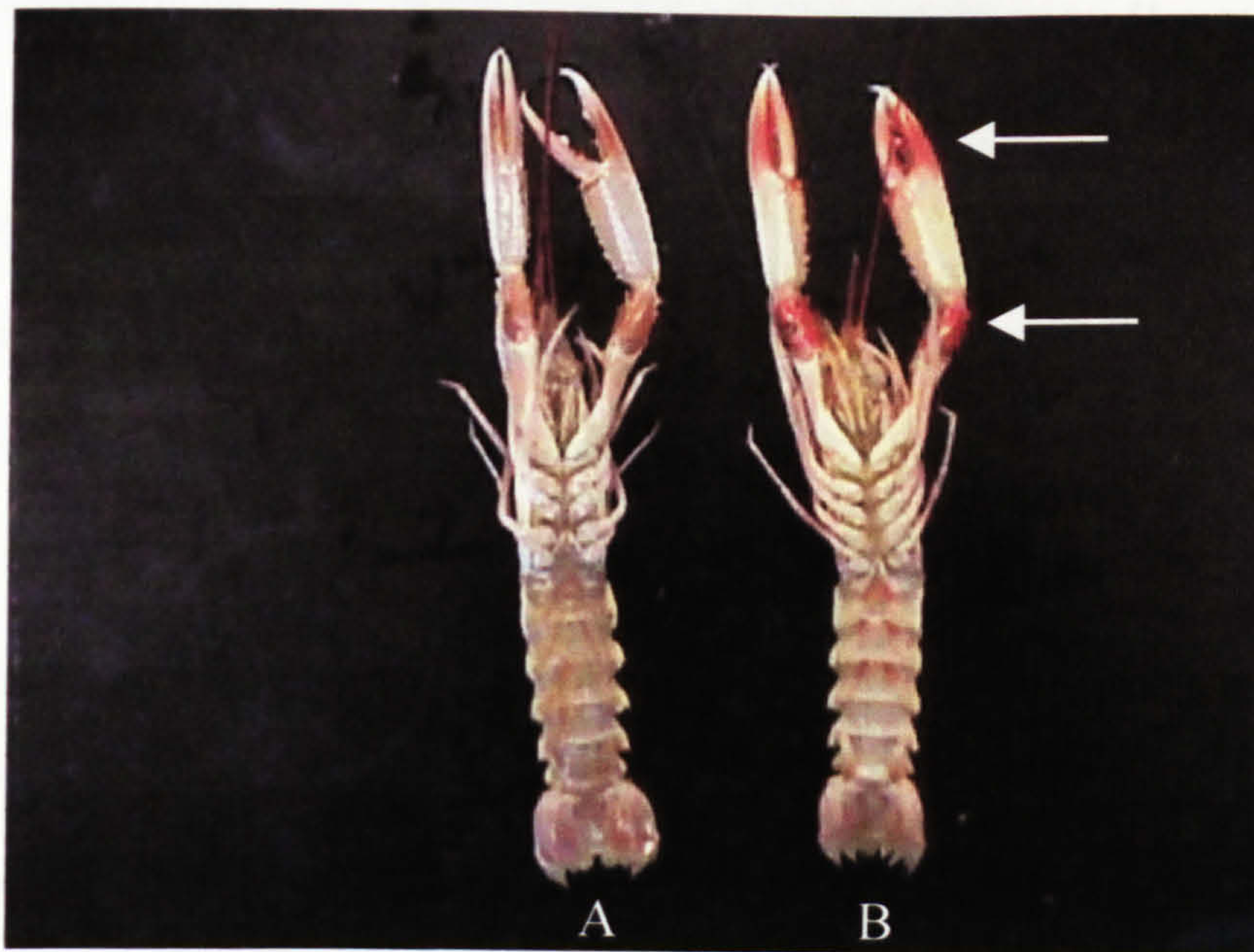
- The discovery and identification of a ciliate parasite infecting *N. norvegicus* is reported (Chapter 7).

- The successful *in vitro* culture of this parasitic ciliate has allowed the investigation of proteolytic enzymes produced by the ciliate (Chapter 8).

Figure 1.1 Photograph showing upper view of healthy (A) and *Hematodinium*-infected (B) *N. norvegicus*. Infected lobsters are a bold orange colour and have a "cooked appearance".

Figure 1.2 Photograph showing underside of healthy (A) and *Hematodinium*-infected (B) *N. norvegicus*. The body and appendages have a bleached appearance apart from a vivid red banding pattern on the claws (arrows).


A
B


A
B

Chapter 2

Development and application of an Enzyme-Linked Immunosorbent Assay (ELISA) for the diagnosis of *Hematodinium* infection in *N. norvegicus* and other crustaceans.

2.1 Introduction

The Norway lobster (*Nephrops norvegicus*) supports a major commercial fishery in the Northeast Atlantic and in the Mediterranean. In Scotland, lobster populations harbour an infection by a parasitic dinoflagellate of the genus *Hematodinium* (Field *et al.*, 1992). Infection of *N. norvegicus* by *Hematodinium* species was initially diagnosed by the dull orange colouration of the carapace and appendages; such animals were also observed to be in a moribund state with milky white haemolymph (Field *et al.*, 1992). However this diagnostic method lacks sensitivity and only the heaviest infections can be identified. A more sensitive method was later developed in which the pleopod is examined under low power light microscopy for the aggregation of parasites in the vasculature (Field and Appleton, 1995). The severity of infection is staged against a five-point scale, from apparently uninfected to a fully patent infection. The pleopod method has proved to be reliable as a field method for identifying advanced infections, but it is unable to detect low-level haemolymph infection, and also requires a degree of training and standardisation.

Immunodiagnostic techniques have been applied to detect several pathogens of marine organisms (Bryant *et al.*, 1999; Poulos *et al.*, 1999; Hsu *et al.*, 2000). An indirect fluorescent antibody technique (IFAT), developed using a polyclonal rabbit antiserum raised against a mixed *in vitro* culture of vegetative forms of *Hematodinium* found infecting *N. norvegicus,* has been used to show that some *N. norvegicus* harbour infections outside of the main infective season (Field and Appleton, 1996). More recently, a Western blot method has been developed using the polyclonal anti-*Hematodinium* antiserum and applied to study the occurrence and progression of infection (Stentiford *et al.*, 2001c). Both immunoassays offer greater sensitivity and specificity than the previous diagnostic methods, but are time-consuming and complex procedures, requiring trained personnel to carry them out. Following a pilot study, an enzyme linked immunosorbent assay (ELISA) using the polyclonal anti-*Hematodinium* antiserum has been further developed and calibrated as part of the present study, and the results are reported in this chapter. It has the potential to screen large numbers of samples in a short time, and has a greater sensitivity than the Western blot procedure. The assay can also be used to screen other crustacean haemolymph samples for the presence of *Hematodinium.*

2.2 Materials and Methods

2.2.1 Collection and maintenance of experimental lobsters

Norway lobsters (*Nephrops norvegicus*) were caught by otter bottom trawl (70-mm mesh size) at locations south of Little Cumbrae in the Clyde Sea Area (55.41° N, 4.56° W). The lobsters were transported in a cool, damp environment after capture, then maintained in a closed seawater system at 10 °C and 33 ppt salinity prior to experimental study.

2.2.2 Haemolymph preparation and development of assay

Haemolymph samples were withdrawn from the base of the fifth pereiopod using a 1 ml disposable syringe and 25-gauge needle, allowed to clot, frozen at –20 °C and thawed once. All subsequent treatments were performed at 22 °C. The haemolymph samples were vortexed and a 15 µl aliquot of each haemolymph sample was then diluted in 285 µl of distilled water. From this, 100 µl was transferred into each of 2 wells of a 96-well microtiter plate (Immulon 4 HBX) so that for each haemolymph sample the ELISA was carried out in duplicate. After an initial incubation for 30 min, plates were washed 4 times with PBS (pH 7.2) with 0.05 % (v/v) Tween 20. Plates were then incubated for 30 min with 100 µl/well rabbit anti-*Hematodinium* antiserum (1/2000 dilution) (see Field and Appleton, 1996 for antiserum production). Plates were again washed 4 times,

followed by a third incubation for 30 min with 100 μl/well goat anti-rabbit horseradish peroxidase conjugated antibody (1/500 dilution)(Diagnostics Scotland). Plates were washed 4 times and 100 μl TMB substrate (3, 3', 5, 5'-tetramethyl benzidine)(Dynex Technologies) applied for colour development by incubation for 20 min in darkness. The optical density (OD) of the wells in the microtiter plate was then measured at 690 nm with an ELISA reader (Titertec Multiscan).

2.2.3 Sensitivity and comparison of assay with other methods

For determining the sensitivity of the ELISA test, a haemolymph sample was taken from a lobster that gave a positive result in the routine ELISA assessment, but displayed no external signs of infection. An aliquot of this sample was diluted 1:1 in marine anticoagulant (450 mM sodium chloride, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA pH 4.6, 1020 mOsm kg^{-1}) and transferred into an Improved Neubauer counting chamber. Parasite cell numbers were counted and the numbers per ml estimated using standard procedures. The sample was frozen to –20 °C, then thawed and six aliquots were serially 2-fold diluted and assayed by the ELISA procedure above. A sample of uninfected haemolymph was assayed by the same method and a comparison of the OD values from infected and uninfected haemolymph was performed by the Mann-Whitney U test. Significance was considered to be at $p<0.05$.

A routine assessment of infection was made on a sub-sample from 2 monthly trawls using both the pleopod staging method of Field and Appleton (1995) and Western blotting of haemolymph samples, as described by Stentiford *et al.* (2001c). These assessments were then compared with the results from the ELISA conducted on the same haemolymph samples. Haemolymph samples from lobsters testing positive for *Hematodinium* infection by the ELISA method alone were assayed by the IFAT technique of Field and Appleton (1996) to confirm infection status. In this instance, the bovine serum albumin (BSA) used during the washing steps was replaced by an equivalent amount of porcine gelatin, because the anti-*Hematodinium* antibody reacts with BSA on Western blots (Stentiford *et al.*, 2001c).

2.2.4 Specificity of antiserum

Cultures of the dinoflagellates *Alexandrium tamarense* (CCAP 1119/5) and *Gymnodinium catenatum* (CCAP 1117/5) were obtained from the UK Culture Collection of Algae and Protozoa (CCAP). Samples of cell suspensions (1 x 10^5 cells) were centrifuged at 400 x g, the resultant cell pellet was resuspended in 100 µl sample buffer (62.5 mM Tris-HCl pH 6.8, 12.5 % glycerol, 1.25 % ß-mercaptoethanol). Haemolymph or hepatopancreas tissue samples were obtained from the following *Hematodinium*-infected and uninfected crustaceans: Norway lobster from the Clyde Sea Area, the North Minch (N. W. Scotland), the Fladen grounds (N. E. Scotland), the Skagerrak (W. Sweden); edible crab (*Cancer*

pagurus) from Guernsey; blue crab (*Callinectes sapidus*) from the East Coast of America; snow crab (*Chionoecetes opilio*) from Newfoundland. A 50 µl haemolymph sample or a 100 mg tissue sample were resuspended in 100 µl sample buffer. The dinoflagellate, haemolymph and tissue samples were then assayed by the Western blotting technique (Stentiford *et al.*, 2001c) to assess reactivity of the polyclonal anti-*Hematodinium* antiserum against other dinoflagellates and other species of *Hematodinium* from different crustaceans.

2.2.5 Application of assay

The ELISA was used to estimate the prevalence of *Hematodinium* in Norway lobsters from the sample site within the Clyde Sea Area by obtaining bi-monthly haemolymph sub-samples from 50 animals from Sept 2002 to July 2003. Samples were obtained and assayed according to section 2.2.2.

2.3 Results

2.3.1 Evaluation, sensitivity and specificity of assay

The ELISA was able to detect the presence of *Hematodinium* in haemolymph samples. There was a degree of antibody binding in uninfected samples but this was considerably lower than for both low-level and advanced infected samples

(Fig. 2.1). Importantly for routine use, the difference between positive (low-level infections and above) and negative samples was visible by eye.

Serial dilutions of a haemolymph sample with a known number of parasites present were used to determine the sensitivity of the assay (Fig. 2.2). The lowest density sample that was significantly different from the uninfected haemolymph sample was taken to represent the limit of detection of the assay (5×10^4 parasites (ml haemolymph)$^{-1}$).

The reactivity of the polyclonal anti-*Hematodinium* antiserum to free-living dinoflagellates and other species of *Hematodinium* infecting different crustaceans was investigated using the Western blot method (Fig. 2.3). The polyclonal antiserum gave positive multiband smears for all *Hematodinium* infected samples from the Norway lobster, edible crab, blue crab and snow crab. Uninfected Norway lobster and crab haemolymph samples as well as *Alexandrium tamarense* and *Gymnodinium catenatum* did not react with the antiserum (Table 2.1).

2.3.2 Comparison of diagnostic methods

The diagnosis of *Hematodinium* species infection in 2 monthly sub-samples of 30 lobsters taken at times before the seasonal peak of infection, assessed by the pleopod, Western blot and ELISA methods, is shown in Table 2.2. By the pleopod method, all lobsters were assessed to be uninfected but by the antibody-based

methods a number of animals were found to be infected. All haemolymph infections identified by Western blotting were also found to be positive by ELISA, but conversely not all infections identified by ELISA were detected by Western blotting. Comparison of the ELISA and IFAT results on these samples however, gave the same positive infection diagnosis (data not shown).

2.3.3 Seasonality of infection

The prevalence of infected *N. norvegicus* assessed by ELISA in bi-monthly trawl catches from the study site in the Clyde during the period September 2002 to July 2003 is shown in Figure 2.4. The results confirm previous prevalence estimates (Field *et al.*, 1992; Stentiford *et al.*, 2001b), with a seasonal pattern of infection that peaks between January and May.

2.4 Discussion

The results obtained show that the ELISA is a sensitive and specific diagnostic test for the presence of *Hematodinium* parasites in the haemolymph of the Norway lobster. In common with the Western blot method of Stentiford *et al.* (2001c), it can detect both low-level and advanced infections. Moreover, it offers significant advantages over the Western blot procedure in terms of its sensitivity, simplicity and the number of samples that can be assayed. Importantly for routine use, the difference between positive and negative samples was visible by eye. The

sensitivity of the ELISA has been determined to be 5 x 10^4 parasites (ml haemolymph) $^{-1}$, making it more sensitive than the Western blot procedure by a factor of four. This is consistent with the observation that several low-level infections identified by the ELISA were not detected by the Western blot.

Similar ELISA tests have been developed for detection of crustacean biomarkers such as lectins (Agundis *et al.*, 2000), agglutins (Jayasree *et al.*, 2000), hyperglycaemic hormone (Chang *et al.*, 1998), and the MrNV nodavirus, which causes white tail disease of commercially important prawns (Romestand and Bonami, 2003). However the method described in this chapter is the first ELISA for a dinoflagellate parasite of crustaceans. The ability of the ELISA to test multiple samples within a short period, without sophisticated analytical equipment, is a significant development. Previous immunological methods such as the IFAT and Western blot are complex and time-consuming. The ELISA requires only a small volume of haemolymph to conduct the test; host lobsters need not be damaged, and if necessary the sampled animals can be kept alive for further observation under laboratory conditions.

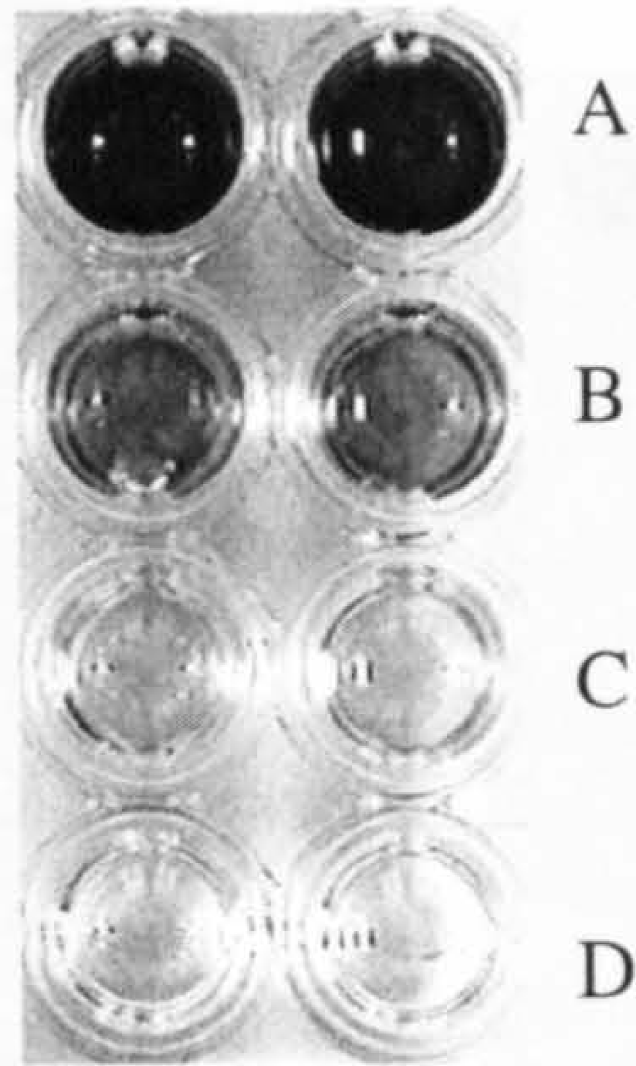
The ELISA will be particularly useful in the identification of *Hematodinium* species infection in previously unexamined *N. norvegicus* stocks as specificity experiments show that the polyclonal antiserum reacts to *Hematodinium* infection in *N. norvegicus* from four geographically separate fishing grounds; the Clyde Sea Area, North Minch, Fladden and the Irish Sea. In addition, the ELISA could also

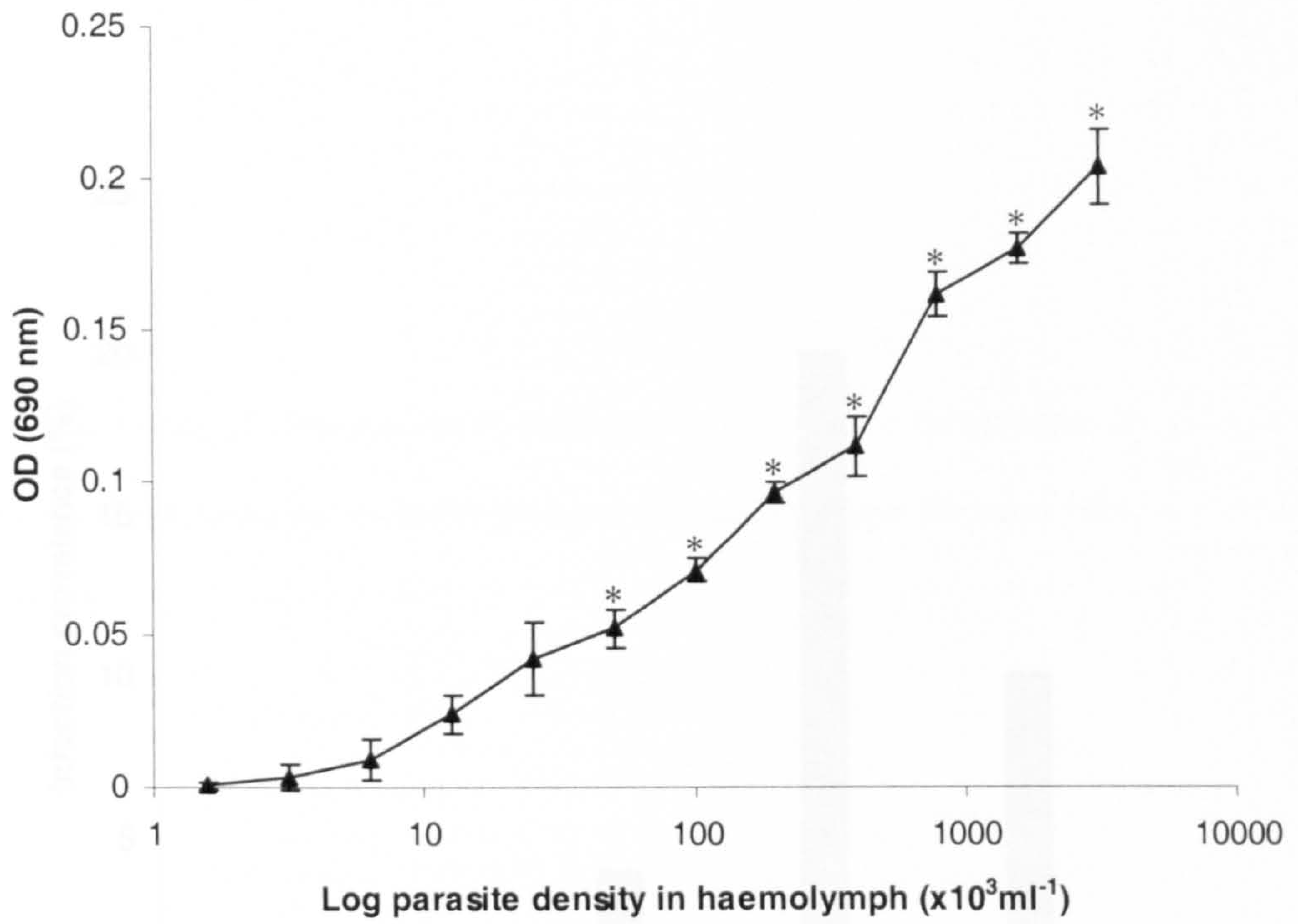
be used to identify *Hematodinium* infections in other crustacean species as the primary anti-*Hematodinium* antibody is also reactive to *Hematodinium* species infecting edible crabs, blue crabs and snow crabs (Table 2.2).

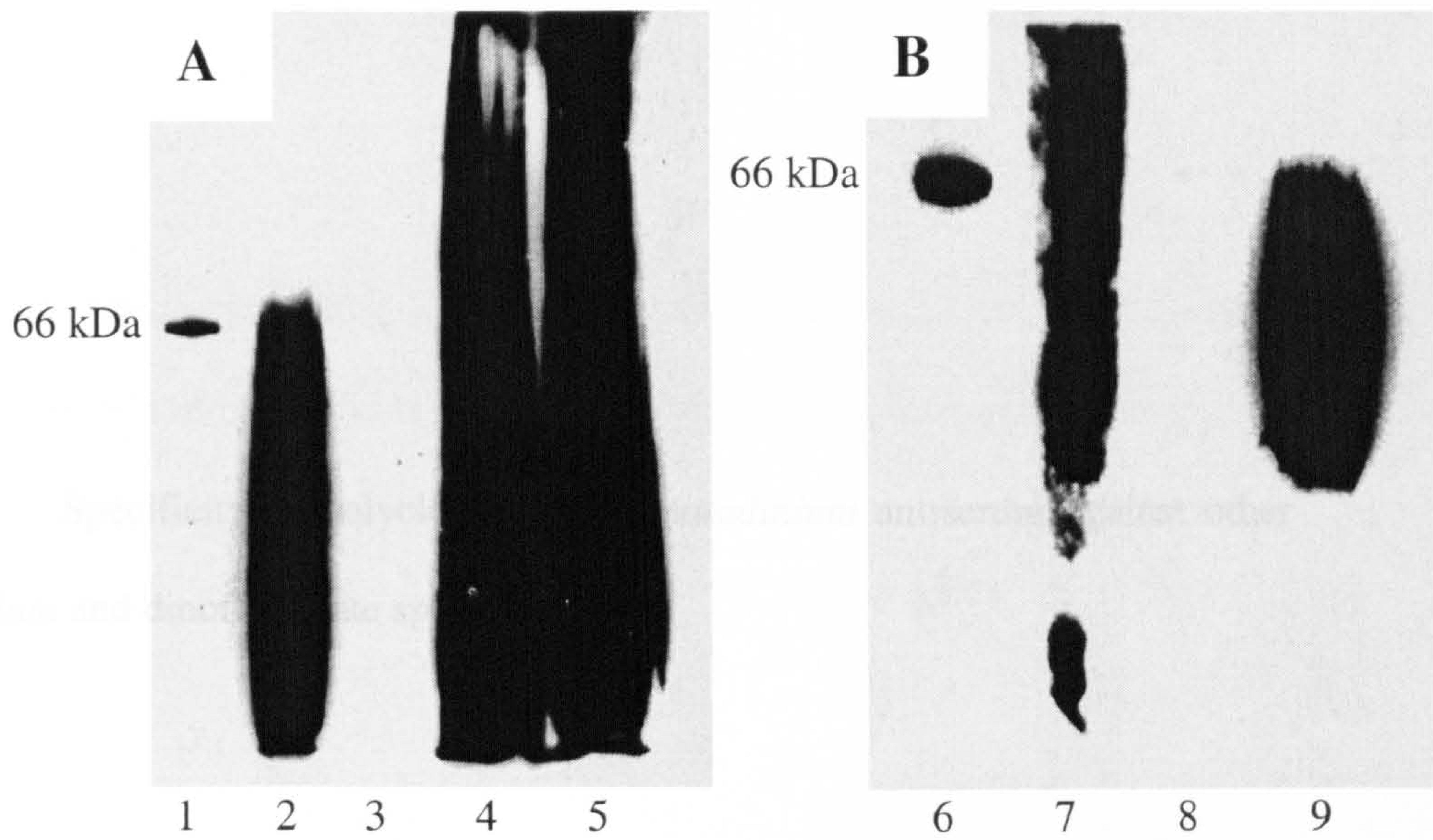
The finding by Bushek *et al.* (2002) that the polyclonal anti-*Hematodinium* antiserum used in this study is reactive against antigens of the oyster pathogen *Perkinsus marinus* raises interesting issues concerning the phylogenetic relatedness of *Hematodinium* and *Perkinsus.* However this does not affect the usefulness of the antiserum in detecting *Hematodinium* in crustaceans as *Perkinsus marinus* is exclusively a mollusc pathogen. In addition, the antiserum has been tested against two free-living dinoflagellates (*Alexandrium tamarense* and *Gymnodinium catenatum*) with no positive cross-reaction being evident.

Dinoflagellate/haemolymph/tissue samples	Reaction with polyclonal anti-*Hematodinium* antiserum
Hematodinium infected *N. norvegicus* from the Clyde Sea area, North Minch, Fladen, Irish Sea and Sweden	+
Hematodinium Infected *C. pagurus, C. sapidus, C. opilio*	+
Uninfected *N. norvegicus, C. pagurus, C. sapidus, C. opilio*	–
A. tamarense (CCAP 1119/5) *G. catenatum* (CCAP 1117/5)	–

Date lobster caught	Pleopod (% prevalence)	Western blot (% prevalence)	ELISA (% prevalence)
10/10/00	0	13.3	26.6
06/11/00	0	16.6	20.0


A
B
C
D


0.25
0.2
0.15
0.1
0.05
0
OD (690 nm)
1
10
100
1000
10000
Log parasite density in haemolymph (x10³ml⁻¹)


A
B
66 kDa
66 kDa
1
2
3
4
5
6
7
8
9

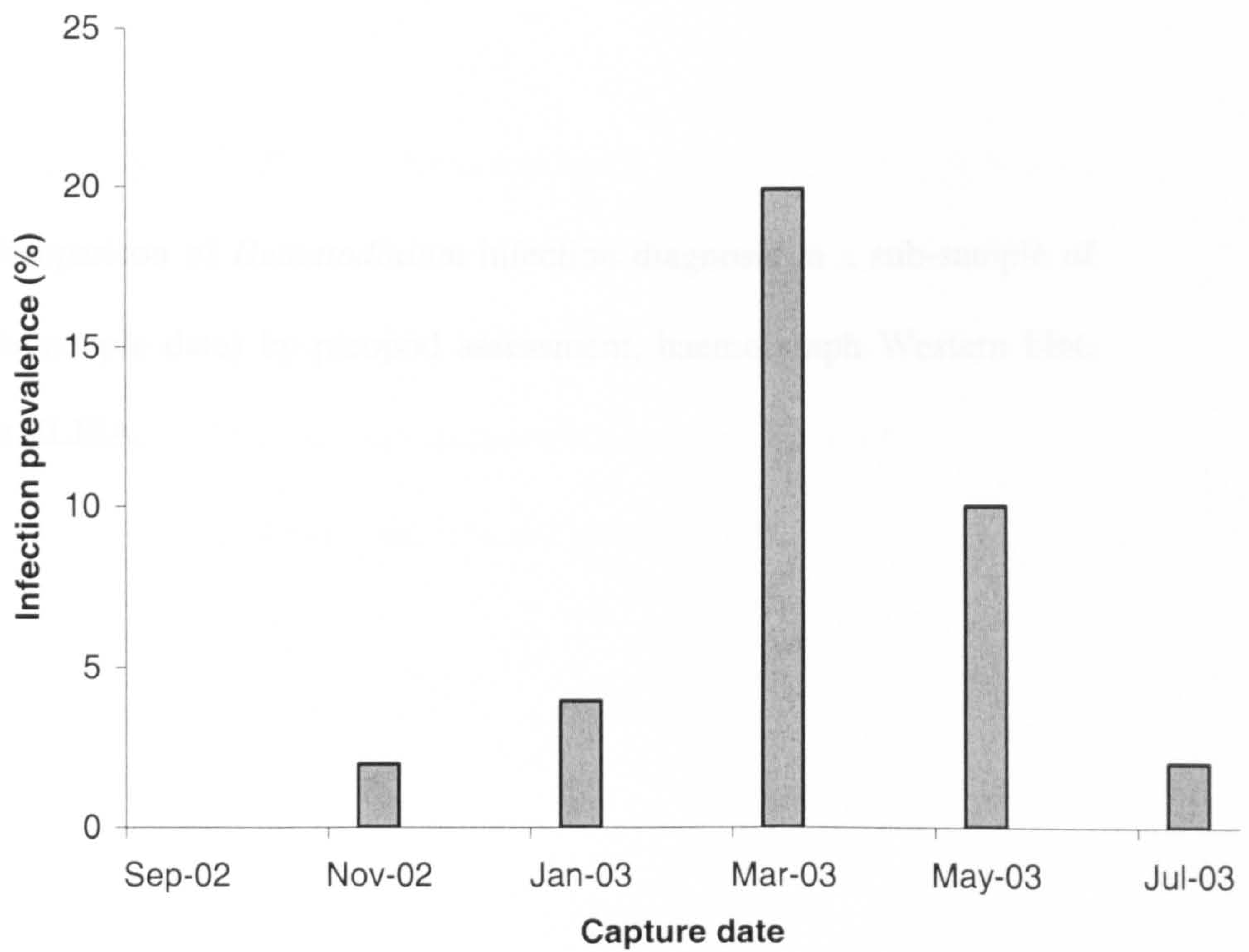

25
20
15
10
5
0
Infection prevalence (%)
Sep-02
Nov-02
Jan-03
Mar-03
May-03
Jul-03
Capture date

Table 2.1 Specificity of polyclonal anti-*Hematodinium* antiserum against other *Hematodinium* and dinoflagellate species.

Table 2.2 Comparison of *Hematodinium* infection diagnosis in a sub-sample of 30 lobsters (each sample date) by pleopod assessment, haemolymph Western blot, and haemolymph ELISA.

Figure 2.1 Section of ELISA plate showing advanced infection (A), low-level infection (B), uninfected (C), and negative control (D) ELISA reactions.

Figure 2.2 Sensitivity of ELISA for *Hematodinium*. Infected haemolymph from an infected Norway lobster was serially 2-fold diluted and assayed by the ELISA. Uninfected haemolymph assayed by the ELISA had a mean OD value of 0.037 with a standard deviation (SD) of 0.008. Data points represent means ± SD (n=6).

* Indicates $P<0.05$ between uninfected and infected haemolymph.

Figure 2.3 Western blots of hepatopancreas samples from *N. norvegicus* from Sweden (A) and *C. pagurus* from the English Channel (B) using anti-*Hematodinium* antiserum. Lanes 1 and 6: molecular weight marker; lanes 2 and 9: positive control infected *N. norvegicus* hepatopancreas; lanes 3 and 8: uninfected *N. norvegicus* and *C. pagurus* samples, respectively; lanes 4 and 5: infected *N. norvegicus* samples; lane 7; infected *C. pagurus* sample.

Figure 2.4 ELISA-derived infection prevalence for sample site in the Clyde Sea Area from bi-monthly haemolymph samples from 50 lobsters.

Chapter 3

Development and application of a PCR-based assay and DNA probes for the diagnosis of *Hematodinium* infection in *N. norvegicus* and in other crustaceans.

3.1 Introduction

Infection of the Norway lobster (*N. norvegicus*) by a parasitic dinoflagellate of the genus *Hematodinium* has been described from a number of locations around the West Coast of Scotland and the Irish Sea (Field *et al.*, 1992; Briggs and McAliskey, 1996; Appleton *et al.*, 1997; Field *et al.*, 1998). Recently, the edible crab *Cancer pagurus* in UK waters has also been found to harbour *Hematodinium* (Stentiford *et al.*, 2002). Parasitic *Hematodinium* species have been reported infecting a number of decapod crustaceans including *Callinectes sapidus* (Newman and Johnson, 1975; Messick, 1994; Messick and Shields, 2000), *Chionoecetes bairdi* (Meyers *et al.*, 1987; Love *et al.*, 1993), *Chionoecetes opilio* (Taylor and Khan, 1995), *Necora puber* (Wilhelm and Mialhe, 1996), *Ovalipes ocellatus* (MacLean and Ruddell, 1978), and *Portunus pelagicus* (Hudson and Shields, 1994). In each of the above examples, infection by *Hematodinium* species has been shown to have serious consequences for the host decapod population.

Diagnosis of infection by *Hematodinium* in the Norway lobster has previously been made by the pleopod method of Field and Appleton (1995), and by a number of immunoassays (Field and Appleton 1996; Stentiford *et al.*, 2001c; Small *et al.*, 2002; Chapter 2 of this thesis). All are useful techniques, but each has drawbacks in terms of ease of use, the low number of samples that can be assayed, or the sensitivity of the assay. In addition, the polyclonal antibody that is used in the immunoassays was raised against an *in vitro* culture of *Hematodinium*, introducing the possibility that the antibody may not recognise other life cycle stages of the parasite that may exist in a host animal.

The expansion of molecular diagnostic methods, such as the polymerase chain reaction (PCR) and DNA probes, which display levels of sensitivity and specificity that are far greater than pre-existing techniques, and detect all the life cycle stages, has permitted the identification of several marine pathogens. These include *Marteilia sydneyi* in the Sydney rock oyster (Kleeman and Adlard, 2000), *Perkinsus marinus* in the Eastern oyster (Reece *et al.*, 1997; Robledo *et al.*, 1999), *Haplosporidium nelsoni* in oysters (Stokes and Burreson, 1995), and *Hematodinium* in decapod crustaceans (Hudson and Adlard, 1994). Hudson and Adlard (1996) found substantial sequence variation within the first internal transcribed spacer (ITS1) of ribosomal DNA (rDNA) among different species of *Hematodinium* infecting *N. norvegicus*, *C. bairdi*, *C. sapidus*, and also other dinoflagellates. This provides a sound basis for the development of specific PCR primers that can be used to identify *Hematodinium* infection in *N. norvegicus* as

the primers used in the Hudson and Adlard (1994) study were based on conserved rDNA sequences (SSU and 5.8S). This chapter describes the use of these in the development and application of a PCR-based assay and DNA probes for the diagnosis of *Hematodinium* infection in *N. norvegicus* and other crustaceans.

3.2 Materials and Methods

3.2.1 Specimen preparation

Norway lobsters *Nephrops norvegicus* were caught as described in section 2.2.1. Haemolymph samples were assayed for the presence of *Hematodinium* by ELISA (Chapter 2). Haemolymph samples were taken from infected lobsters and frozen at –20 °C prior to DNA extraction. For lobsters having a low level infection as indicated by ELISA, a sub-sample of the same haemolymph was used to estimate parasite cell numbers per ml of haemolymph using an Improved Neubauer counting chamber. Tissue samples from infected and uninfected lobsters were retained for DNA extraction and also fixed in Davidson's seawater fixative (20 ml formalin (40 %), 10 ml glycerol, 10 ml glacial acetic acid, 30 ml 100% ethanol, 30 ml seawater) for 24 hours, dehydrated in ethanol and embedded in paraffin wax. Several other samples of haemolymph and tissue from crustacean species common in the UK were also retained for DNA extraction. Tissues from a shore crab *Carcinus maenas* potentially infected with *Hematodinium* from the English Channel were also fixed in Davidson's seawater fixative and embedded in

paraffin. To investigate possible secondary hosts of *Hematodinium*, amphipods (*Orchomene nanus*) were captured in baited traps from the Fairlie channel area of the Clyde Sea Area (55.45.9° N, 4.52.5° W) according to the method of Moore and Wong (1995). Individual *O. nanus* were either frozen at –20 °C or preserved in 100 % ethanol prior to DNA extraction from whole animals.

3.2.2 DNA extraction

Genomic DNA was extracted from 100 µl haemolymph samples and 100 mg tissue samples taken from crustacean species used in this study, and from individual *O. nanus*, according to standard procedures (Sambrook *et al.*, 1989). Briefly, samples of haemolymph, tissue, or whole amphipod, were homogenised in 250 µl extraction buffer (50 mM Tris, 5 mM EDTA, 100 mM NaCl, pH 8), 100 µl of 10 % SDS (w/v) and Proteinase-K (0.28 ng μl^{-1}) and incubated at 56 °C for 18-24 h. DNA from an *in-vitro* culture of *Hematodinium* was also extracted. 5 ml of *in vitro* culture (approx 1 x 10^6 cells) was centrifuged at 1000 x g for 4 min at 4 °C. The resulting cell pellet was resuspended in 250 µl extraction buffer, 100 µl of 10 % SDS (w/v) and 10 µl Proteinase-K (10 µg ml^{-1}) and incubated at 56 °C for 18-24 h. DNA was purified by a single step standard phenol/chloroform (1:1) extraction, precipitated in 550 µl 100% ethanol using 20 µl 5M NaCl, and resuspended in 50 µl sterile deionised water. DNA concentrations and purity were estimated by measuring the 260/280 optical density ratios using a

spectrophotometer (Gene Quant II, Pharmacia Biotech) and adjusted to be between 0.1 and 100 ng for experimental use.

3.2.3 Primary amplification, cloning and sequencing

The first internal transcribed spacer (ITS1) and flanking 3' region of the 18S rDNA complex were amplified independently from two *Hematodinium* genomic DNA templates, using the forward primer 5' GTT CCC CTT GAA GGA GGA ATT C 3' and reverse primer 5' CGC ATT TCG CTG CGT TCT TC 3'. Primer sequences and amplification conditions were as described by Hudson and Adlard (1994). 680 bp amplification products were run on 1.5 % (w/v) agarose gels, stained with ethidium bromide and viewed under UV illumination; images were obtained using a gel documentation system (Appligene). Each amplification product of approximately 680 bp was excised from the agarose gel and purified by the use of a QIA-quick gel extraction kit (Qiagen). Purified amplification products were ligated into the pGEMT-Easy plasmid vector (Promega) (7 μl amplification product, 1 μl vector, 10 μl ligation buffer, 1 μl DTT solution, 1 μl T4 DNA ligase, 2 Hr at 16°C), and used to transformed *Escherichia coli* (strain JM 109) by heat shock (8 μl ligation reaction, 100 μl cell suspension, 42 °C for 45 sec) according to the manufacturer's instructions. Transformed cells were plated onto LB agar (bacto-agar (1.5 % w/v, in LB medium (NaCl, 10 g l^{-1}; bacto-tryptone, 10 g l^{-1}; yeast extract, 5 g l^{-1}, pH 7.5)), containing ampicillin (50 μg ml^{-1}), isopropyl-beta-D-thiogalactopyranoside (IPTG)(40 μg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-

beta-D-galactopyranoside (X-gal)(100 μg ml^{-1}) and grown overnight at 37 °C. Positive transformations were identified by blue/white selection and selected colonies were grown overnight in LB medium containing ampicillin (50 μg ml^{-1}). Recombinant plasmids were purified using a miniprep kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA concentrations and purity were estimated by measuring the 260/280 optical density ratios. Ligation of correct product was confirmed by restriction enzyme digestion (Eco RI) and analysis of products on agarose gels. Bi-directional sequencing of 2 clones from a single PCR reaction from each template was performed by MWG-AG Biotech (Germany).

3.2.4 Primer and probe design

The nucleotide sequences obtained were aligned using the software programmes ClustalX 1.81 (Thompson *et al.*, 1994) and BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Sequences were compared for similarity to those of other dinoflagellates by BLAST in Genbank. Suitable priming regions for PCR exhibiting specificity for the *Hematodinium* sp. from *N. norvegicus* were identified from the sequence alignments (Fig. 3.1), and by comparison with previously published *Hematodinium* sp. sequences (Hudson and Adlard, 1996). Four oligonucleotides, designated 18S F1, 18S F2, 18S R1, and ITS R1 (Table 3.1) were chosen for PCR and construction of PCR-generated DNA probes. These were synthesised commercially (MWG-AG Biotech, Germany).

3.2.5 PCR sensitivity and specificity

The sensitivity of the *Hematodinium*-specific primers 18S F2 and ITS R1 for use in total genomic DNA sample screening by PCR was assessed by serial dilution of a genomic DNA sample from an infected lobster. The number of *Hematodinium* cells in the infected haemolymph sample was estimated using an improved Neubauer haemocytometer, and total DNA was extracted by previously described methods (section 3.2.2). The amplification reaction mixtures contained 0.1-100 ng genomic DNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCL, 0.1 % Triton X-100 (v/v), 1.5 mM $MgCl_2$, 100 μM of dNTPs, 10 pmol each primer, 1 unit of Taq polymerase (Promega), and sterile deionised water to a final volume of 20 μl. Reactions were overlaid with 10 μl of mineral oil. Thermal cycling conditions were as follows: denaturation at 94 °C for 30 sec; primer annealing at 57 °C for 1 min; chain extension at 72 °C for 1 min; repeated for 35 cycles, with a final cycle incorporating a 7 min extension. A 10 μl aliquot of each PCR reaction was checked for amplification products by 1.5 % (w/v) agarose gel electrophoresis and ethidium bromide staining. Images were captured by a UPV gel documentation system.

PCR primers were tested for specificity against genomic DNA samples isolated from *Hematodinium*-infected and uninfected *Nephrops norvegicus* and *Cancer pagurus*, several other crustacean species common to the UK (detailed in Table 3.2), a *Mesanophrys*-like ciliate found infecting *N. norvegicus*, the free-living

dinoflagellates *Gymnodinium catenatum* (CCAP 1117/6) and *Alexandrium tamarense* (CCAP 1119/5), and the protozoan parasites *Leishmania major, Plasmodium falciparum* and *Trypanosoma brucei.*

3.2.6 Verify PCR assay and secondary host investigation

Samples of haemolymph were obtained from *N. norvegicus* sampled from the Clyde Sea Area, the Fladen and North Minch fishing grounds in Scotland, the Irish Sea, and the Swedish Skagerrak fishing grounds. Total genomic DNA from 100 μl haemolymph samples was extracted as described in section 3.2.2, and PCR reaction conditions were the same as described in section 3.2.5. Heart, hepatopancreas, gill and claw tissue samples were taken from low level-infected and advanced-*Hematodinium*-infected lobsters, genomic DNA was extracted from 100 mg tissue samples and the PCR assay was performed under the conditions described above. Total genomic DNA was extracted from individual amphipods (*Orchomene nanus*) and the PCR assay was carried out as described in section 3.2.5.

3.2.7 Labelling of DNA probes

The DNA probes were synthesized by incorporation of digoxygenin-11-dUTP (Roche) during PCR using primer sets 18S F2/ITS R1 and 18S F1/18S R1, and 100 ng of genomic DNA template (extracted from parasite cells from an *in vitro*

culture of *Hematodinium*). Locations and sequences of the primers used to synthesize the probes are given in Table 3.1 and Figure 3.2. Reaction conditions were followed as suggested by the manufacturer, with annealing temperatures of 52 °C and 56 °C (based on G + C content) being used with primers 18S F2/ITS R1 and 18S F1/18S R1, respectively. Incorporation of digoxygenin (DIG) was indicated by an increase in molecular mass as analysed on ethidium bromide-stained agarose gels, and the labelled PCR product was gel-extracted and purified using a QIAquick gel extraction kit (Qiagen). Probe concentration was estimated by side-by-side comparison of a diluted series of the probes and a DIG-labelled control in a spot test on nylon membranes.

3.2.8 DIG-labelled probe specificity

The specificity of Probe 1 (constructed using primers 18S F2 and ITS R1 located in the 18S and ITS1 rDNA regions) was determined by dot-blot hybridisations. Samples of 100 ng (2 μl) of genomic DNA from *Hematodinium*-infected haemolymph from *N. norvegicus*, *C. sapidus* and *C. opilio* were heat-denatured (95 °c, 5 min), spotted onto nylon membranes and fixed by UV crosslinking (120,000 μ joules/ cm^2 for 30 sec). Genomic DNA extracted from *N. norvegicus* itself, and a *Mesanophrys*-like ciliate found infecting *N. norvegicus* were also heat-denatured and spotted onto nylon membranes and fixed by UV crosslinking. Membranes were prehybridised in 10 ml of 2 x SSC (20 x SSC = 3 M NaCl, 0.3 M Na-citrate, pH 7.0), 50 % (v/v) formamide, 5 x Denhardt's solution and 100 µg

ml^{-1} herring sperm DNA at 42 °C for 2 h in 35mm x 300mm glass bottles in a rotating hybridisation oven. The prehybridisation buffer was replaced with 10 ml hybridisation buffer (2 x SSC, 50 % (v/v) formamide, 5 x Denhardt's solution, 100 μg ml^{-1} herring sperm DNA and 1 % Dextran sulphate (v/v)) containing 50 ng ml^{-1} DIG-labelled DNA probe and was incubated with the membrane overnight at 42 °C in 35mm x 300mm glass bottles in a rotating hybridisation oven. Removal of unhybridised probe was achieved by two 5 min washes in 2 x SSC at room temperature and two 15 min washes at 42 °C with 0.1 x SSC (all washing volumes were 20 ml). Following equilibration in 20ml maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5), membranes were blocked for 30 min at room temperature in 10 ml blocking buffer (maleic acid buffer plus 1 % (w/v) blocking reagent: Roche). Membranes were incubated at room temperature with anti-DIG-alkaline phosphatase antibody (Roche) diluted 1:5000 in blocking buffer followed by removal of unbound antibody with two 15 min washes in washing buffer (20 ml, maleic acid buffer plus 0.3 % (v/v) Tween 20). After equilibration in 10ml detection buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the membrane was incubated at room temperature in the dark for 2-6 h in 5 ml 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBT/BCIP) diluted in 5ml detection buffer (1/50 dilution of stock solution). The reaction was stopped with a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) wash. All washing, immunodetection and resolution steps were performed in plastic cassettes.

3.2.9 *In situ* hybridisation

Paraffin-embedded tissue sections from *Hematodinium*-infected *N. norvegicus* and *C. maenas* were cut at 6 µm, placed on salinized slides and baked for 45 min at 60 °C. Sections were deparaffinized (xylene, 2 min), rehydrated in an ethanol series (1 min each ethanol grade, 100, 90 and 70%), washed in distilled water and permeabilized with 10-50 µg ml^{-1} Proteinase-K (0.5 ml) in TNE buffer (50 mM, Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.4) for 30 min at 37 °C in a humid chamber. Proteolysis was inactivated by two 1 min washes in 20ml 1 x PBS followed by equilibration in 20ml 2 x SSC. Samples were prehybridised in 500 µl prehybridisation buffer (see section 3.2.8 for details of prehybridisation and hybridisation buffers) in a humid chamber for 60 min at 37 °C. The prehybridisation buffer was replaced with 50-100 µl hybridisation buffer containing 0.1 ng μl^{-1} heat-denatured DIG-labelled probe. After applying glass coverslips, sections were placed on a heating block at 95 °C for 5 min to denature the target DNA, then immediately put on ice for 5 min and allowed to hybridise overnight in a humid chamber at 42 °C. Post-hybridisation washes included 2 x SSC at room temperature, twice for 5 min, and 0.1 x SSC at 42 °C once for 10 min, followed by equilibration in maleic acid buffer (all 20 ml, see section 3.2.8). Sections were blocked with 500 µl blocking buffer (see section 3.2.8) at 37 °C for 15 min followed by incubation for 1 h at 37 °C with 500 µl of dilute anti-DIG-alkaline phosphatase antibody (Roche) diluted 1:1000 in blocking buffer.

Unbound antibody was removed by two 5 min washes in 20 ml washing buffer followed by one 5 min wash in 20ml detection buffer (see section 3.2.8). NBT/BCIP was diluted (1/50 dilution of stock solution) in detection buffer and 200 µl added to sections and incubated at room temperature in the dark for 2-6 h. The reaction was stopped with a 20 ml TE buffer wash. Slides were washed in double distilled H_2O and counterstained with 1 % (w/v) eosin for 1 min, followed by ethanol dehydration (1 min each ethanol grade, 100, 90 and 70%), and mounted in aqueous mounting medium (histomount). Hybridisation conditions were optimised by varying the concentration (10-50 µg ml^{-1}) and length of incubation (15-60 min) of Proteinase-K, and the concentration of DIG-labelled DNA probes (0.1-1 ng μl^{-1} heat-denatured DIG-labelled probe). Negative controls included samples without the addition of DIG-labelled probe and uninfected tissue sections.

3.3 Results

3.3.1 *Hematodinium* rDNA sequences

Efficient amplification of *Hematodinium* ribosomal DNA was achieved using the nucleotide primers previously described by Hudson and Adlard (1994). The 680 bp products from PCR amplifications (using template DNA samples from the *Hematodinium in vitro* culture and infected haemolymph) were successfully cloned into a plasmid vector, sequenced and aligned (Fig. 3.1). The 3' end of the

18S gene was almost conserved between both isolates, apart from one nucleotide variation at position 213 bp. The 5' region of ITS1 showed a number of nucleotide variations between the isolates, and this sequence variation is addressed in Chapter 4.

3.3.2 PCR primer design, sensitivity and specificity

A new forward primer was synthesised (18S F2) which was specific to an area within the V9 domain of the 3' end of the 18S gene (80-102 bp upstream of the 18S/ITS1 boundary) (Fig 3.1). The V9 domain had previously been shown to be highly conserved between *Hematodinium* species when compared with other dinoflagellates (Hudson and Adlard, 1996). A new reverse primer was also synthesised (ITS R1), specific to an area within the ITS1 sequence 256-277 bp downstream of the 18S/ITS1 boundary. The positions and sequences of primer sets are shown in Figures 3.1, 3.2 and Table 3.1. Amplification of DNA from *Hematodinium*-infected *N. norvegicus* haemolymph using the primer pair 18S F2 and ITS R1 led to the production of a diagnostic band of 380 bp from 1 ng or more DNA (Fig. 3.3). This band was not produced using a sample of 100 ng DNA from uninfected haemolymph. Based on initial cell counts of the parasite numbers in the haemolymph, 1 ng genomic DNA is equivalent to 6 parasite cells. The *Hematodinium*-specific PCR primer pair did not generate a PCR product of appropriate size when any of the genomic DNA preparations from two dinoflagellate species, a *Mesanophrys*-like ciliate found infecting *N. norvegicus*,

several protozoan parasites and a number of crustacean species were used in the PCR assay (Table 3.2). However the primer pair did generate an appropriate signal when PCR reactions were performed with genomic DNA templates prepared from *Hematodinium*-infected *N. norvegicus* haemolymph and *Hematodinium*-infected *C. pagurus* hepatopancreas tissue.

3.3.3 Verify PCR assay for *Hematodinium*

Hematodinium infection in *N. norvegicus* haemolymph samples from the Clyde Sea Area, N. Minch and Fladen were detectable by PCR (Fig. 3.4). *Hematodinium* infection was also detected in *N. norvegicus* haemolymph samples from the Irish Sea and the Swedish Skagerrak. Variation in PCR product intensity was observed between haemolymph samples from the different locations, probably reflecting differences in parasite loading of the samples (as rDNA sequences were identical over the oligonucleotide primer regions used, see Ch 4). To assess the ability of the PCR-based assay to detect *Hematodinium* in tissues of infected lobsters, genomic DNA preparations from heart, hepatopancreas, gill and claw tissue were evaluated by the PCR assay. Claw tissue consistently produced a very intense PCR product from both low-level and advanced infections (Fig. 3.5).

3.3.4 *Hematodinium* infection in *Orchomene nanus*

Amphipods (*O. nanus*) were collected from the Hunterston channel in the Clyde Sea Area on August 2001, March 2003 and August 2003. Genomic DNA samples were prepared from each animal and assayed by PCR for the presence of *Hematodinium.* The PCR assay on the August 2000 samples indicated that 5/13 amphipods were potentially infected with the *Hematodinium* parasite (Fig. 3.6), however, when this was repeated with a larger sample number obtained in March 2003 and August 2003 no indication of infection was observed by PCR (Table 3.3).

3.3.5 DNA Probe 1 specificity

Using dot-blot hybridisations, 50 ng ml^{-1} DIG-labelled DNA Probe 1 produced a strong signal for *Hematodinium* species from *N. norvegicus.* A very slight signal was produced for *Hematodinium* samples from *C. sapidus* and *C. opilio* (Fig. 3.7). However, no signal was observed for DNA samples from *N. norvegicus* or from a *Mesanophrys*-like ciliate infecting lobsters.

3.3.6 *In situ* hybridisation

DNA Probes 1 and 2 hybridised to parasite cells present in paraffin-embedded myocardial heart tissue sections, prepared from *Hematodinium*-infected *N.*

norvegicus (Fig. 3.8). There was negligible background hybridisation observed for both DNA Probes used. A marked increase in signal intensity was observed when using Probe 2 compared to Probe 1 against parasites in heart tissues (Fig. 3.8), and several other *Hematodinium*-infected *N. norvegicus* tissues. Alteration of DIG-labelled Probe 1 concentration and incubation time did not enhance hybridisation and, as a result, signal intensity. Because of this, only Probe 2 was used in further hybridisation studies. No signal was observed for negative control samples without DNA Probe 2 in the hybridisation buffer (Fig. 3.9). Probe 2 hybridised well with parasite cells in the haemal space of the hepatopancreas and gill filaments from infected *N. norvegicus* (Fig. 3.10). Probe 2 also hybridised well to presumptive *Hematodinium* cells present in haemal space of the hepatopancreas and gill filament tissue sections from *C. maenas* (Fig. 3.11).

3.4 Discussion

Methods developed for the diagnosis of *Hematodinium* infection in the Norway lobster include the examination of lobsters for signs of gross infection by carapace discoloration, aggregation of parasite cells in the pleopods, and several immunoassays. In this study molecular probes were developed and applied for the diagnosis of *Hematodinium* infection in *N. norvegicus* and other crustaceans. The rDNA gene repeat was chosen as target region for the design of molecular probes based on Hudson and Adlard's (1996) finding that there was a significant degree of sequence variation between *Hematodinium* sp. However, the rDNA sequences

obtained were never deposited in GenBank or any other databases, hence amplification and sequencing of *Hematodinium* isolates from *N. norvegicus* was carried out as part of this study. Cloning of the 3' end of the 18S and 5' end of the ITS1 rDNA genes from *Hematodinium* species infecting *N. norvegicus* revealed that the 3' end of the 18S gene was conserved (apart from one polymorphic nucleotide site) between the two isolates used in the study, but the ITS1 sequences showed some nucleotide variation. This issue is addressed in Chapter 4. The primer set 18S F2 and ITS R1 efficiently amplified parasite DNA in the presence of host DNA, resulting in the production of a diagnostic band of 380 bp from genomic DNA samples of at least 1 ng, equivalent to 6 parasite cells. The PCR assay was further validated by amplification of parasite DNA from samples of *Hematodinium*-infected *N. norvegicus* haemolymph from geographically separate areas in UK waters, and from Sweden. The differences in PCR product intensity (Fig. 3.4) may represent different levels of infection, or a different seasonal pattern of infection from the Clyde Sea Area, as the host moult period has been implicated in *Hematodinium* infection seasonality (Field *et al.*, 1992), and *N. norvegicus* moulting is thought to vary between geographical location in UK waters (J. Atkinson, personal communication). Sequence analysis in Chapter 4 confirms that no substantial intraspecific variation of parasite rDNA occurs between the sample locations, which would affect oligonucleotide binding and PCR amplification success. The primers used did not produce any amplification signal when DNA templates prepared from several other crustacean species were used, indicating that these primers can be used to investigate whether the

Hematodinium species infecting *N. norvegicus* is found in other crustaceans. The finding that genomic DNA samples derived from claw tissue repeatedly gave a very strong PCR product from both low-level and advanced infections suggests that this should be the tissue of choice to use when conducting infection prevalence screening in *N. norvegicus* using the PCR assay. Furthermore, it also suggests that the parasite may reside in claw muscle tissues during early infection, before entering the haemolymph and infecting other tissues.

The DIG-labelled DNA Probe 1 constructed using the same primers as used for the PCR assay spanning the 18S and ITS1 rDNA regions is a sensitive probe that is able to selectively detect *Hematodinium* DNA from *N. norvegicus*, compared with that of *Hematodinium* sp. from *C. sapidus* or *C. opilio*. However, the level of *Hematodinium* infection in samples of *C. sapidus* or *C. opilio* was not quantified, and the apparent probe specificity may be due to parasite DNA template availability. During *in situ* hybridisation the probe was able to localise individual parasites in lobster tissues. However, the signal from the probe was weak and could not be improved by either incubating the section with a higher concentration of probe or by increasing the incubation time. In contrast to this, Probe 2 constructed using primers 18S F1 and 18S R1, which amplify conserved 18S rDNA only, gave a very intense signal when hybridising to parasite cells within paraffin-embedded sections suing the same reaction conditions at Probe1. Kleeman *et al.* (2002) reported variations in sensitivity and signal intensity between different 18S/ITS1-based DIG-labelled probes for *Martelia sydneyi* and

M. refringens. This suggests that the observed difference in signal intensity between *Hematodinium* Probes 1 and 2, may reflect the availability of target sequence, since ITS regions are excised from the mRNA in the cell cytoplasm prior to ribosomal construction, and as such are not available for probe hybridisation. Alternatively, the shorter length of Probe 2 may assist in increased binding to target sequences and result in an increased signal. Probe 2, based on 18S rDNA conserved between all species of *Hematodinium*, efficiently hybridised to parasites in the hepatopancreas and gill tissues of *N. norvegicus* and *C. maenas*. Consequently, Probe 2 could be used to confirm and investigate *Hematodinium* infections in a wide range of crustaceans once possible cross reactivity with host tissues are eliminated.

The PCR primers developed were used to investigate *O. nanus* as a possible secondary host or transmission vector for *Hematodinium*. This amphipod species is known to be a generalist scavenger, with a preference for crustacean carrion (Moore and Wong, 1995), and as such will feed on deceased *N. norvegicus* with large numbers of parasite cells present. Furthermore, the lack of success in transmission experiments with cultured *Hematodinium* cells and infected haemolymph (Vickerman, 1994) indicates that an undiscovered intermediate host may be required for completion of the parasite life cycle and its ability to infect *N. norvegicus* (Appleton and Vickerman, 1998). Some amphipods are known to predate on crustaceans, alive or dead (Templeman, 1954; Scarratt, 1965), and have previously been reported to be infected by dinoflagellates (Johnson, 1986).

Messick and Shields (2000) also suggested that several amphipods collected during prevalence studies for *Hematodinium* in *C. sapidus* were potentially infected with this parasite. Analysis of samples of *O. nanus* collected from the Clyde Sea Area and assayed by PCR revealed that in August 2001, 5/13 amphipods were positive for the presence of *Hematodinium*. However, no individual amphipods were retained for *in situ* hybridisation to localise the parasite. The positive signal produced by PCR was most probably produced by infective *Hematodinium* cells in *O. nanus* tissues as opposed to the gut contents, as amphipods were held alive for 72 h in order for the stomach contents to be purged. However the possibility of parasite cells adhering to the exterior surface of the amphipod cannot be ruled out. When amphipod sampling and the PCR assay were repeated in March 2003 and August 2003, no *Hematodinium* DNA could be detected.

The combined use of the PCR primers and DNA Probe 2 will prove valuable in elucidating the life cycle of *Hematodinium* in *N. norvegicus*. Several life history stages of *Hematodinium* from *N. norvegicus* have been described from *in vitro* cultures (Appleton and Vickerman, 1998), but of these only a few forms have been observed during natural infection in the lobster. It has been suggested by Appleton and Vickerman (1998), that ingestion of *Hematodinium* dinospores takes place during suspension feeding by the lobster (Loo *et al.*, 1993), and that initiation of infection takes place after penetration of the gut wall by the dinospores. Others have suggested that damaged cuticle tissues of crustaceans

during moulting may be the site of parasite entry (Eaton *et al.*, 1991). The portal of entry of PKX (Phylum Myxozoa) in salmonids has recently been identified by *in situ* hybridisation (Morris *et al.*, 2000), supporting the use of DNA probes developed for *Hematodinium* to address this question by looking at low-level naturally and experimentally infected lobsters, and also to identify life history stages previously unseen.

Table 3.1 Oligonucleotide primer sequences and annealing positions, designed to bind to *Hematodinium* 18S and ITS1 regions of the rDNA gene complex for use in PCR and construction of DNA probes.

Table 3.2 Use of *Hematodinium*-specific PCR primer set 18S F2 and ITS1 R1 against other dinoflagellate, ciliate, protozoa and decapod crustacean DNA samples. + = Single amplification product of 380 bp; - = no amplification product or amplification product of incorrect size.

Table 3.3 Comparison of possible *Hematodinium* infection prevalence in secondary host *O. nanus* assessed by the PCR assay.

Primer	Sequence 5'-3'	Position	Purpose
18S F1	GTTCCCCTTGAAGGAGGAATTC	216-238 bp upstream 18S/ITS1 boundary	Probe 2
18S F2	CAGTTTCTGGAAGTGGCAGCTG	80-102 bp upstream 18S/ITS1 boundary	PCR and probe 1
18S R1	AGCTGCCACTTCCAGAAACT	81-101 bp upstream 18S/ITS1 boundary	Probe 2
ITS R1	GAAGGGAAGGGGAGAAGAAGC	256-277 bp downstream 18S/ITS1 boundary	PCR and probe 1

Genomic DNA template	PCR Diagnosis
Alexandrium tamarense CCAP 1119/5	-
Gymnodinium catenatum CCAP 1117/6	-
Mesanophrys-like ciliate	-
Plasmodium falciparum 3D7	-
Trypanosoma equiperdium wt	-
Leishmania major wt	-
Carcinus maenas	-
Necora puber	-
Cancer pagurus	-
Maja squinado	-
Liocarcinus depurator	-
Pagurus bernhardus	-
Hematodinium-infected *N. norvegicus*	+
Hematodinium-infected *C. pagurus*	+

Date (mo/yr)	Infection prevalence in *O. nanus* by PCR
08/2001	5/13
03/2003	0/40
08/2003	0/40

Figure 3.1 Alignment of amplified *Hematodinium* nucleotide sequences consisting of (A) the 3' end of the 18S and (B) the 5' end of the first internal transcribed spacer (ITS1) region of the ribosomal DNA gene complex from 2 isolates. Isolate NnHem1; *Hematodinium* from a continuous *in-vitro* culture first isolated in 1992, NnHem2; *Hematodinium*-infected *N. norvegicus* haemolymph sample from 2000. Nucleotide region underlined in 18S sequence indicates V9 domain. Bold shading indicates PCR primers 18S F2 and ITS R1. Dots represent conserved nucleotide, dashes represent missing nucleotide.

Figure 3.2 Diagram showing position of oligonucleotide primers for use in PCR and construction of DNA probes.

A

```
NnHem1   GTTCCCCTTGAAGGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGTGCTGATTACGTC
NnHem2   ............................................................

         CCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAGTGATCCGGTGAATAATTC
         ............................................................
                                 18S F2
         GGACGGCAGCCTTTTCCAGTTTCTGGAAGTGGCAGCTGGAAGTTTAGTGAACCTTATCAC
         ............................................................

         TTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
         ................................A.........................
```

B

```
NnHem1   CGCACGAATAATCAATAAAAAACACCGTGAACCTTGGCCATTAGCACGAGCAAAAAAGCG
NnHem2   ............................................................

         CATGCGCATGCTGCATGCCCCCGCCGCCGCCGCCGCCTCCGCTGTGTGTGTGTGTGTGTG
         ............................................................

         --GGGGTGTTTGTGTGTGCGCGTTCGTGCTACTAAGGGCTGTGAGAGATGGGGAACCACC
         TG...........................................T..............

         TCTCCAAATATTTCTCCAGGCCCACGTTTGTTTTCCTTATAATAACTCTCTAATTTCACT
         ...................-........................................
                                   ITS R1
         TATTCAATTATAACTAAGCTTCTTCTCCCCTTCCCTTCTTCGTCCAGAAGAAGAAGAAGG
         ............................................................

         AGGAGGAGGAGGAGGAGGGAGGCTATATATATAATTTTCAATTTAGAAA
         G...................A.T..........................
```

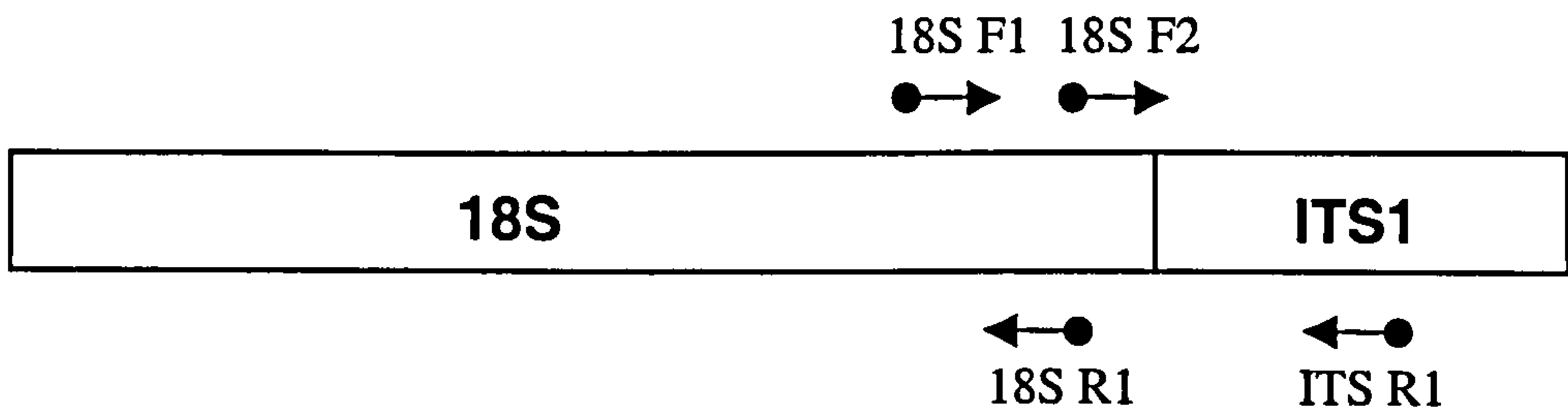
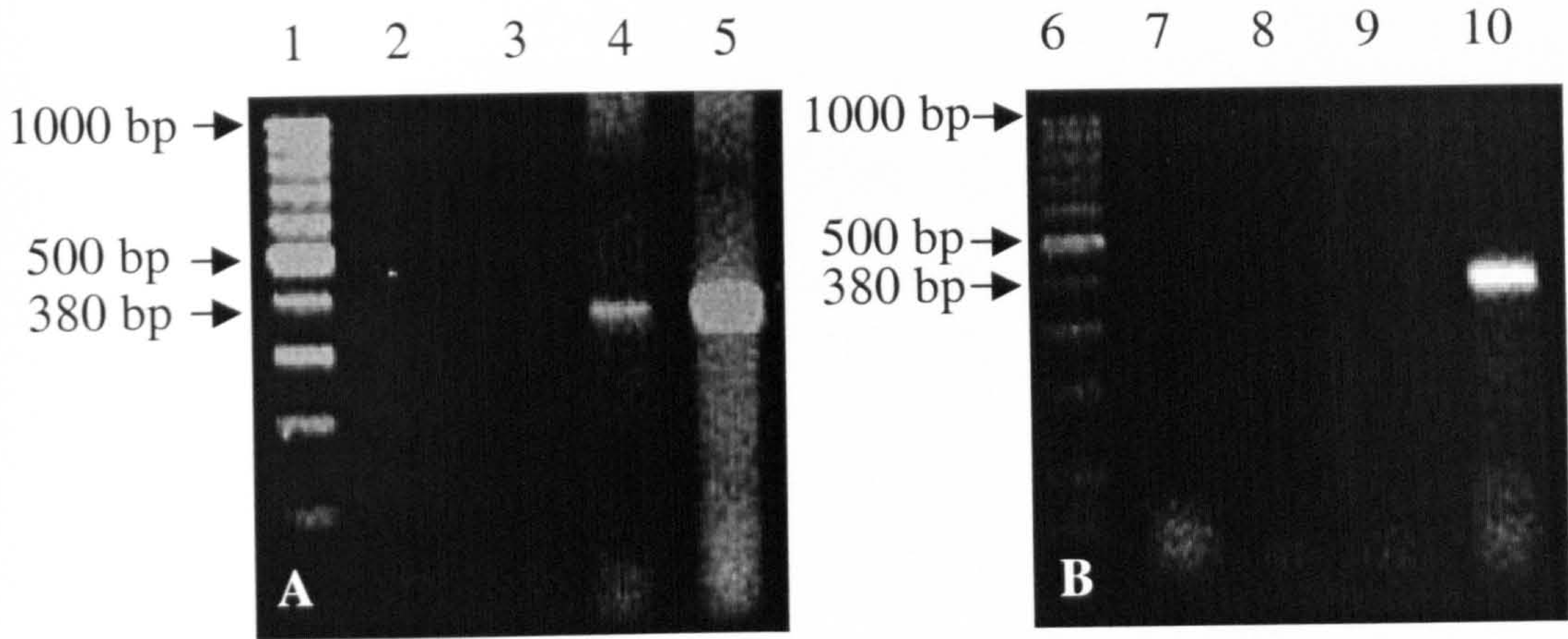
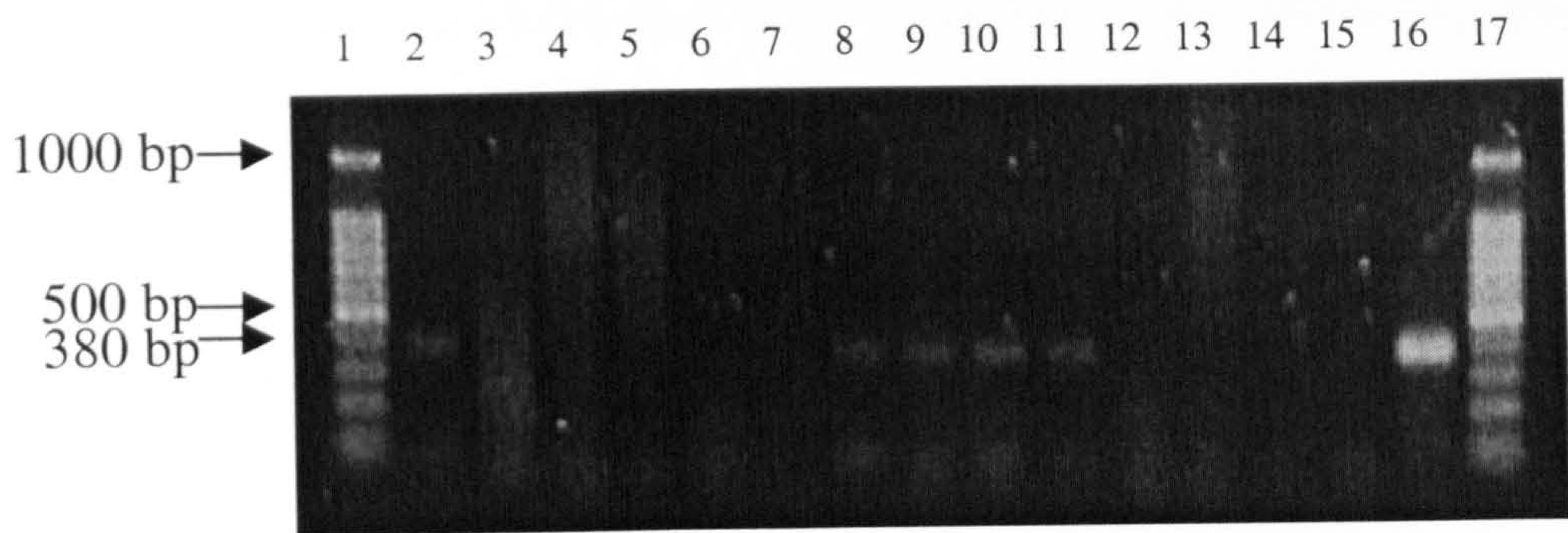


Figure 3.3 Sensitivity of PCR assay for detection of *Hematodinium* infecting *N. norvegicus*. Lane 2: 100 ng μl^{-1} *N. norvegicus* host DNA control; Lanes 3-7: Infected haemolymph DNA template concentrations, 100, 50, 10, 1, 0.1 ng μl^{-1}; Lanes 1 and 8: 100 bp molecular weight marker.

Figure 3.4 Verification of PCR assay on *Hematodinium*-infected *N. norvegicus* haemolymph samples from different geographical locations. Lane 1, 100 bp molecular weight marker; lanes 2-4, 100 ng μl^{-1} total genomic DNA from the Clyde Sea, Fladen and North Minch, respectively. Lane 5, *N. norvegicus* host DNA control (100 ng μl^{-1}); lane 6, *Hematodinium* DNA control from *in vitro* culture (50 ng μl^{-1}).


1 2 3 4 5
6 7 8 9 10
1000 bp
500 bp
380 bp
1000 bp
500 bp
380 bp
A
B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
1000 bp
500 bp
380 bp

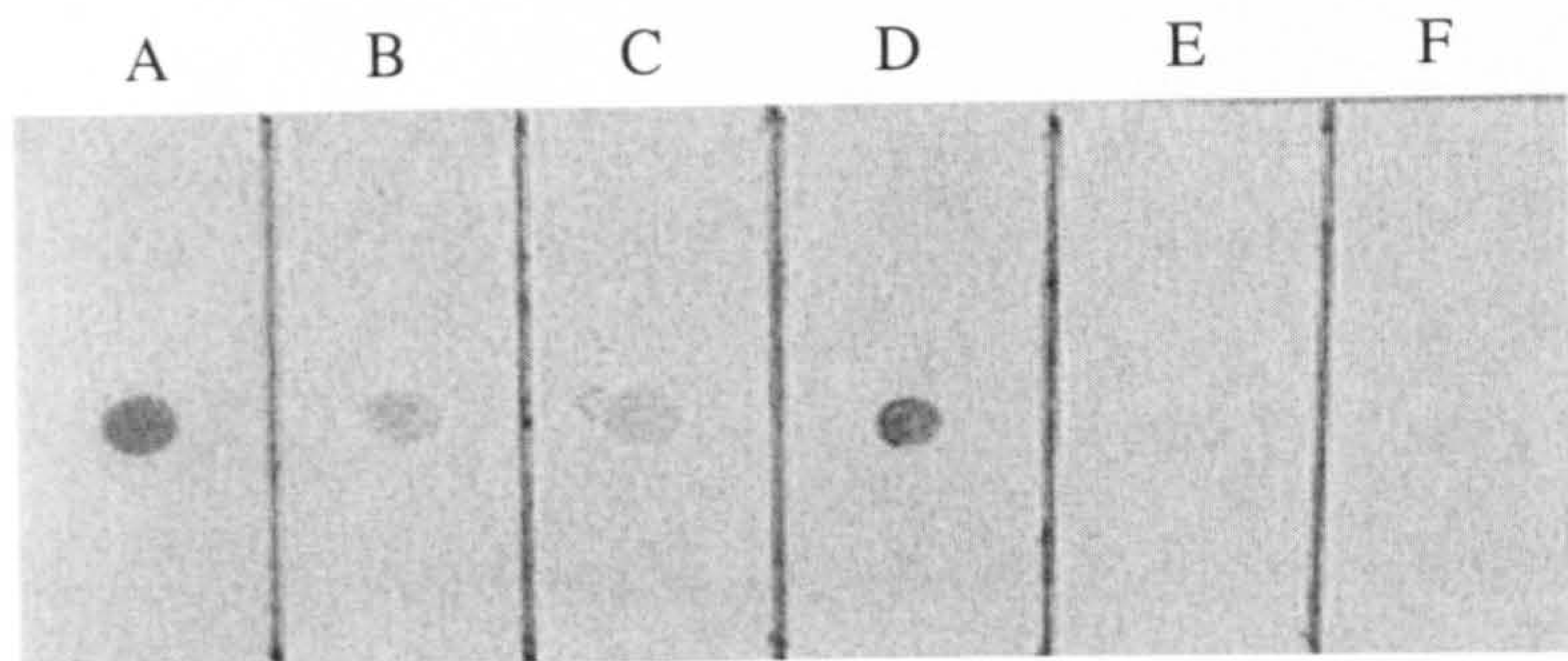
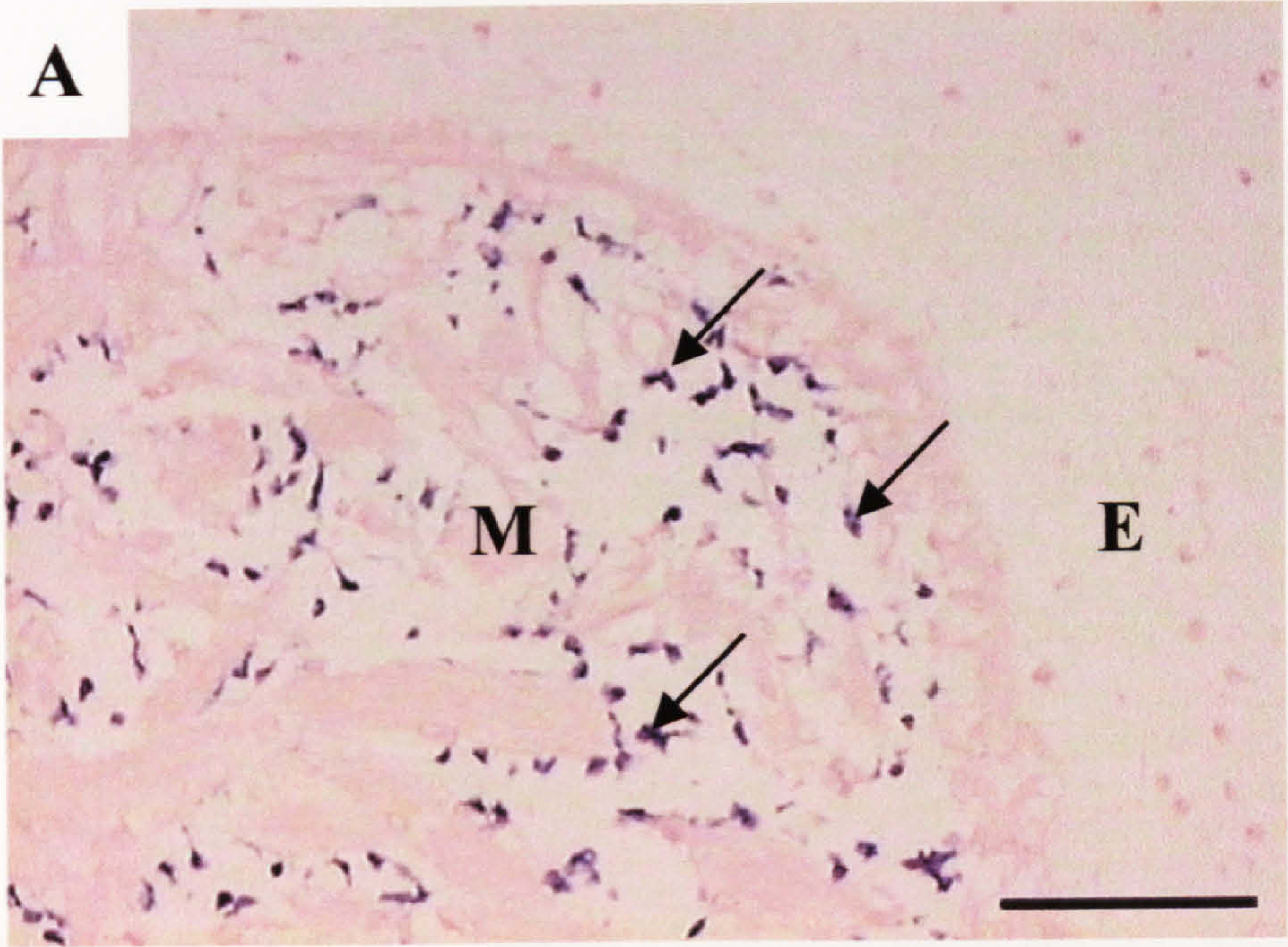

A B C D E F

Figure 3.8 Hybridisation of Probe 1 (A) and Probe 2 (B) to *Hematodinium* parasites (arrows) in myocardial heart tissue sections of *N. norvegicus*. M = myocardium; E = epicardium. Scale bar 100 µm.


A
M
E

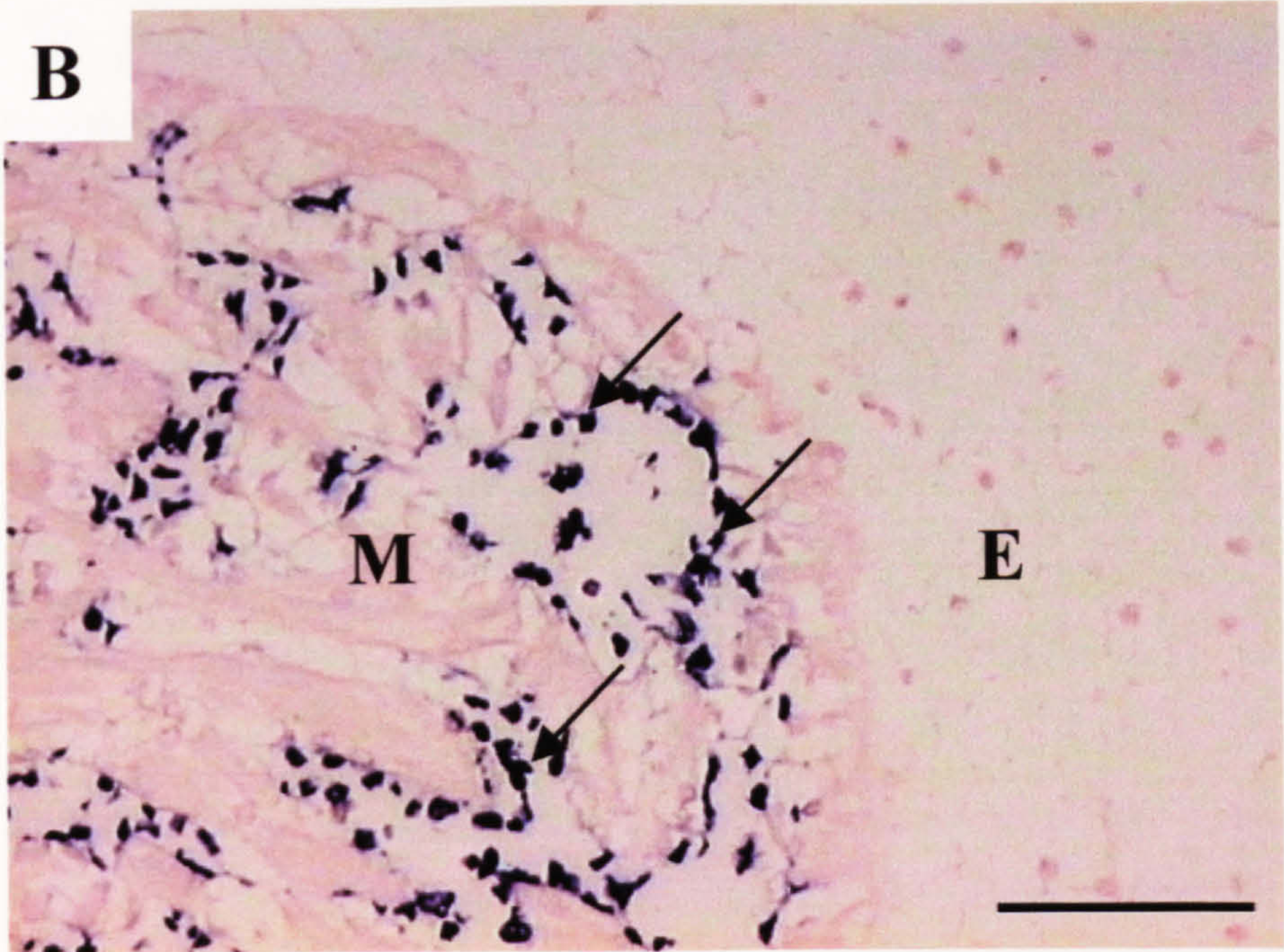
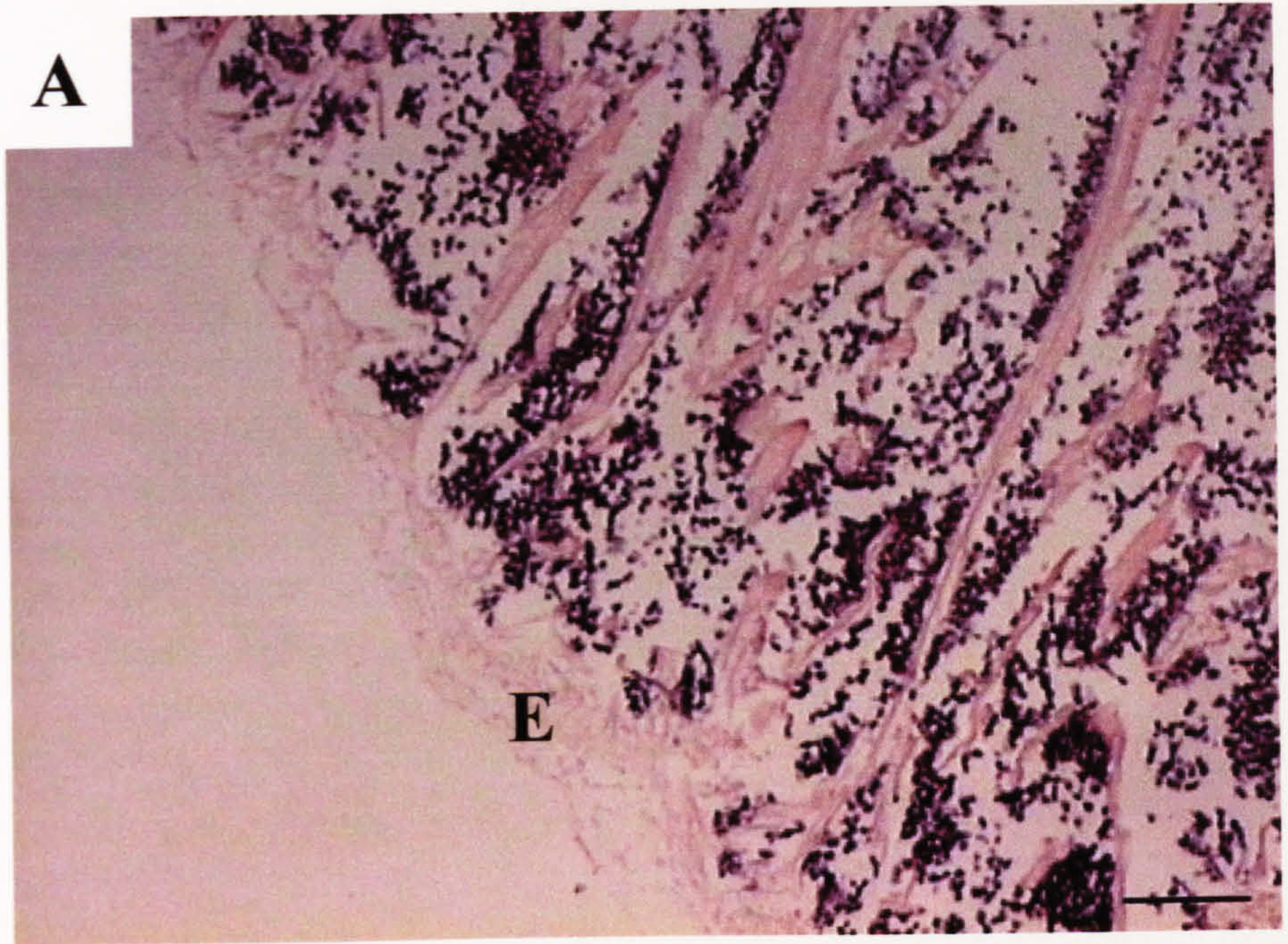

B
M
E

Figure 3.9 *In situ* hybridisation control reactions on *Hematodinium*-infected *N. norvegicus* heart tissue sections when Probe 2 is present (A) and absent (B) from hybridisation buffer. M = myocardium; E = epicardium. Scale bar 100 μm.


A
E

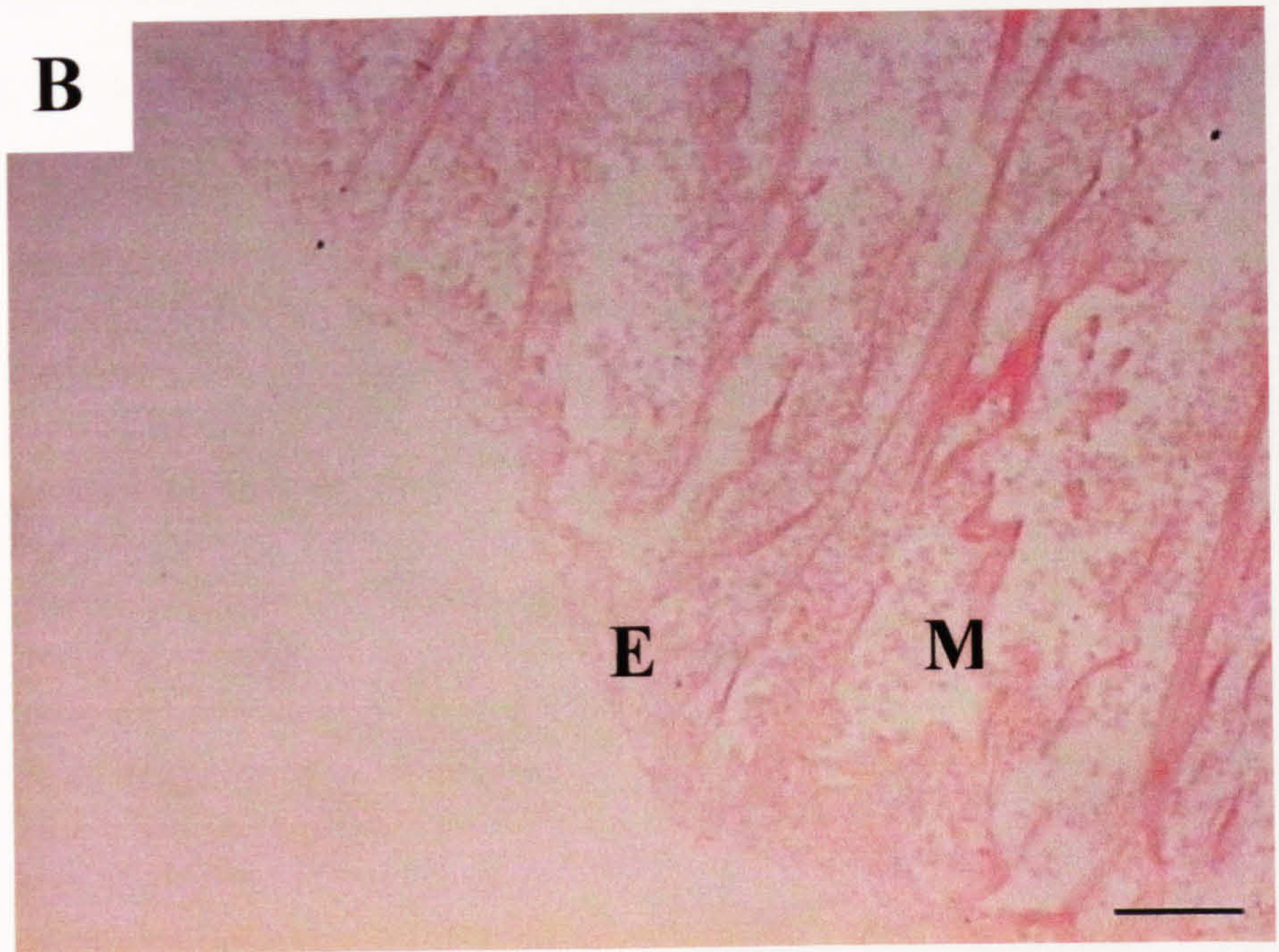
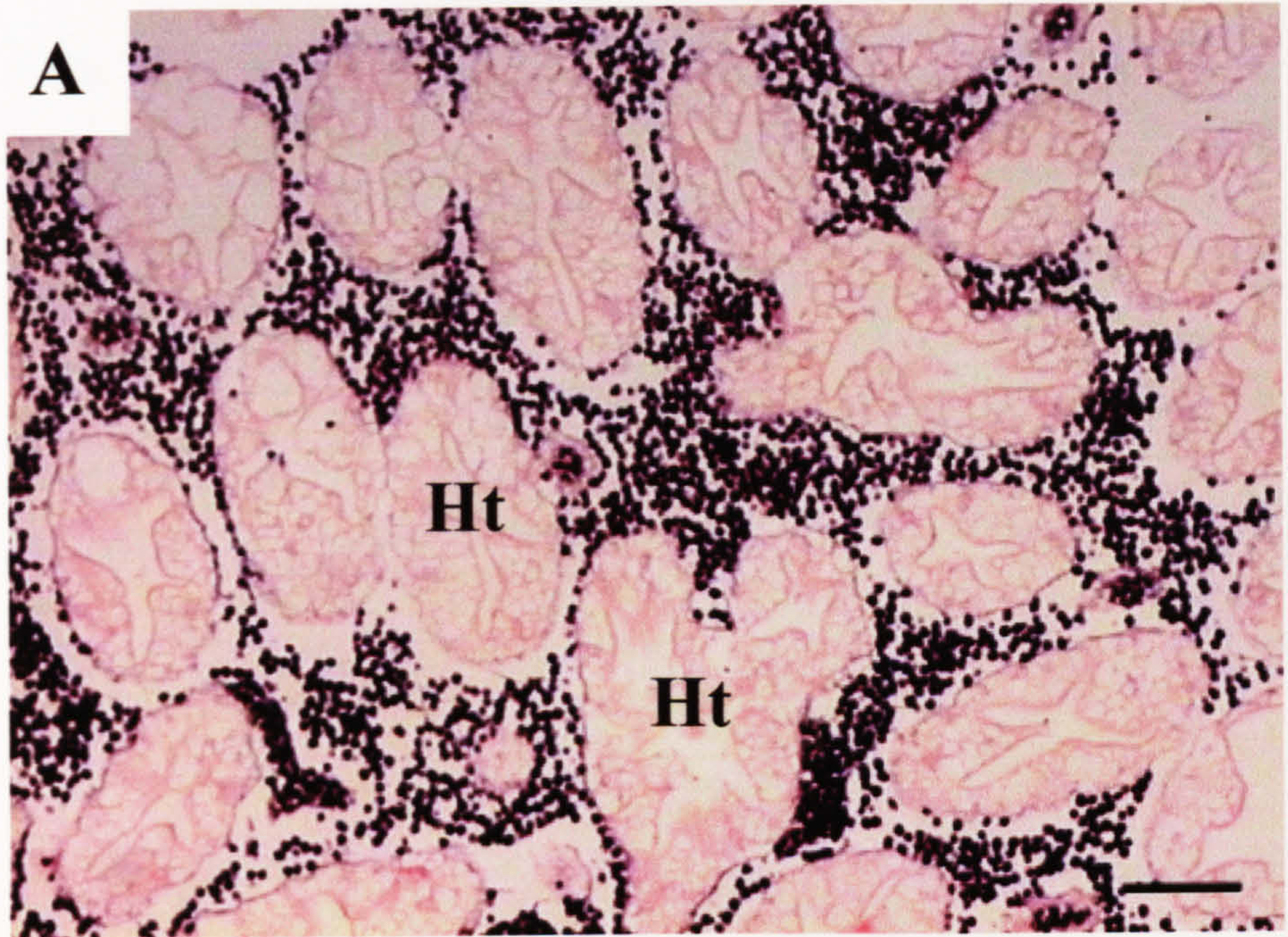

B
E
M

Figure 3.10 Hybridisation of Probe 2 to *Hematodinium* cells in the haemal space of hepatopancreas (A) and in the gills (B) of *N. norvegicus* tissue sections. Ht = hepatopancreatic tubule; arrows = parasite cells. Scale bar 100 μm.


A
Ht
Ht

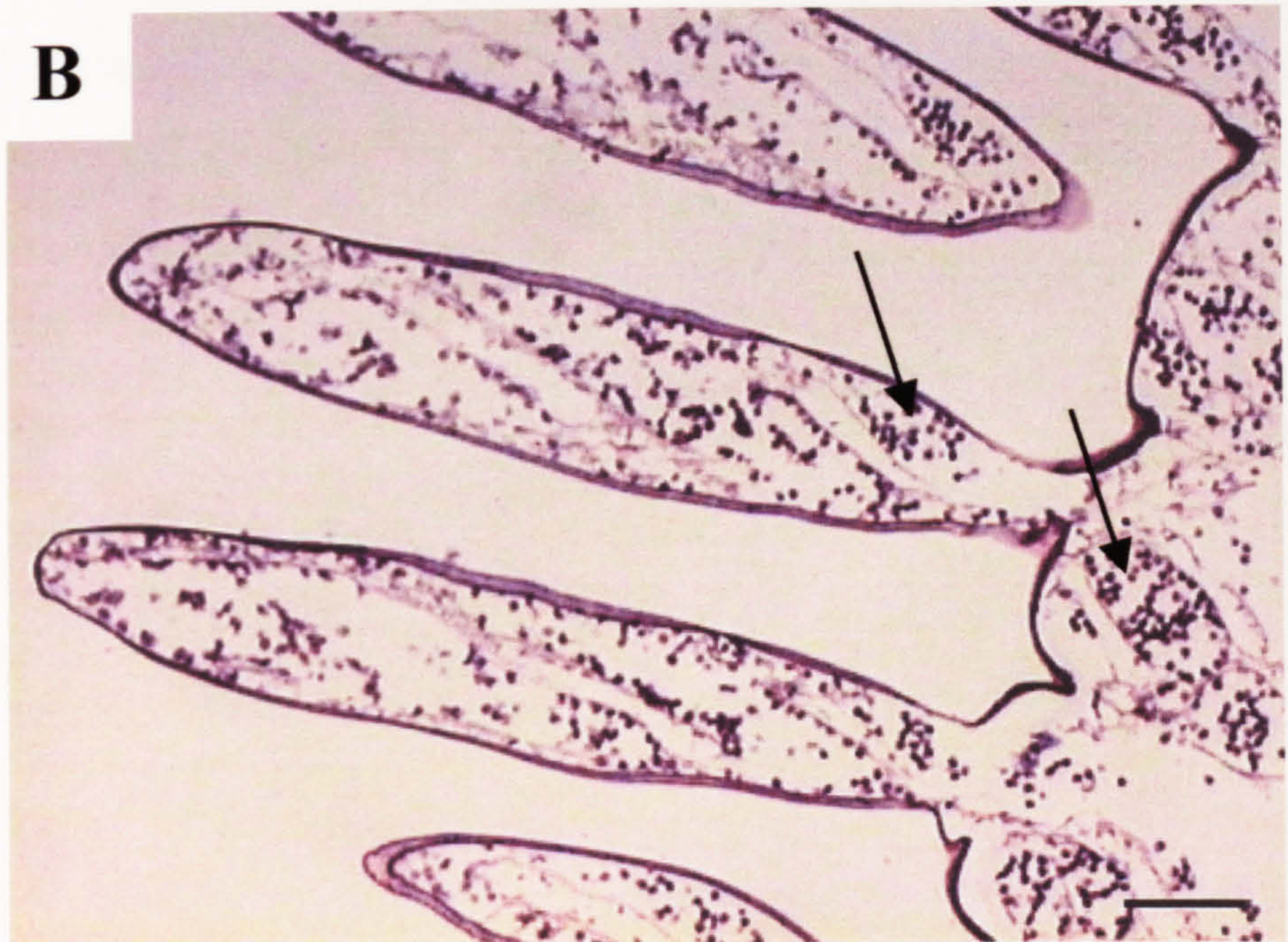
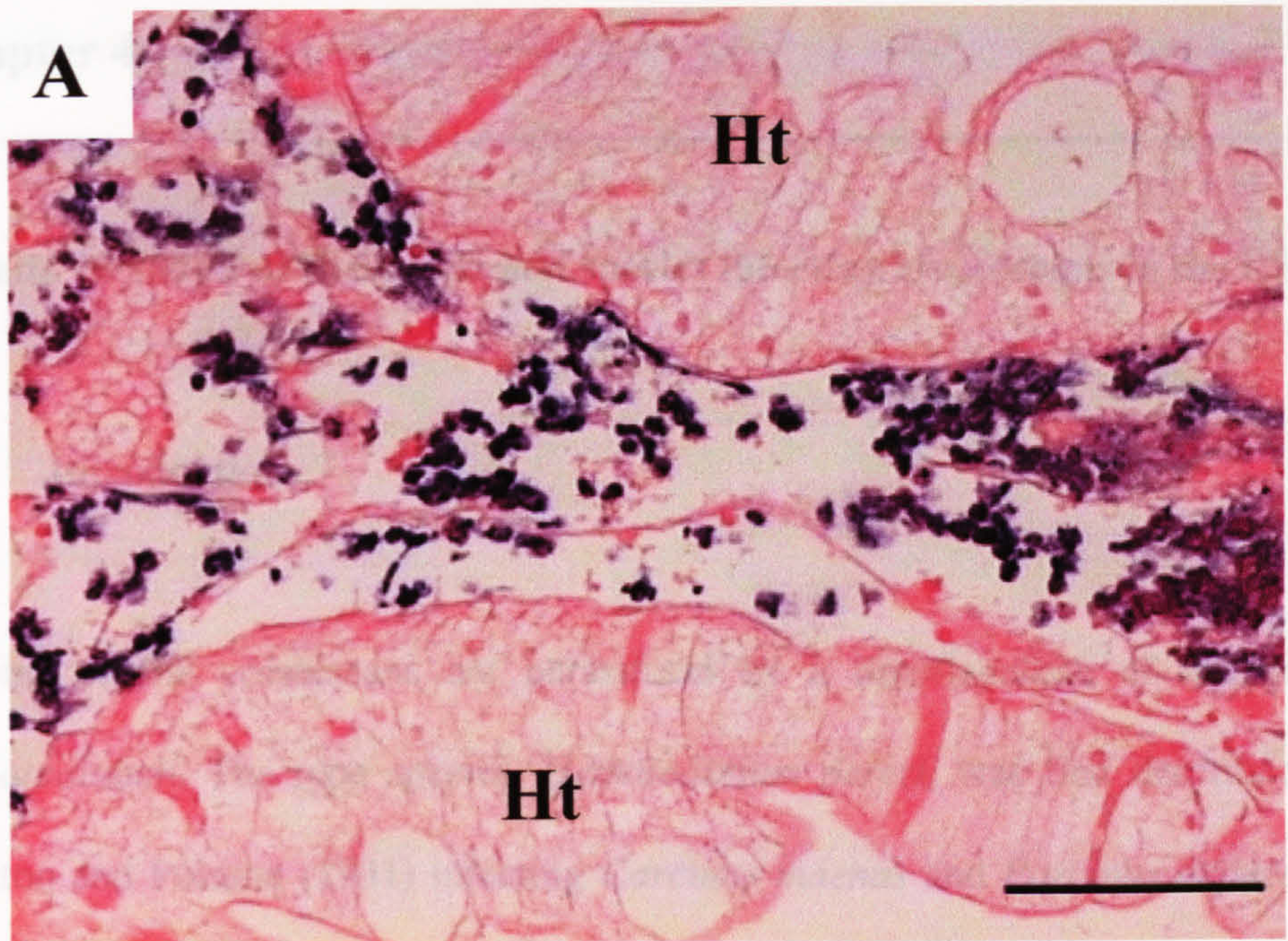
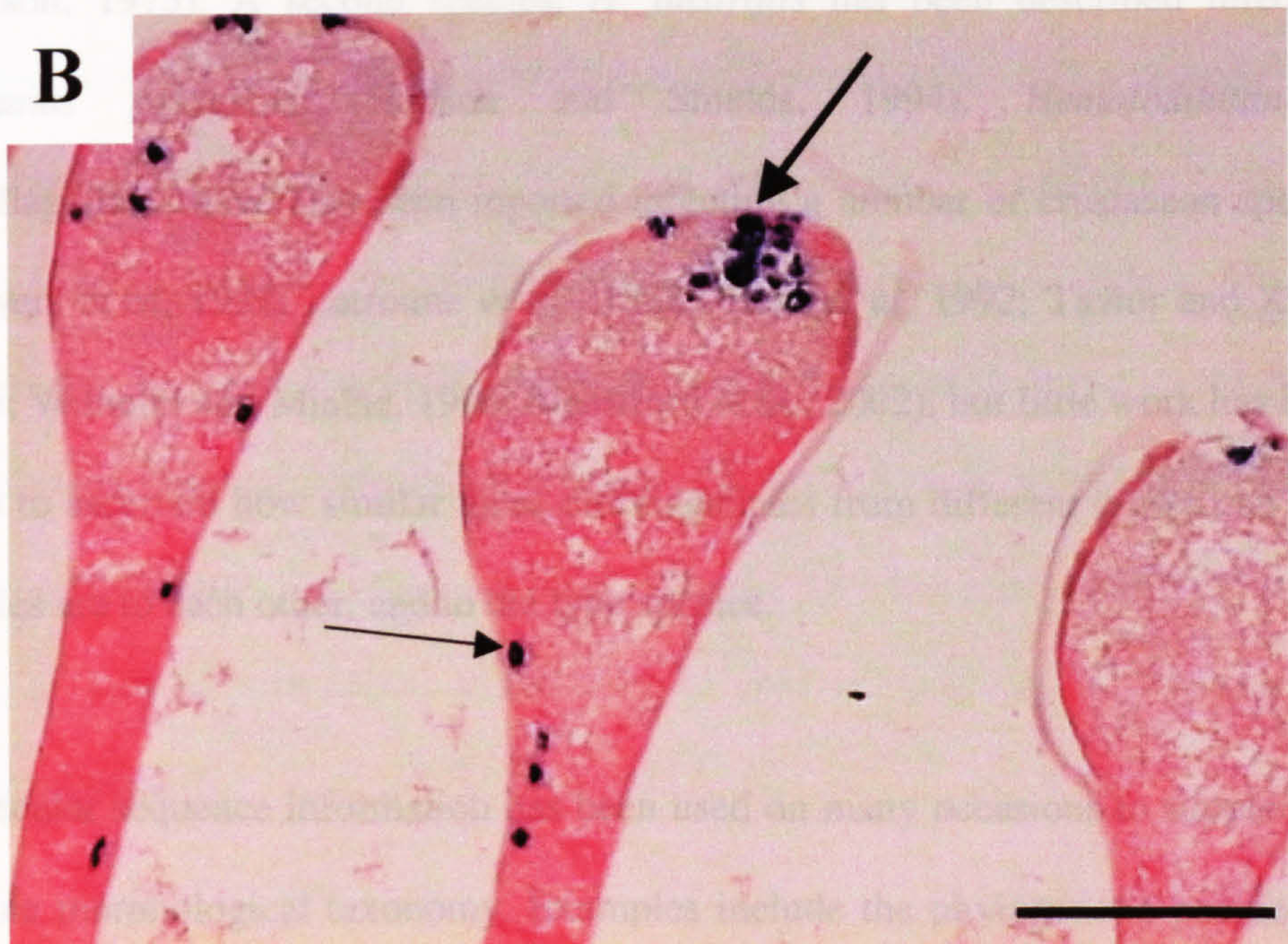

B

Figure 3.11 Hybridisation of Probe 2 to *Hematodinium* cells in the haemal space of hepatopancreas (A) and in the gill tips (B) of *Carcinus maenas* tissue sections. Material supplied by Dr. G. Stentiford, CEFAS Weymouth Laboratory. Ht = hepatopancreatic tubule; arrows = parasite cells. Scale bar 10 μm.


A
Ht
Ht


B

Chapter 4

Genetic variation of *Hematodinium* species infecting crustaceans in the UK.

4.1 Introduction

Several marine crustaceans are parasitized by a dinoflagellate of the genus *Hematodinium*. The type species, *Hematodinium perezi*, was first described by Chatton and Poisson (1931) infecting *Carcinus maenas* and *Portunus depurator*. *H. perezi* has since been recorded from *Callinectes sapidus* (Newman and Johnson, 1975). A second species, *H. australis* has been described infecting *Portunus pelagicus* (Hudson and Shields, 1994). *Hematodinium*-like dinoflagellates have also been reported infecting a number of crustacean species (Meyers *et al*, 1987; Latrouite *et al.*, 1988; Field *et al*, 1992; Taylor and Khan, 1995; Wilhelm and Mialhe, 1996; Stentiford *et al.*, 2002), but little work has been done to ascertain how similar these dinoflagellates from different crustacean host species are to each other, and to the type species.

Molecular sequence information has been used on many occasions to supplement marine morphological taxonomy. Examples include the phylogenetic position of *Perkinsus* (Goggin and Barker, 1993), *Marteilia refringens* (Berthe *et al.*, 2000) and several dinoflagellates (Fensome *et al.*, 1999). The non-coding internally transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) complex are

generally assumed to evolve faster than the transcribed small subunit (SSU) rDNA gene (Hillis and Dixon, 1991; Li and Graur, 1991). Sequence variation in the ITS region has been reported between species of trematodes (Anderson and Barker, 1993), fungi (Lee and Taylor, 1992) and Apicomplexa (Goggin, 1994). Ribosomal DNA sequence variation has also been successfully used to discriminate between toxic and non-toxic *Alexandrium tamarense* and *Pseudonitzschia* species (Scholin *et al.*, 1994; Higman *et al.*, 2001), the geographical location and virulence of *Perkinsus marinus* strains (Robledo *et al.*, 1999; Reece *et al.*, 2001), and for the identification of cold and warm water strains of *Cryptocaryon irritans* (Diggles and Adlard, 1997).

Hudson and Adlard (1996) analysed partial SSU and ITS1 rDNA sequences of *Hematodinium* species from *Nephrops norvegicus*, *Callinectes sapidus*, *Chionoecetes bairdi* and *C. opilio*. They reported substantial sequence variation within the ITS1 region between samples from different hosts, and suggested that the *Hematodinium* sp. infecting *Nephrops norvegicus* was distinct from the others. However, sequence variation between different samples from the same species host, or sequence variation in and between similar geographical locations was never addressed.

In this study the SSU and ITS1 rDNA sequences from isolates of *Hematodinium* infecting *N. norvegicus* and a number of other crustacean species in UK waters

are compared to assess the level of nucleotide sequence variability in relation to geographic location, host species and sample history.

4.2 Materials and Methods

4.2.1 Isolates

Ten isolates of *Hematodinium* sp. were obtained: five infected haemolymph samples from the Norway lobster, *Nephrops norvegicus*; one infected tissue sample from the edible crab *Cancer pagurus*; two *in vitro* culture samples of *Hematodinium* sp. from *N. norvegicus* and the hermit crab, *P. bernhardus*, respectively; a *potentially* infected amphipod, *Orchomene nanus*; and a sample of *H. perezi*-infected haemolymph from a blue crab. All isolates originated from crustaceans in UK waters apart from the blue crab sample from Virginia on the East coast of the USA (Fig. 4.1 and Table 4.1). Infected haemolymph, hepatopancreas tissue and cell culture samples were collected and stored as described in Chapters 2 and 3.

4.2.2 DNA extraction, and amplification

DNA was extracted from the samples (Table 4.1) according to the methods described in section 3.2.2. Amplification of the 3' end of the SSU, the complete ITS1 and the 5'end of the 5.8S gene was achieved using the forward primer (5'

GTT CCC CTT GAA GGA GGA ATT C 3') and reverse primer (5' CGC ATT TCG CTG CGT TCT TC 3') previously described by Hudson and Adlard (1994). The amplification reaction mixtures contained 100 ng genomic DNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCL, 0.1 % Triton X-100 (v/v), 1.5 mM $MgCl_2$, 100 µM of dNTPs, 10 pmol each primer, 1 unit of Taq polymerase (Promega), and sterile deionised water to a final volume of 20 µl. Reactions were overlaid with 10 µl of mineral oil. Thermal cycling conditions were as follows: denaturation at 94 °C for 1 min; primer annealing at 52 °C for 1 min; chain extension at 72 °C for 3 min; repeated for 35 cycles, with a final cycle incorporating a 7 min extension. Amplification products were run on a 1.5 % (w/v) agarose gel, stained with ethidium bromide and viewed under a UV light source.

4.2.3 Cloning and sequencing

Cloning and sequencing of PCR amplification products was as described in section 3.2.3. Two PCR clones of each isolate were generated and sequenced independently. Multiple sequence alignments were constructed using the software programmes ClustalX 1.81 (Thompson *et al.*, 1994) and BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

4.3 Results

4.3.1 Sequence alignment

The ten *Hematodinium* isolates were subjected to sequence analysis of the 3' end of the SSU and the ITS1 of the ribosomal DNA complex. A single fragment of approximately 680 bp was amplified from all ten parasite isolates, independent of host origin. The nucleotide sequence of the 3' end of the SSU and ITS1 were aligned (Fig. 4.2). The position of the 3' end of the SSU was determined by comparison with *Hematodinium* isolates previously sequenced (Hudson and Adlard, 1996). The end of the ITS1 region was determined by comparison with an alignment of *H. perezi* and several *Perkinsus* and *Prorocentrum* species (K. Hudson and K. Reece, VIMS, personal communication). This facilitated the alignment of rDNA ITS1 sequences from UK isolates of *Hematodinium* (Fig. 4.3). The lengths of the ribosomal DNA regions are therefore approximations.

4.3.2 Analysis of the ribosomal SSU region

All sequences were similar, having at least 98.62 % homology. The 3' end of the SSU sequences of *Hematodinium* isolates were 216 bp in length, except for the hermit crab (*Pagurus bernhardus*) *in vitro* culture isolate (PbHem1), which was 215 bp due to a deletion at nucleotide position 7 (Fig 4.2). The sequence from PbHem1 also showed a transversion (A>T) at nucleotide position 13 compared to

the other isolate sequences. The isolates NnHem2, NnHemNM1 and OnHem1 showed transitions at nucleotide positions 92 and 167 (A>G, G>A) over the SSU 3' sequence, whereas the isolates NnHem1, PbHem1, NnHem2 and CsHem1 had a transversion at nucleotide position 204 (A>C) when compared to the other isolate sequences. The 3' SSU sequence from *Callinectes sapidus* showed a transition (T>C) at nucleotide position 129. The G + C (guanine + cytosine) content of the SSU sequences varied between 50.5 % and 51.4 %, although in the majority of isolates it was 50.5 % or 50.9 % (Table 4.2).

4.3.3 Analysis of the ribosomal ITS1 region

The ITS1 region of different *Hematodinium* isolates varied in length between 330 and 352 bp (Table 4.2). The G + C content also varied between 46.0 % and 47.8 %. The aligned ITS1 sequences of *Hematodinium* isolates from the UK showed several nucleotide insertions, transitions and transversions, with nucleotide deletions and insertions being the most common source of sequence dissimilarity (Fig. 4.3). The similarity matrix revealed that the overall sequence similarity was high, with isolates CpHem1 and NnHemF1 being 100 % identical, as were the isolates NnHemF2 and OnHem1 (Table 4.3). The isolates NnHemF1 and NnHemF2 exhibited 99.72 % homology, differing only by 1 transversion, as did NnHemF2 and CpHem1. The ITS1 sequence of *H. perezi* was very different from all UK-originating *Hematodinium* isolates and differed by 60.2 % over its length.

4.4 Discussion

This study represents the first analysis of SSU and ITS1 rDNA sequences from *Hematodinium sp.* parasites infecting several crustacean species common in UK waters. It has previously been documented that the 3' end of the SSU rDNA gene was completely conserved between several *Hematodinium* species from different crustacean hosts and geographic origins (Hudson and Adlard, 1996). However, rDNA sequence examination has revealed several differences in nucleotide sequences at the 3' end of the SSU between groups of *Hematodinium* isolates used in this study (Fig. 4.2). It is unlikely that these are sequencing errors as duplicated PCR and sequencing reactions for each isolate gave identical results. In addition, the nucleotide differences observed are common to several isolates that were amplified, cloned and sequenced at different times during this study.

In contrast to the relatively high homology within the SSU rDNA, the non-transcribed spaces (ITS) of rDNA genes can show great variation. ITS regions have been observed to vary among species within a genus or among populations (Lee and Taylor, 1992). Hudson and Adlard (1996) previously reported that the ITS 1 sequence of the *Hematodinium* sp. from *N. norvegicus* was only 22.7 % similar to ITS1 sequences from *H. perezi* from *C. sapidus.* This large variation is consistent with the many different morphological features observed between both species of *Hematodinium.* The ITS1 sequence of *H. perezi* used in this study was only 39.8 % similar to the other *Hematodinium* spp. sequenced, supporting the

finding of Hudson and Adlard (1996) that it is distinct from other *Hematodinium* species.

Hematodinium infection of *N. norvegicus* has been reported from several locations on the West Coast of Scotland (Field *et al.*, 1992). Analysis of rDNA ITS1 sequences from Norway lobsters from different locations (Fig. 4.1) has established no significant pattern in the geographical distribution of genetic strains of the parasite from geographically separate sites of the Clyde, North Minch and Fladen. None of the lobster-derived isolates were 100 % identical to each other and they had varying homologies between 92.94 % and 99.72 %. However, the two isolates from the Fladen were 99.72 % similar to each other, having only 1 nucleotide difference.

Hematodinium infection has also been reported from the edible crab *Cancer pagurus* from the English Channel (Stentiford *et al.*, 2002). Morphologically, the parasite cells from *N. norvegicus* and *Cancer pagurus* are very similar, and are present in host crustaceans as uni-, bi and multinucleate forms of comparable size. Parasite cells have comparable condensed chromatin profiles, lipid droplets, trichocysts, mitochondria and speckled matrix organelles. The pathology of infection is similar in both crustacean hosts, with several tissues infiltrated by large numbers of parasite cells. The claw muscle of *N. norvegicus* shows a large parasite infiltration, although Z-lines of the sarcomeres remain intact (Stentiford *et al.*, 2000b). In contrast, the sarcomeres from claw muscle fibres of *C. pagurus*

showed severe disorganisation of filaments in the region of the Z-line, indicating differences in parasite virulence or host susceptibility. This study indicates that the species of *Hematodinium* infecting *C. pagurus* from the English Channel is the same as *Hematodinium* species infecting *N. norvegicus* from locations on the West Coast of Scotland. Consequently, the differences seen in pathology between the crab and lobster may be due to factors of host susceptibility rather than parasite strain difference.

The discovery of a number of *Hematodinium*-infected amphipods of the species *Orchomene nanus*, detailed in Chapter 3, section 3.2.6, and the confirmation of infection via sequence analysis in this chapter may be highly significant with regard to the life cycle of the parasite. Scavenging amphipods have previously been reported to harbour dinoflagellate parasites (Johnson, 1986; Messick and Shields, 2000), and may act as a reservoir or intermediate host. The *Hematodinium* isolate from *O. nanus* from the Clyde was 100 % identical to an *N. norvegicus* isolate from the Fladen, and very similar to other *N. norvegicus* isolates. The fact that *O. nanus* and *N. norvegicus* inhabit the same environment supports this conclusion. Further studies are warranted to investigate prevalence of *Hematodinium* infection in *O. nanus* and its possible involvement in disease transmission.

Prolonged culture of *Hematodinium* may result in genetic divergence similar to the natural variation observed in isolates obtained from field samples of infected

animals. The *in vitro* culture samples of *Hematodinium* from the Norway lobster (NnHem1), and the hermit crab (PbHem1), serially cultured for 9 and 5 years, respectively, showed diverse ITS1 genetic variation when compared to recent isolates from *N. norvegicus* (Table 4.3). The *Hematodinium* trophonts found infecting *P. bernhardus* had a nucleus, caseiform organelles and speckled matrix organelles similar in structure to those in the *Hematodinium* species from *N. norvegicus* (Appleton *et al.*, 1997). During culture however, life cycle stages of the parasite were noticeably different to the *Hematodinium* species from the *N. norvegicus* (K. Vickerman, Glasgow University, personal communication), consistent with the sample having the highest ITS1 sequence divergence in this study. Consequently, genetic differences of the parasite may be reflected in variations in virulence or morphology. On the other hand, repeated passage in the laboratory may select for a certain genetic variant that can adapt well to culture conditions. Sequencing of several field isolates prior to and after prolonged culture is necessary to elucidate whether strain selection takes place.

In order to investigate fully the extent of *Hematodinium* species diversity, many isolate sequences from each sample locality are needed to examine variation within the population, and then between populations. This study was limited in sample size, but ITS1 rDNA sequences obtained indicate that there is no systematic geographical distribution of genetic strains of the parasite in crustaceans dwelling in UK waters. Furthermore, it appears that the same *Hematodinium* species can infect different crustaceans in different localities. As

this study has shown a high ITS sequence similarity between all UK *Hematodinium* isolates, targeting other gene sequences and the utilisation of techniques such as restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) may be useful in future studies to facilitate genetic characterization of samples and interpret the apparent limited genetic variation of *Hematodinium* sp. in UK waters.

Table 4.1 Origin and details of *Hematodinium* isolates used in this study.

Table 4.2 Sequence length and G+C content (%) variation of the 3' end of the SSU and ITS1 regions of ribosomal DNA sequences from *Hematodinium* isolates.

Abbreviation	Crustacean host	Sample location	Date obtained	Infection diagnosis method	Parasite sample source
NnHem1	*N. norvegicus*	Clyde	Spring 1992	Pleopod	*In vitro* culture
NnHem2	*N. norvegicus*	Clyde	May 2001	ELISA	Haemolymph
NnHem3	*N. norvegicus*	Clyde	June 2001	ELISA	Haemolymph
NnHemF1	*N. norvegicus*	Fladen	June 2001	ELISA	Haemolymph
NnHemF2	*N. norvegicus*	Fladen	June 2001	ELISA	Haemolymph
NnHemNM1	*N. norvegicus*	N. Minch	June 2001	ELISA	Haemolymph
CpHem1	*C. pagurus*	Guernsey	Winter 2001	Histology/Western blot	Hepatopancreas tissue
PbHem1	*P. bernhardus*	Clyde	July 1996	IFAT	*In vitro* culture
OnHem1	*O. nanus*	Clyde	August 2001	PCR	Whole amphipod
CsHem1	*C. sapidus*	USA	November 2001	Haemolymph smear	Haemolymph

Sample	3' SSU		ITS1	
	Length	G+C	Length	G+C
NnHem1	216	50.9	347	47.8
NnHem2	216	50.9	330	46.1
NnHem3	216	50.5	348	47.7
NnHemF1	216	50.5	350	47.4
NnHemF2	216	50.5	350	47.4
NnHemNM1	216	50.5	344	47.7
CpHem1	216	50.5	350	47.4
PbHem1	215	51.2	331	46.2
OnHem1	216	50.5	350	47.4
CsHem1	216	51.4	352	46.0

Table 4.3 Similarity matrix (% homology) for aligned ITS1 DNA sequences shown in Fig. 4.3.

	NnHem1	NnHem2	NnHem3	NnHemF1	NnHemF2	NnHemNM1	CpHem1	PbHem1	OnHem1
NnHem1	100.00	93.50	98.59	98.02	97.74	98.02	98.02	93.22	98.59
NnHem2		100.00	93.50	93.22	92.94	94.35	93.22	99.44	92.94
NnHem3			100.00	98.87	98.59	97.74	99.15	92.94	98.59
NnHemF1				100.00	99.72	98.02	100.00	92.66	99.72
NnHemF2					100.00	98.02	99.72	92.37	100.00
NnHemNM1						100.00	97.74	93.79	98.31
CpHem1							100.00	94.35	99.72
PbHem1								100.00	92.37
OnHem1									100.00

Figure 4.1 Geographical location of sample collection sites in the UK waters.

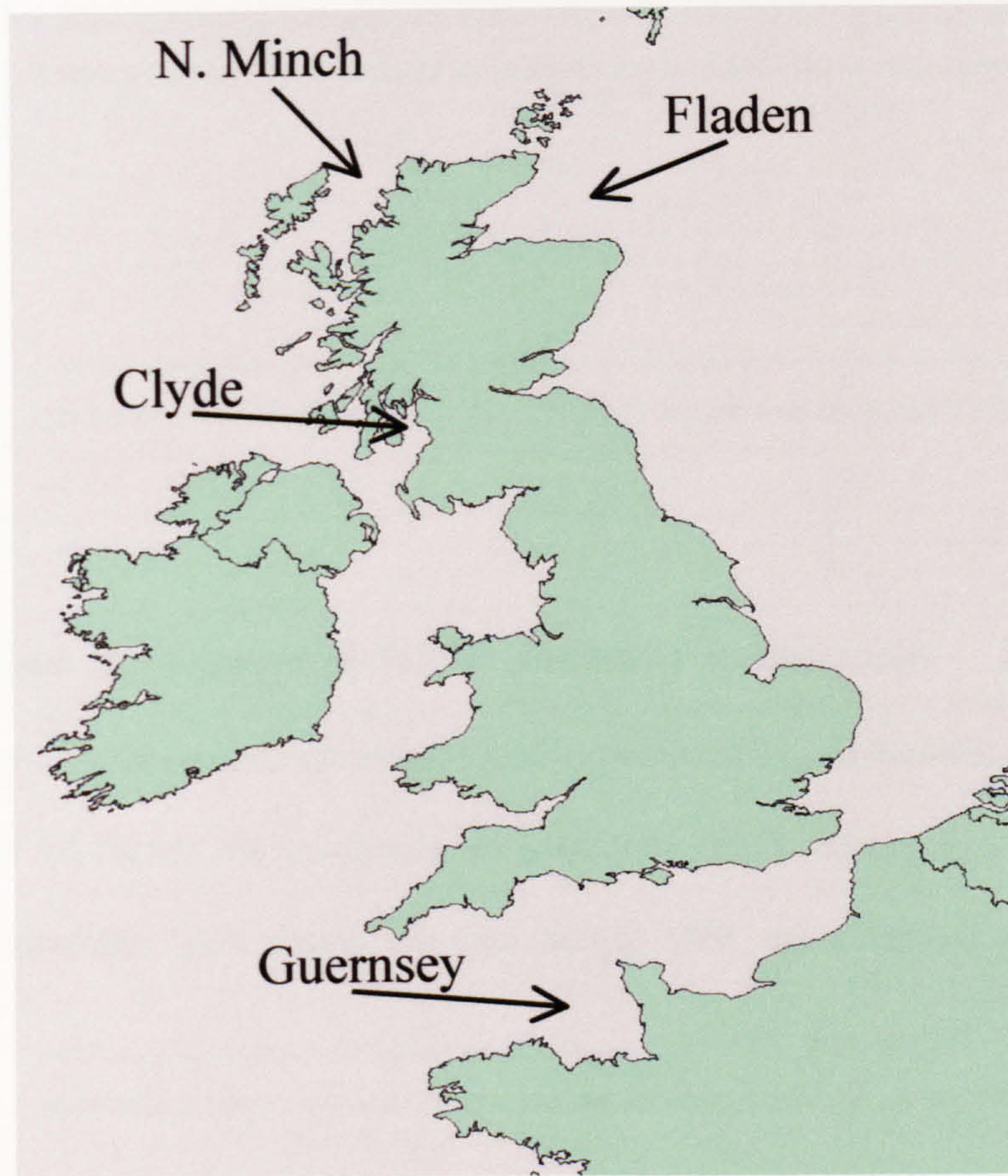

N. Minch
Fladen
Clyde
Guernsey

Figure 4.2 Alignment of consensus nucleotide sequences of amplified SSU and ITS1 ribosomal DNA from all *Hematodinium* isolates. Dots represent conserved nucleotides. Gaps generated by alignment are shown by dashes. The single line indicates the SSU region and the double line represents the 5.8S region.

```
NnHem3      1 CTAGTAAGCGCGAGTCATCAGCTCGTGCTGATTACGTCCCTGCCCTTTGTACACACCGCC
CpHem1      1 ............................................................
NnHemF1     1 ............................................................
NnHemF2     1 ............................................................
OnHem1      1 ............................................................
NnHemNM1    1 ............................................................
NnHem1      1 ............................................................
PbHem1      1 ......-.....T...............................................
NnHem2      1 ............................................................
CsHem1      1 ............................................................

NnHem3     61 CGTCGCTCCTACCGATTGAGTGATCCGGTGAATAATTCGGACGGCAGCCTTTTCCAGTTT
CpHem1     61 ............................................................
NnHemF1    61 ............................................................
NnHemF2    61 ...............................G............................
OnHem1     61 ...............................G............................
NnHemNM1   61 ...............................G............................
NnHem1     61 ............................................................
PbHem1     60 ............................................................
NnHem2     61 ............................................................
CsHem1     61 ............................................................

NnHem3    121 CTGGAAGTGGCAGCTGGAAGTTTAGTGAACCTTATCACTTAGAGGAAGGAGAAGTCGTAA
CpHem1    121 ............................................................
NnHemF1   121 ............................................................
NnHemF2   121 ................................................A...........
OnHem1    121 ................................................A...........
NnHemNM1  121 ................................................A...........
NnHem1    121 ............................................................
PbHem1    120 ............................................................
NnHem2    121 ............................................................
CsHem1    121 .......C....................................................

NnHem3    181 CAAGGTTTCCGTAGGTGAACCTGAGGAAGGATCATTCGCACGAATAATCAATAAAAAA-C
CpHem1    181 ..........................................................-.
NnHemF1   181 ..........................................................-.
NnHemF2   181 ..........................................................-.
OnHem1    181 ..........................................................-.
NnHemNM1  181 ..........................................................-.
NnHem1    181 .......................C..................................-.
PbHem1    180 .......................C..................................A.
NnHem2    181 .......................C..................................-.
CsHem1    181 .......................C....................G..A-.....T.T.-T

NnHem3    240 ACCGTGAACCTTGGCCATTAGCACGAGCAAAAAAGCGCATGCGCATGCTGC-ATGCCCCC
CpHem1    240 ...................................................-........
NnHemF1   240 ...................................................-........
NnHemF2   240 ...................................................-........
OnHem1    240 ...................................................-........
NnHemNM1  240 ...................................................-........
NnHem1    240 ...................................................-........
PbHem1    240 ...................................................-........
NnHem2    240 ...................................................-........
CsHem1    239 TTTA.T.TTT.C.CA..CA.A..TTCA.CGTG..C.TT.GC.ATTA...A.G.C.A.TA.

NnHem3    299 GCCGCCGCCGCCGCCTCCGCTGTGTGTGTGTGTGTGTGTG--GGGG-----TGTTTGTGT
CpHem1    299 ........................................TG....-----.........
NnHemF1   299 ........................................TG....-----.........
NnHemF2   299 ........................................TG....-----.........
OnHem1    299 ........................................TG....-----.........
NnHemNM1  299 ..................................------TG....-----.........
NnHem1    299 ....................................----TG....-----.........
PbHem1    299 ......------....................----------....-----.........
NnHem2    299 ......------....................----------....-----.........
CsHem1    299 TAGCTA..TA.T.AG.GG.GC.GTG......TG..TAC.ACT.CTACTTCT.AC.C..AG
```

Figure 4.2 Continuation.

```
NnHem3     352 GTG---CGCGTTCGTGCTACTAAGGG-CTGTGAGTGATGGGGAACCACCTCTCCAAATAT
CpHem1     354 ..---....................-.................................
NnHemF1    354 ..---....................-.................................
NnHemF2    354 ..---....................-.................................
OnHem1     354 ..---....................-.................................
NnHemNM1   348 ..---....................-.................................
NnHem1     350 ..---....................-.......A.........................
PbHem1     338 ..---....................-.................................
NnHem2     338 ..---....................-.................................
CsHem1     359 C..AACT..ACA.ACA...G..CCCCT..C.TGC..G.A..AG.AGTAGCT..T.CGGGG

NnHem3     408 TTCTCCAG-CCCACGTTTGTTTTC-CTTATAAT--AACTCTCTAATTTCACTTATTCAAT
CpHem1     410 ........-...............-........--.........................
NnHemF1    410 ........-...............-........--.........................
NnHemF2    410 ........-...............-........--.........................
OnHem1     410 ........-...............-........--.........................
NnHemNM1   404 ........-...............-........--.........................
NnHem1     406 ........G...............-........--.........................
PbHem1     394 ........-...............-........--..........C..............
NnHem2     394 ........-...............-........--.........................
CsHem1     419 .GTGAGG.TA.GGT.G.A..ACA.G.C..CC.CTG.....CTCC..CC...G.T.G.TT.

NnHem3     464 TATA--ACTAAGCTTCTTCTCCCCTTCCCTTCTTCGTCCAGAAGAAGAAGAAGGGGGAGG
CpHem1     466 ....--................................................A.....
NnHemF1    466 ....--................................................A.....
NnHemF2    466 ....--.................................T..............A.....
OnHem1     466 ....--.................................T..............A.....
NnHemNM1   460 ....--.................................T..............A.....
NnHem1     463 ....--................................................A.....
PbHem1     450 ....TA............................................------....
NnHem2     450 ....TA............................................------....
CsHem1     479 CCATAA..AC.A.A...CTAAT--...AGC.A...A..TT.CTCTGCTCC----------

NnHem3     522 AGGAGGAGGAGGGAAGTTATATATATAATTTTCAATTTAGAAAATTTTAGCGATGAATGC
CpHem1     524 ..............G.............................................
NnHemF1    524 ..............G.............................................
NnHemF2    524 ..............G.............................................
OnHem1     524 ..............G.............................................
NnHemNM1   518 ..............G.........G...................................
NnHem1     521 ..............G.C...........................................
PbHem1     504 ..............G.............................................
NnHem2     504 ..............G.............................................
CsHem1     527 CTTTC.C..G.AT.G.GCT.TCT.CA..CG.ATG.C.-......................

NnHem3     582 CTTGGCTCGGGTTACGAT
CpHem1     584 ..C...............
NnHemF1    584 ..C...............
NnHemF2    584 ..C...............
OnHem1     584 ..C...............
NnHemNM1   578 ..C...............
NnHem1     581 ..C...............
PbHem1     564 ..C...............
NnHem2     564 ..C...............
CsHem1     586 ..C...............
```

Figure 4.3 Alignment of consensus nucleotide sequences of amplified ITS1 ribosomal DNA from UK isolates of *Hematodinium*. Dots represent conserved nucleotides. Gaps generated by alignment are shown by dashes.

```
NnHemF2     1 CGCACGAATAATCAATAAAAAA-CACCGTGAACCTTGGCCATTAGCACGAGCAAAAAAGC
OnHem1      1 ......................-.....................................
NnHemNM1    1 ......................-.....................................
NnHem1      1 ......................-.....................................
NnHemF1     1 ......................-.....................................
CpHem1      1 ......................-.....................................
NnHem3      1 ......................-.....................................
NnHem2      1 ......................-.....................................
PbHem1      1 ......................A.....................................

NnHemF2    60 GCATGCGCATGCTGCATGCCCCCGCCGCCGCCGCCGCCTCCGCTGTGTGTGTGTGTGTGT
OnHem1     60 ............................................................
NnHemNM1   60 .......................................................-----
NnHem1     60 .........................................................---
NnHemF1    60 ............................................................
CpHem1     60 ............................................................
NnHem3     60 ...........................................................-
NnHem2     60 ................................------.................-----
PbHem1     61 ................................------.................-----

NnHemF2   120 GTGTGGGGGTGTTTGTGTGTGCGCGTTCGTGCTACTAAGGGCTGTGAGTGATGGGGAACC
OnHem1    120 ............................................................
NnHemNM1  115 -...........................................................
NnHem1    117 -...............................................A...........
NnHemF1   120 ............................................................
CpHem1    120 ............................................................
NnHem3    119 -...........................................................
NnHem2    109 -----.......................................................
PbHem1    110 -----.......................................................

NnHemF2   180 ACCTCTCCAAATATTTCTCCAG-CCCACGTTTGTTTTCCTTATAATAACTCTCTAATTTC
OnHem1    180 ......................-.....................................
NnHemNM1  174 ......................-.....................................
NnHem1    176 ......................G.....................................
NnHemF1   180 ......................-.....................................
CpHem1    180 ......................-.....................................
NnHem3    178 ......................-.....................................
NnHem2    164 ......................-.....................................
PbHem1    165 ......................-.................................C...

NnHemF2   239 ACTTATTCAATTATA--ACTAAGCTTCTTCTCCCCTTCCCTTCTTCGTCCTGAAGAAGAA
OnHem1    239 ...............--...........................................
NnHemNM1  233 ...............--...........................................
NnHem1    236 ...............--.................................A.........
NnHemF1   239 ...............--.................................A.........
CpHem1    239 ...............--.................................A.........
NnHem3    237 ...............--.................................A.........
NnHem2    223 ...............TA.................................A.........
PbHem1    224 ...............TA.................................A.........

NnHemF2   297 GAAGGAGGAGGAGGAGGAGGAGGGAGGTTATATATATAATTTTCAATTTAGAAA
OnHem1    297 ......................................................
NnHemNM1  291 ...................................G..................
NnHem1    294 ...........................C..........................
NnHemF1   297 ......................................................
CpHem1    297 ......................................................
NnHem3    295 .....G...................A............................
NnHem2    283 .------...............................................
PbHem1    284 .------...............................................
```

Chapter 5

Isolation and *in vitro* culture of *Hematodinium perezi* from the blue crab *Callinectes sapidus*.

5.1 Introduction

In 1997, the blue crab, *Callinectes sapidus* (Fig. 5.1) supported the largest commercial fishery within the Chesapeake Bay, Virginia, USA, and the second largest fishery in Virginia, USA (Kirkeley, 1997). However, major reductions in crab catches have occurred since 1998. These have been attributed to fishing pressure, environmental processes and disease. *Hematodinium perezi* is a parasitic dinoflagellate that infects *C. sapidus* along the East Coast of the United States. Epizootics have been reported from high salinity waters in Florida (30 % prevalence, Newman and Johnson, 1975), from Maryland and Virginia (70-100 % prevalence, Messick 1994; Messick and Shields, 2000) and from Georgia (Lee, 2000). In all cases these authors reported a detrimental effect on the local blue crab fishery. Experimental infection of blue crabs has shown that the parasite is highly pathogenic and multiplies quickly to kill its host (Shields and Squyars, 2000).

Many free-living dinoflagellates (some of which are involved in harmful algal blooms) have been isolated, cultured *in vitro* and documented (Usup *et al.*, 1994;

Jensen and Moestrup, 1997). However, only one parasitic dinoflagellate, a *Hematodinium* species from the Norway lobster, *Nephrops norvegicus*, has been maintained in continuous culture. This was achieved using a balanced salt solution (*Nephrops* saline) supplemented with 10 % (v/v) foetal calf serum (Appleton and Vickerman, 1998). In culture, the parasite undergoes a series of developmental changes, suggested to correspond to those taking place in the infected host, which culminate with the production of macrospores and microspores (Fig. 5.2). Efforts to infect lobsters experimentally using different culture-derived life cycle stages have so far been unsuccessful (Vickerman, 1994). A partial progression of the life cycle of a *Hematodinium* species infecting Alaskan tanner crabs, *Chionoecetes bairdi*, has also been reported. This consisted of single, binucleate, and multinucleate plasmodia parasite cells which differentiate into vegetative cells, later giving rise to dinospores (Meyers *et al.*, 1987).

Although the species of *Hematodinium* infecting the blue crab, *H. perezi,* has been maintained within host individuals for over 7 months by sequential injection of infected haemolymph (Shields and Squyars, 2000), no *in vitro* cultures have yet been established. Since long-term passage of *H. perezi* requires substantial space, time and logistical support, the development of *in vitro* cultures offers several advantages for studies of the life cycle of this parasite. A collaborative visit to the laboratory of Dr. J. Shields to study *H. perezi* in blue crabs offered the opportunity to initiate and maintain cultures of *H. perezi*, and hence to examine its

life cycle, and compare it with that of the *Hematodinium* species isolated from *N. norvegicus*.

5.2 Materials and Methods

5.2.1 Crab collection

Blue crabs were caught in Wachapreague Creek, Virginia, USA, using otter trawls or commercial crab pots. Crabs were transported to the Virginia Institute of Marine Science (VIMS) in ice-chilled coolers.

5.2.2 Diagnosis of infection

Crab haemolymph was withdrawn from the arthrodial membrane at the junction of the basis and the ischium of the 5th pereiopod using a sterile disposable 25½-gauge needle and a 1 ml syringe, following sterilization of the cuticle with 70 % (v/v) ethanol. One drop of haemolymph was mixed (1:1) with neutral red solution (0.25 % (w/v) in isolation medium). The isolation medium consisted of a physiological saline buffer (MAM: Modified Appleton Medium) adapted from Appleton and Vickerman, (1998), containing NaCl 19.31 g l^{-1}; KCl 0.65 g l^{-1}; $CaCl_2$ 1.38 g l^{-1}; $MgSO_4$ 1.73 g l^{-1}; Na_2SO_4 0.38 g l^{-1}; HEPES 0.82 g l^{-1}, adjusted to pH 7.8 and autoclaved. To this, glucose (1.0 mg ml^{-1}) was added, along with penicillin (500 mg l^{-1}) and streptomycin (500 mg l^{-1}) to suppress bacterial

contamination, and the isolation medium was sterile filtered (0.2 µm). The stained cell suspension was then examined for *Hematodinium* as a wet smear at 200 x magnification. Infected crabs identified by this technique were then held individually in aquaria at 20 °C to 21 °C, and 24 ppt salinity.

5.2.3 Parasite isolation

Hematodinium cells were separated from host haemocytes by the addition of 1 ml infected haemolymph to 9 ml MAM buffer (detailed above) in sterile 25 cm^2 tissue culture flasks, and were incubated for 30 min at 23 °C. Previous attempts at *in vitro* culture using this method of parasite cell isolation and purification (J. Shields, VIMS, unpublished data) indicated that during initial incubations, crab haemocytes adhere to the plastic surfaces of culture flasks, leaving only parasite cells in suspension in the media. This finding was exploited in the present procedure. Parasite cell suspensions were transferred into new sterile culture flasks and incubated at 23 °C for a further 30 min. The remaining parasite cell suspensions in the culture flasks were pooled, and centrifuged at 400 x g for 10 min at 22 °C. The resulting parasite cell suspension was washed three times with MAM buffer. For the initial parasite isolation, a minimum of 3 flasks were prepared from each crab.

5.2.4 Parasite culture

Isolated parasites were resuspended in MAM buffer supplemented with 10 % (v/v) foetal calf serum (FCS) (sterile filtered), glucose and antibiotics. Cell density was assessed using a haemocytometer and adjusted to give 1 x 10^6 cells ml^{-1}. Cell suspensions (1 ml) were incubated in 24 well culture plates at 23 °C. Over a period of 12 days, different life cycle stages from different isolates were observed. These were photographed under transmitted light or Hoffman modulation contrast using an Olympus IX50 microscope mounted with a Nikon DXM 12000 digital camera.

5.3 Results

5.3.1 Initial isolation of *H. perezi*

Parasites found in the haemolymph of 3 infected blue crabs were used to initiate multiple cultures from each isolate. Parasite cells in the haemolymph were round in appearance with several (>3) refractile granules contained within the cell (Fig. 5.3). The selective uptake of neutral red by *H. perezi* cells in haemolymph samples provided a rapid method of screening blue crab haemolymph samples for the presence of the parasite (Fig. 5.4). Isolation of *H. perezi* cells from infected haemolymph by repetitive short-term culture was effective, with host haemocytes

adhering to the bottom of tissue culture flasks within a few minutes of adding infected haemolymph to the isolation medium.

5.3.2 Parasite forms in early *in vitro* cultures

The following descriptions of life cycle stages of *H. perezi* are based on the terminology used by Appleton (1996) and Appleton and Vickerman (1998) for the developmental forms of *Hematodinium* sp. from the Norway lobster (Fig. 5.2).

The round parasite cells found in blue crab haemolymph are believed to be circulating ovoid plasmodial cells (sporoblasts). These were purified and cultured in isolation medium supplemented with 10 % (v/v) FCS as described above. The cultures had an initial cell density 1 x 10^6 cells ml^{-1} unless otherwise stated. Parasites developed into three different forms over an observation period of 12 days, depending on the original isolate and cell density.

In the first isolate, sporoblasts settled onto the floor of culture wells and began to aggregate into clump colonies (Fig. 5.5). When the density of parasites was increased to 3 x 10^6 cells ml^{-1}, the cells did not aggregate into colonies but formed a matt-like covering of the well floor (Fig. 5.6). Aggregations of sporoblasts formed cytoplasmic extensions from a central mass of the cells (Fig. 5.7). These "gel-like" cytoplasmic extensions were observed to expand and extend in a radial

manner from the central cell mass (Fig. 5.8), thus resembling an arachnoid sporont.

In the second isolate, aggregates of sporoblasts produced thin cytoplasmic threads between colonies, forming a spider-like web or arachnoid stage (Fig. 5.9). The cytoplasmic threads of neighbouring colonies converged and fused, producing a network that covered the base of the culture well. The majority of cells were aggregated, with only a few remaining as single cells in culture. The sporoblast cells appeared to have interdigitated to form a central mass of nucleated cytoplasm (Fig. 5.10). This culture form was very delicate, and any swirling motion of the medium within the culture well caused the cytoplasmic network to disintegrate.

In the third isolate, parasite cells settled onto the bottom of the wells with an even distribution. After 10 days in culture the cells elongated to form filamentous trophonts (Fig. 5.11). This life cycle stage displayed noticeable movement, both horizontally along the surface of the well and also vertically up into the medium. In many instances filamentous trophonts would aggregate into the gorgonlocks form (Fig. 5.12) (so-named by Appleton and Vickerman (1998) because of its resemblance to the head of the mythical gorgon). Over a period of 9-12 days in culture, several parasite cells were found to be differentiating and elongating, indicative of developmental changes to the filamentous trophonts form.

5.4 Discussion

Despite the fact that parasitic syndiniid dinoflagellates of the genus *Hematodinium* can have a devastating impact on several crustacean fisheries (Meyers *et al.*, 1987; Field *et al.*, 1992; Messick, 1994; Wilhelm and Mialhe, 1996; Stentiford *et al.*, 2002), their life cycles are still poorly understood. The successful initiation of cultures of *H. perezi* from the blue crab provides the opportunity to rectify this situation.

The selective uptake of the vital stain, neutral red, by *H. perezi* cells was observed during screening. The stain was localised in the refractile granules contained within the cells, which indicates that these organelles are of endosomal origin. The three different *in vitro* developmental stages of *H. perezi* observed in this study resemble several *in vitro* culture stages of the *Hematodinium* species from the Norway lobster (Appleton and Vickerman, 1998). Aggregations of presumptive sporoblasts formed arachnoid sporonts, then arachnoid networks developed by the fusing of cytoplasmic threads from neighbouring aggregations. Meyers *et al.* (1987), when trying to culture a *Hematodinium* species from the tanner crab, *Chionoecetes bairdi*, observed monolayers which were similar in appearance and description to arachnoid networks, indicating that this may be the common vegetative form of *Hematodinium* species. Moreover, network-like parasite life cycle stages have been observed in abdominal muscle and in

hepatopancreas tissues from infected Norway lobsters (Field and Appleton, 1995; Field and Appleton, 1996).

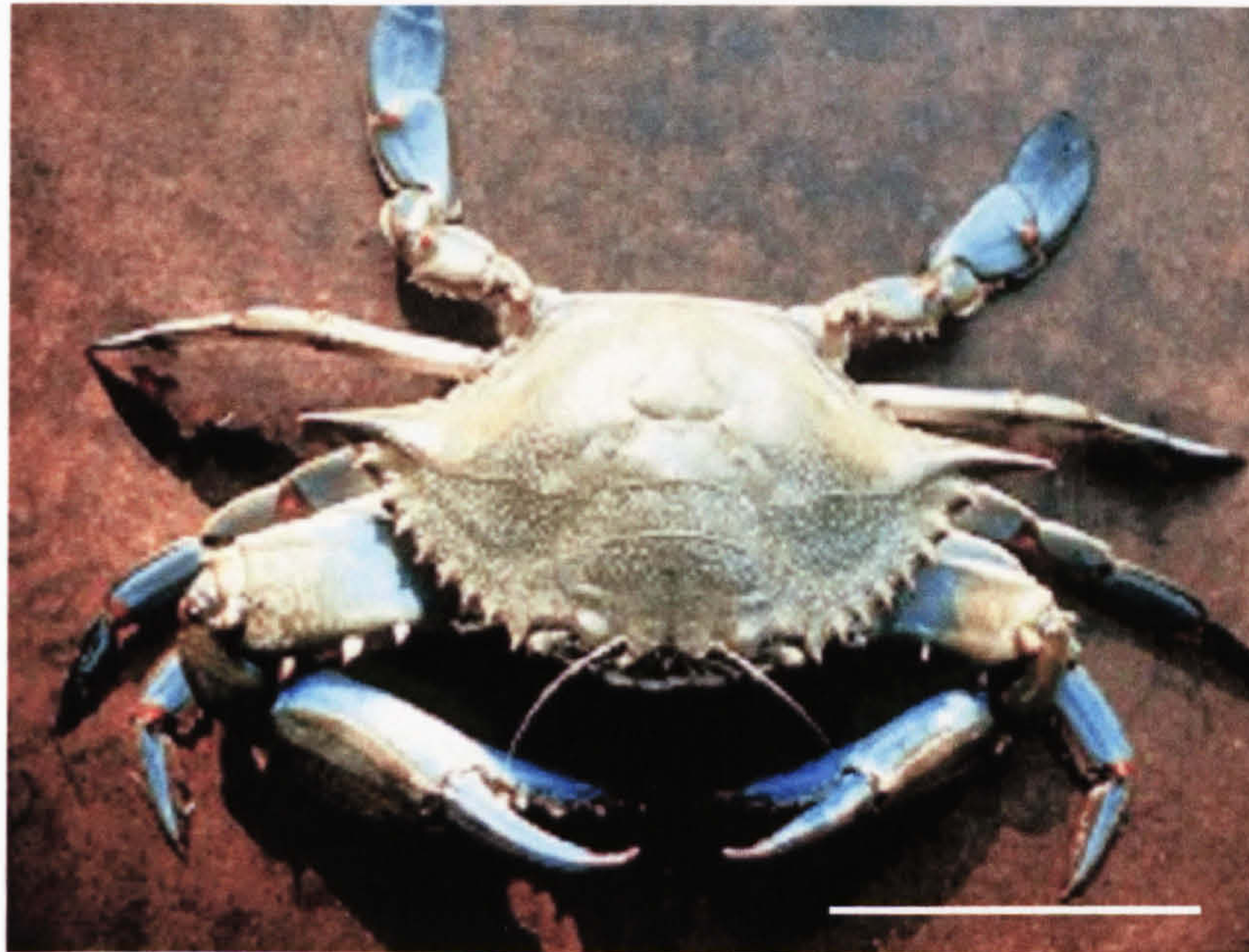
The third stage observed resembles the filamentous trophonts that occur in *in vitro* cultures of *Hematodinium* from the Norway lobster (Appleton and Vickerman, 1998). Aggregation of filamentous trophonts into gorgonlock colonies occurred only after macrospores and microspores germinated, and transformed into multinucleate filaments. Intriguingly, no motile dinospores were observed in the initial parasite cell suspensions used to initiate cultures. Possible explanations for this are that there may have been several post-spore cells in the cell suspension that were able to differentiate into the filamentous trophonts, or the haemolymph may have contained a small population of dinospores from an earlier infection circulating with the sporoblasts, and that these transformed into filamentous trophonts when cultured. Ultrastructural studies of the developmental forms accruing in culture are required to establish the cell structure and morphology in *H. perezi*.

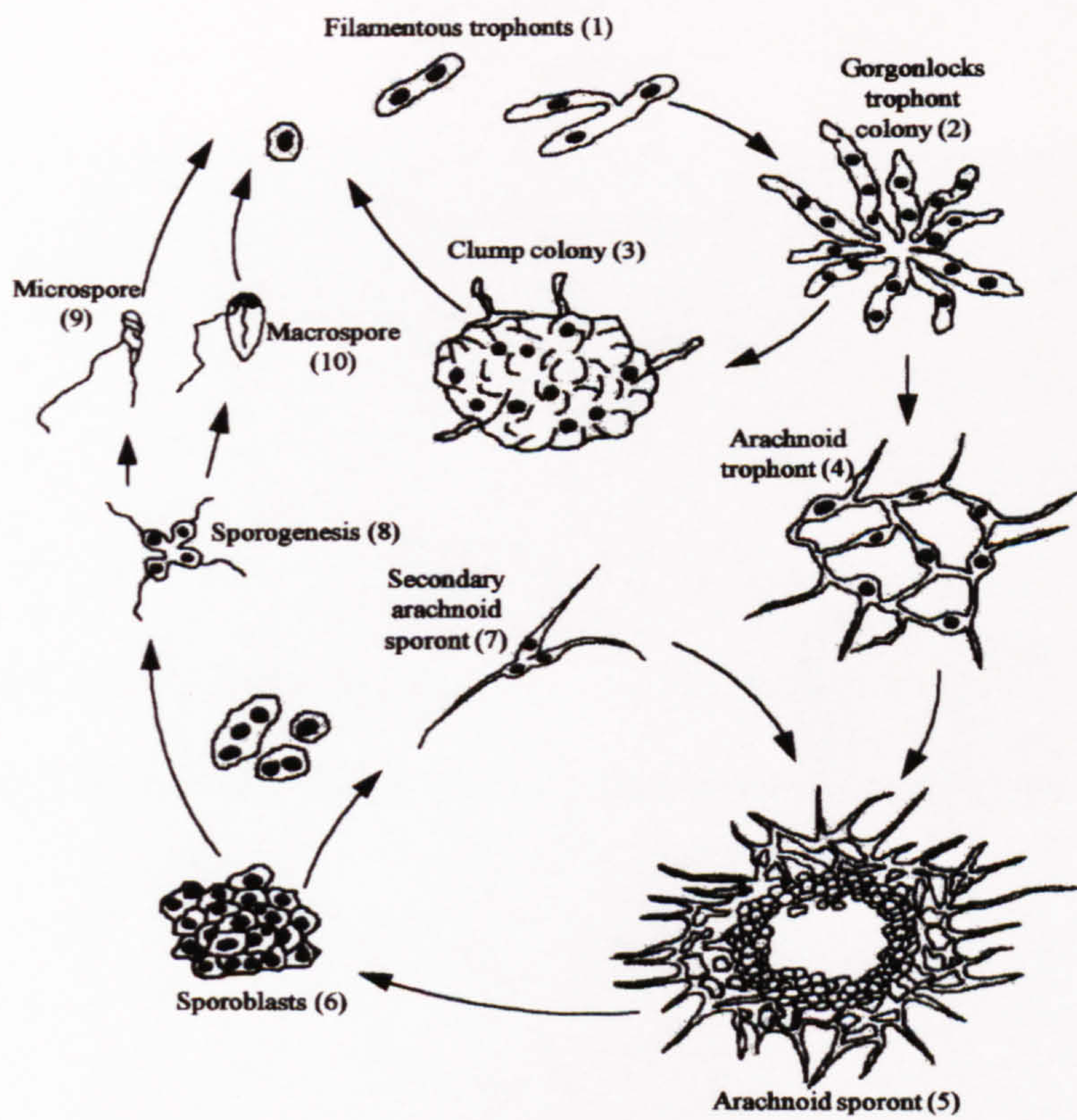
The filamentous form of *H. perezi* observed *in vitro* is similar to the rare vermiform plasmodium stage observed in the haemolymph of infected blue crabs by Shields and Squyars (2000), and arising from dinospore germination in the host. A progression through several other life history stages has subsequently been observed in other *in vitro* cultures of *H. perezi* that have been maintained for longer periods (>60 d) (J. Shields, VIMS, personal communication).

The successful initiation of *in vitro* cultures of *H. perezi* will facilitate the study of many aspects of its life history, morphology, growth characteristics, life cycle and transmission under different temperature and salinity regimes. The cultures will also provide a source of life cycle stages for transmission studies in crabs and other species.

Figure 5.1 The American blue crab *Callinectes sapidus*. Scale bar = 10 cm.

Figure 5.2 Schematic diagram of the proposed life cycle of *Hematodinium sp.* from *Nephrops norvegicus*, based on observations of *in vitro* cultures (Appleton and Vickerman, 1998). The principal multiplicative form *in vitro* is the filamentous trophont (1), which undergoes growth and differentiates into other developmental forms (2-10).




Filamentous trophonts (1)
Gorgonlocks
trophont
colony (2)
Clump colony (3)
Microspore
(9)
Macrospore
(10)
Arachnoid
trophont (4)
Sporogenesis (8)
Secondary
arachnoid
sporont (7)
Sporoblasts (6)
Arachnoid sporont (5)

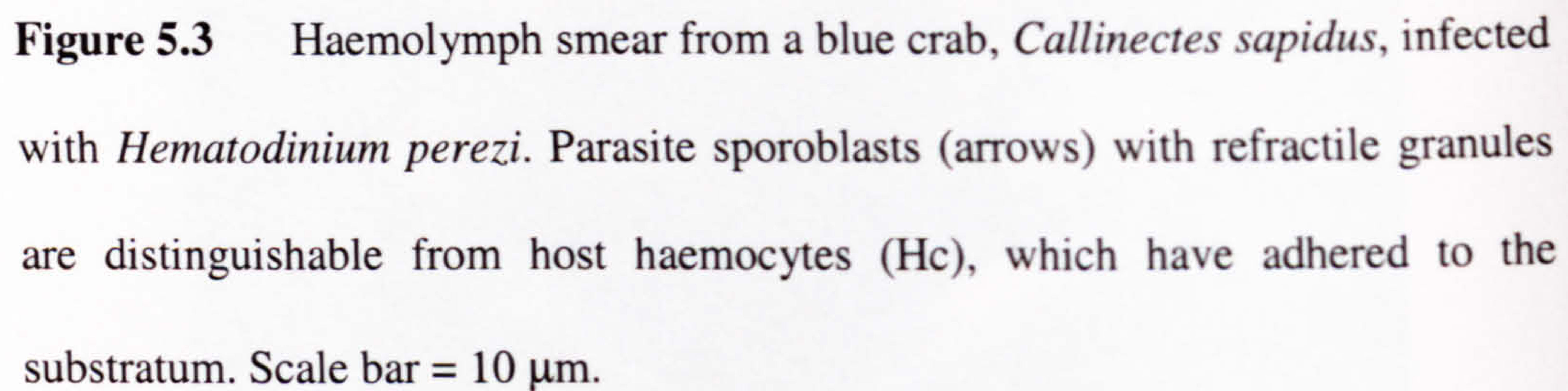


Figure 5.3 Haemolymph smear from a blue crab, *Callinectes sapidus*, infected with *Hematodinium perezi*. Parasite sporoblasts (arrows) with refractile granules are distinguishable from host haemocytes (Hc), which have adhered to the substratum. Scale bar = 10 μm.

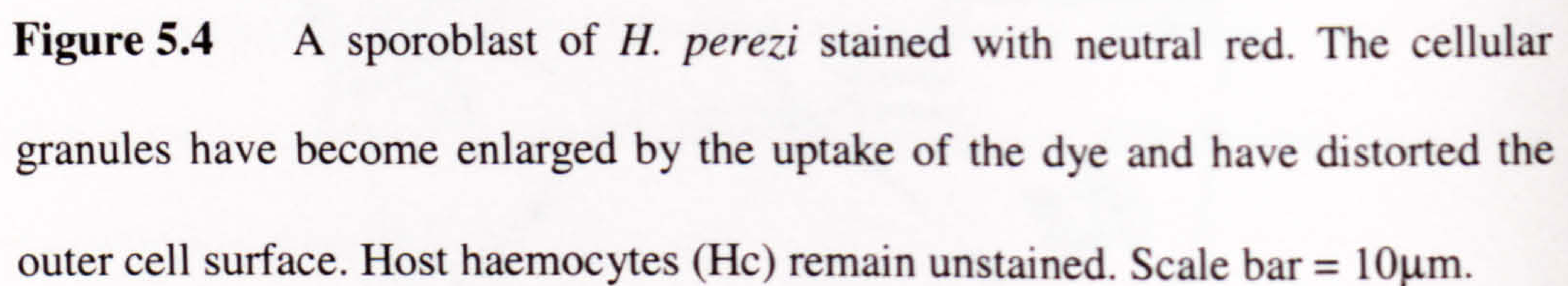
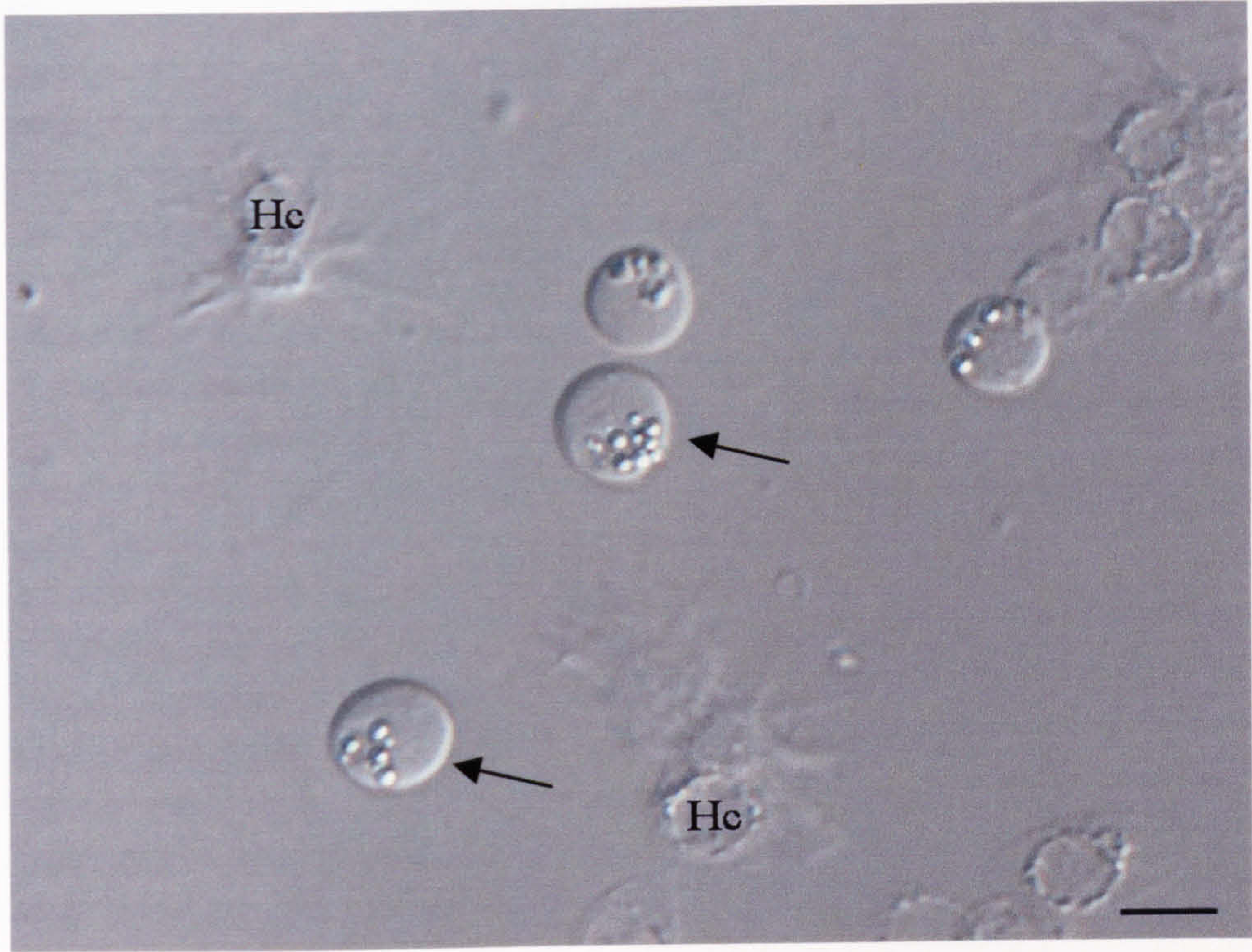


Figure 5.4 A sporoblast of *H. perezi* stained with neutral red. The cellular granules have become enlarged by the uptake of the dye and have distorted the outer cell surface. Host haemocytes (Hc) remain unstained. Scale bar = 10μm.


Hc
Hc

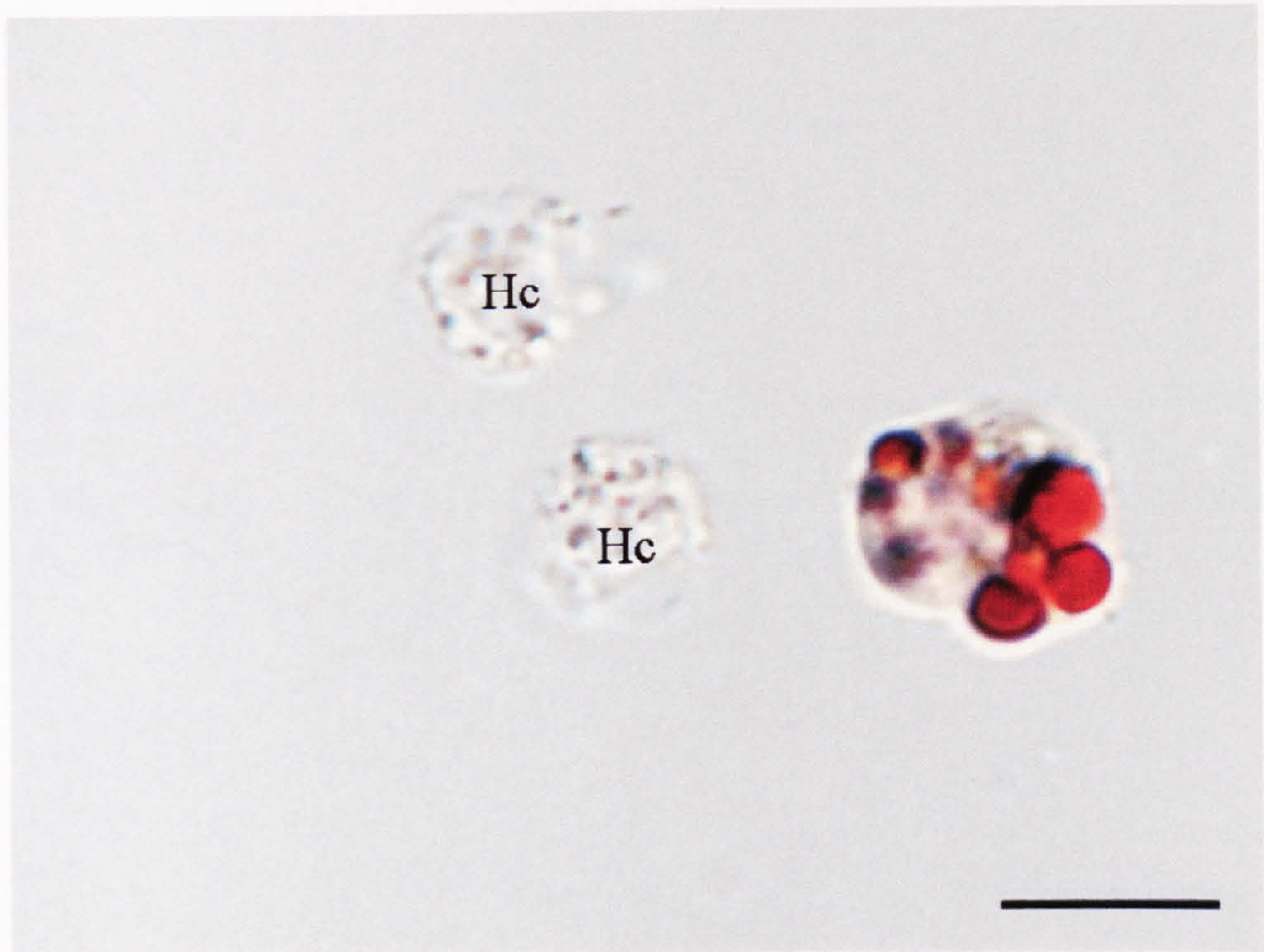
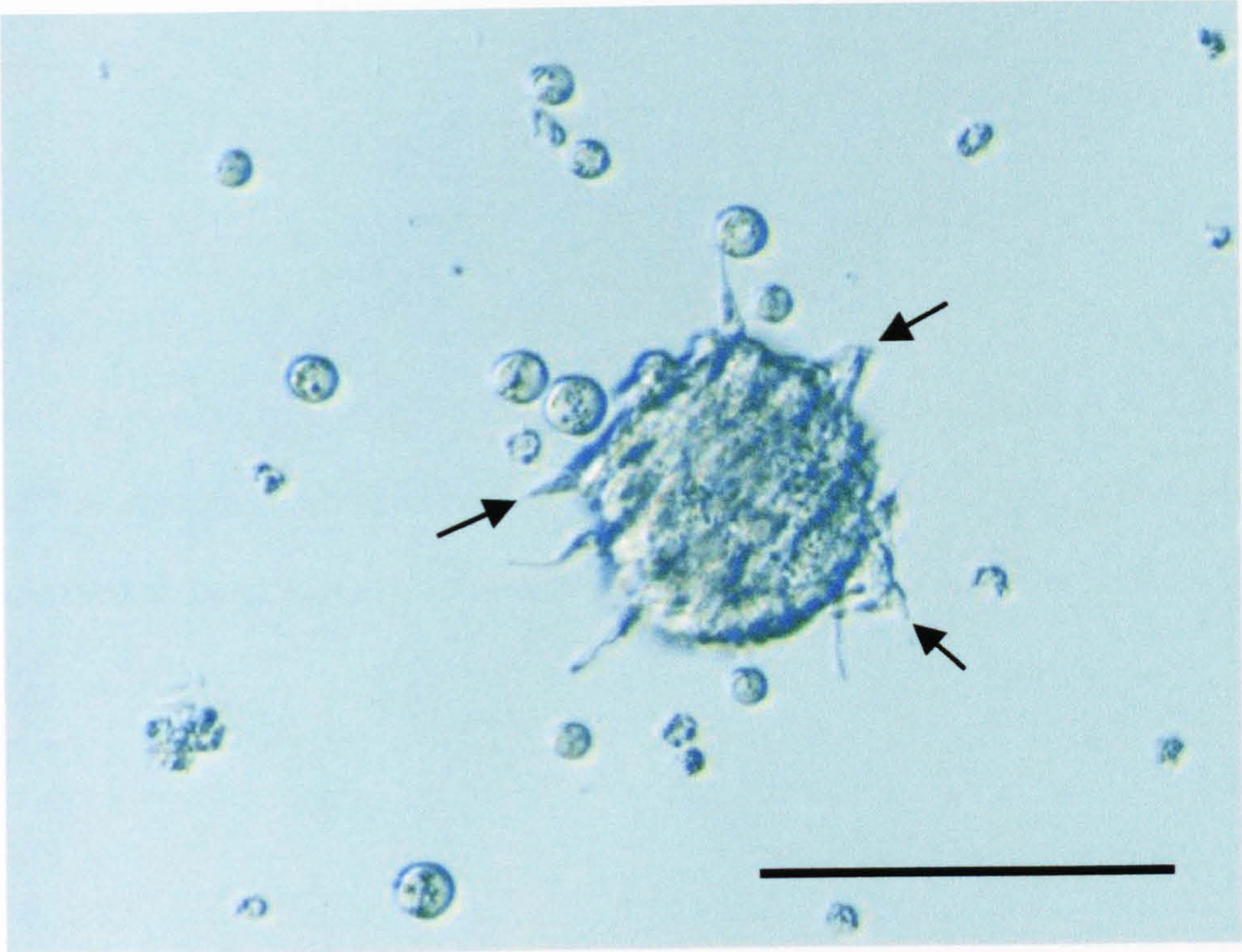

Hc
Hc

Figure 5.7 Cytoplasmic extensions (arrows) beginning to develop from a clump colony of *H. perezi* after 6 days in culture. Scale bar = 200 μm.

Figure 5.8 An arachnoid sporont of *H. perezi* with a radial amoeboid "gel-like" perimeter (arrows) composed of cytoplasmic extensions, after 6 days in culture. Scale bar = 200 μm.



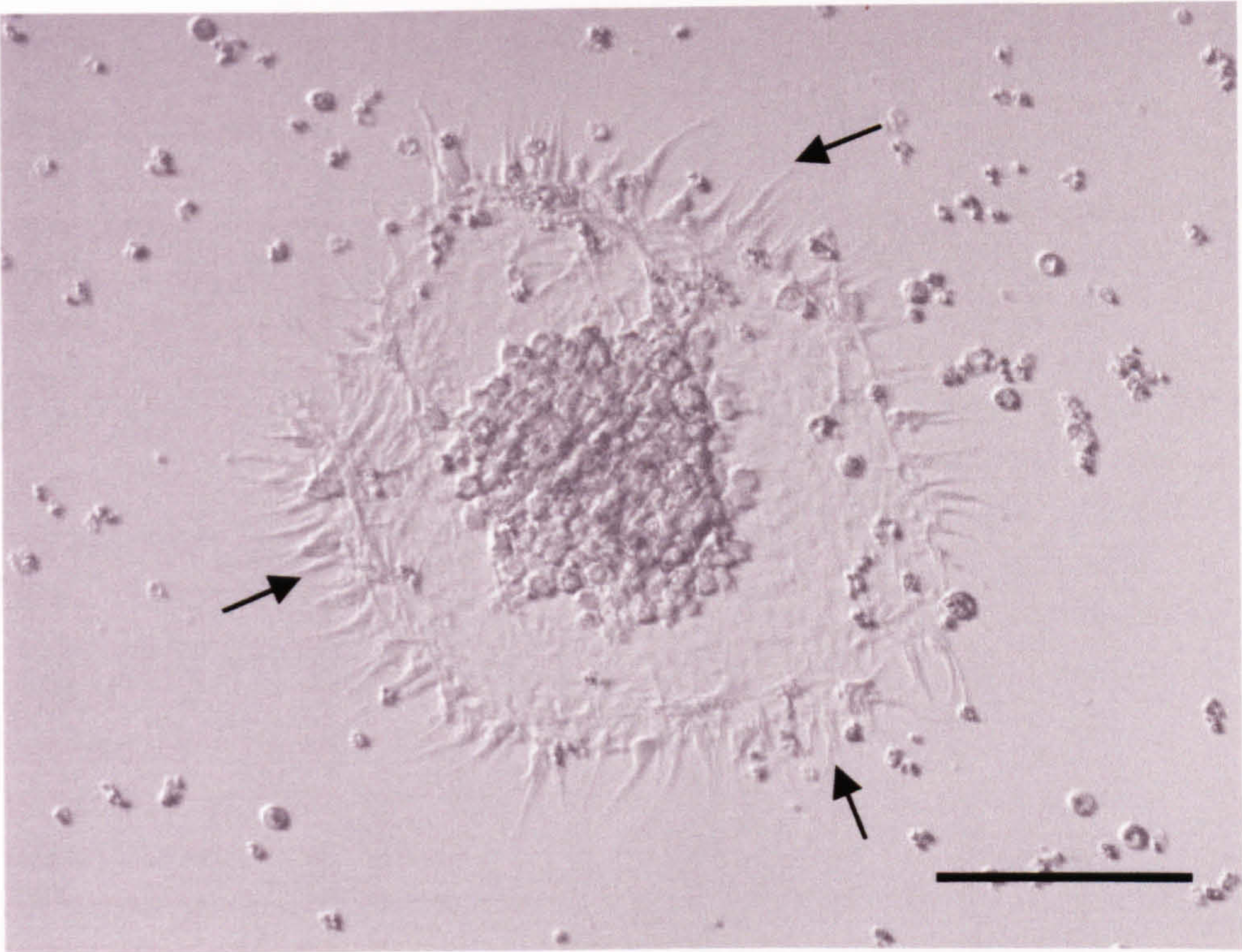
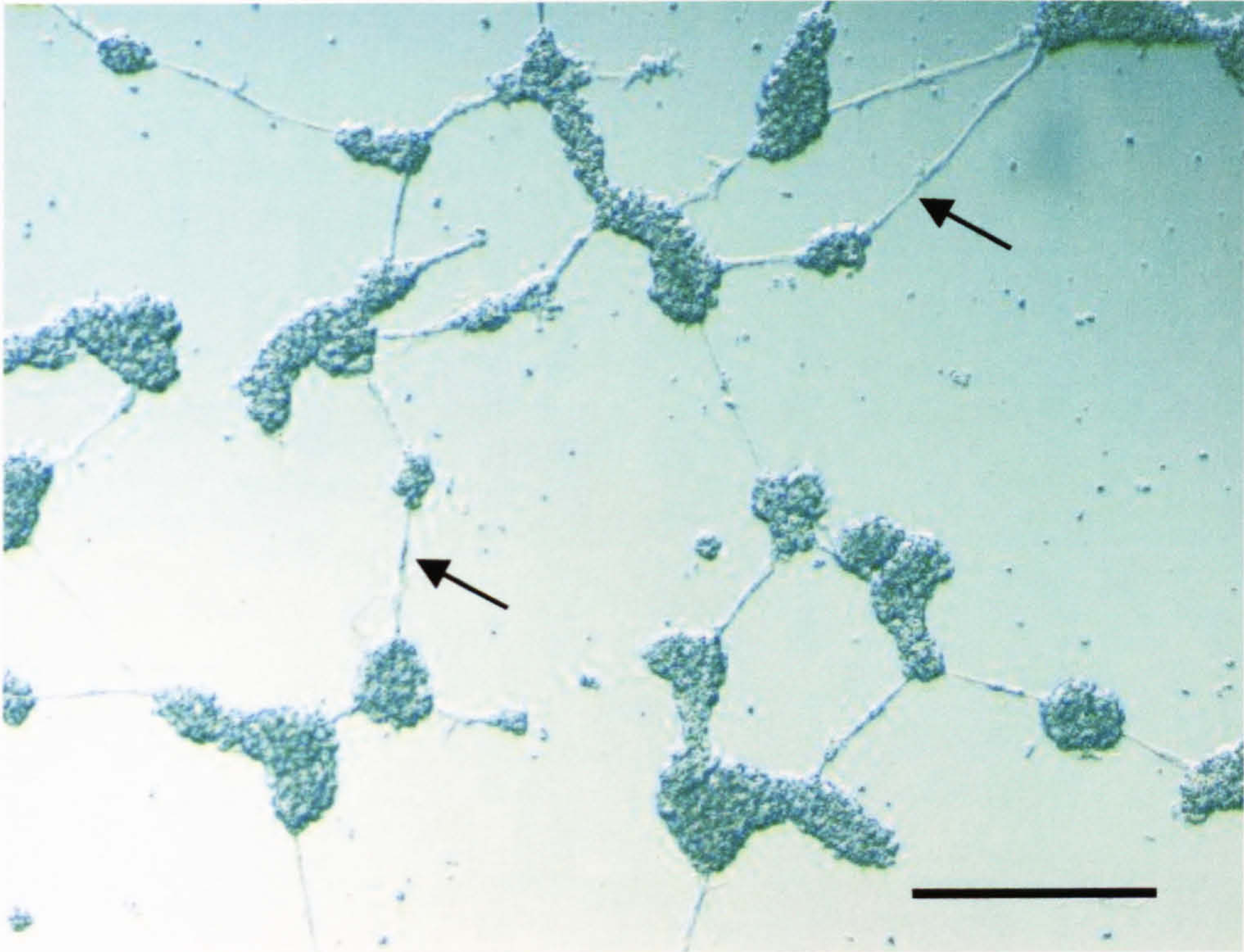


Figure 5.9 Aggregates of *H. perezi* sporoblast cells after 6 days in culture have produced thin cytoplasmic threads (arrows) forming a network between the aggregations. Many of the cytoplasmic threads are not attached to the substratum Scale bar = 500 µm.

Figure 5.10 A cytoplasmic network of *H. perezi* under increased magnification, after 6 days in culture. Scale bar = 50 µm.



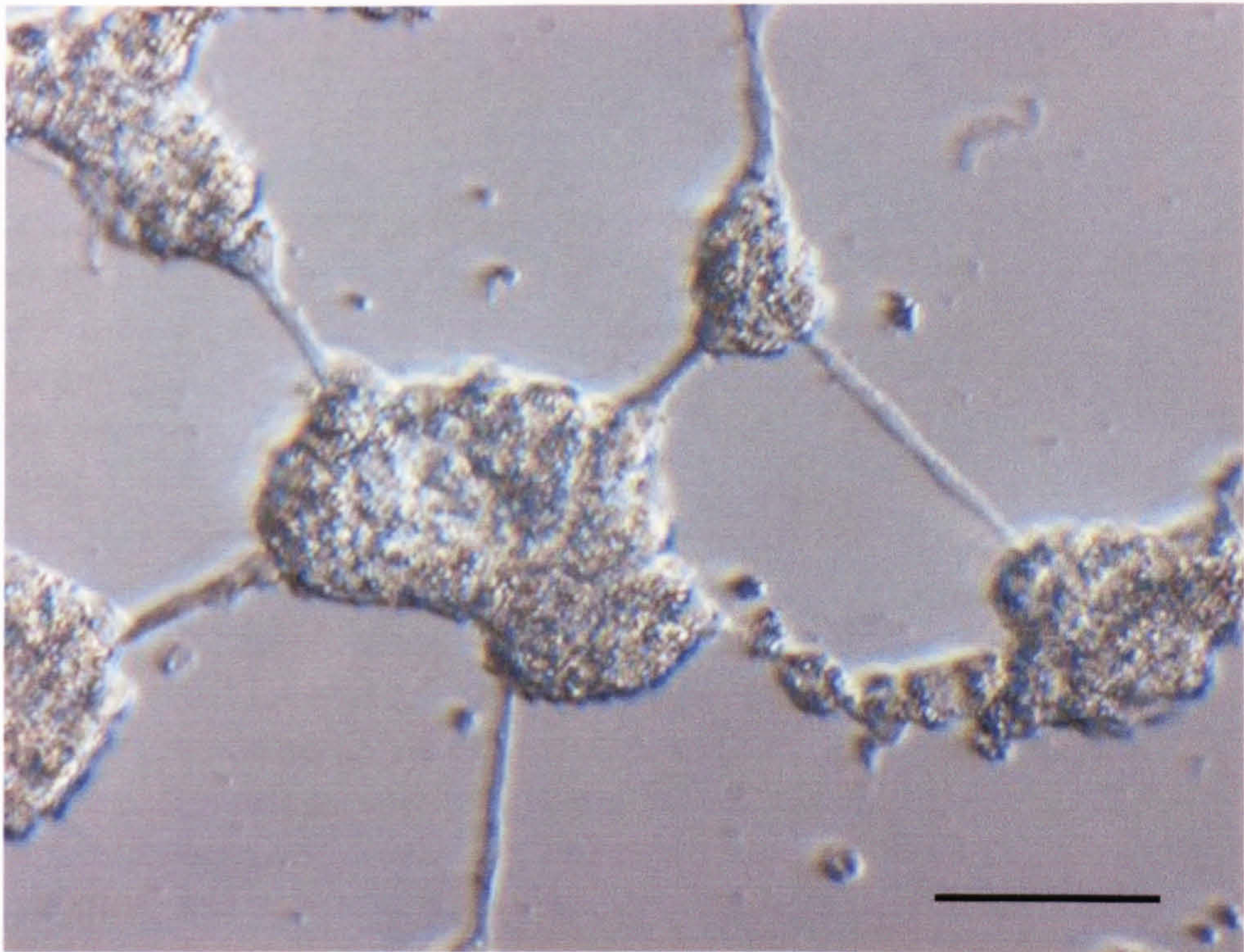
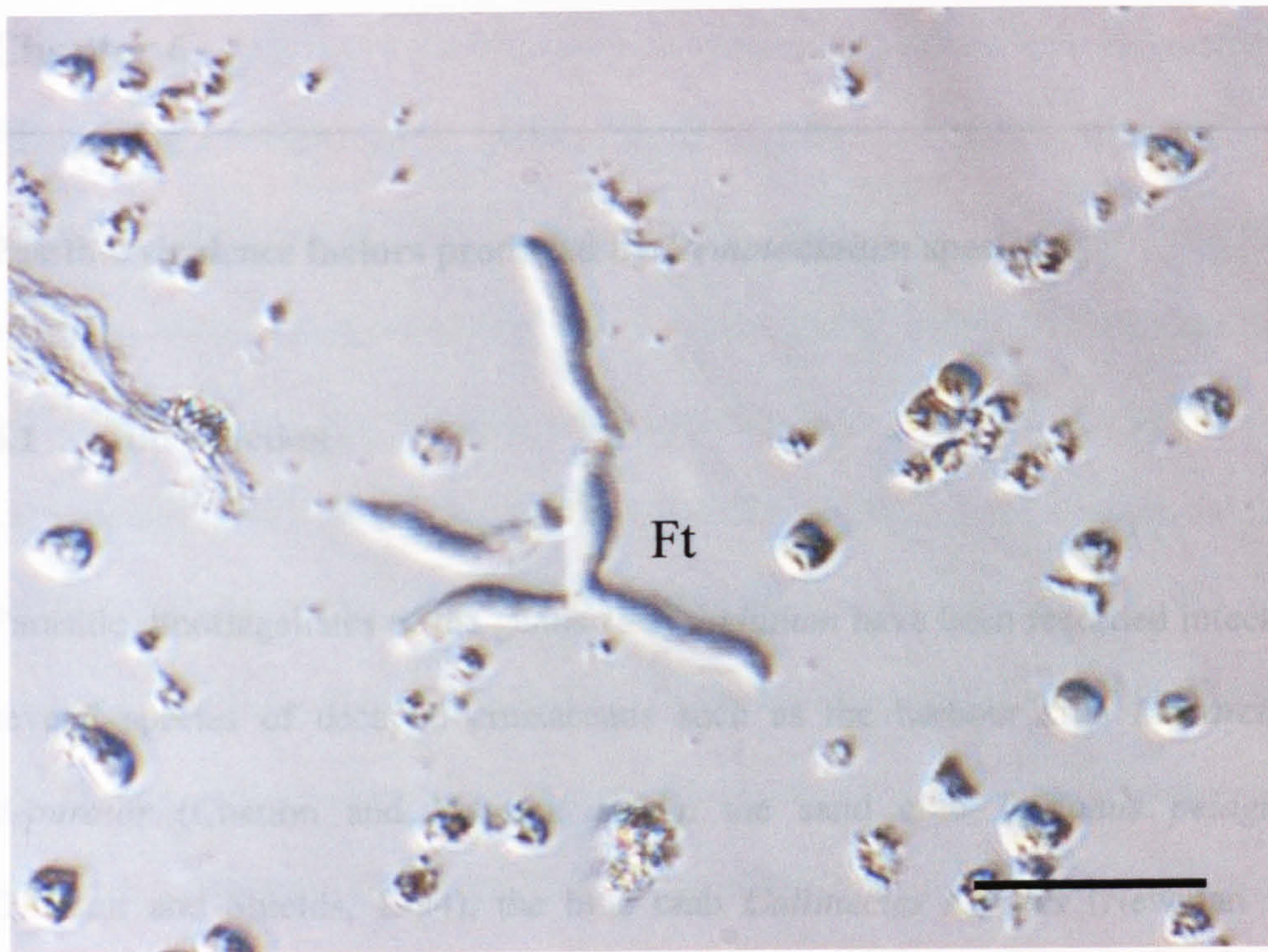
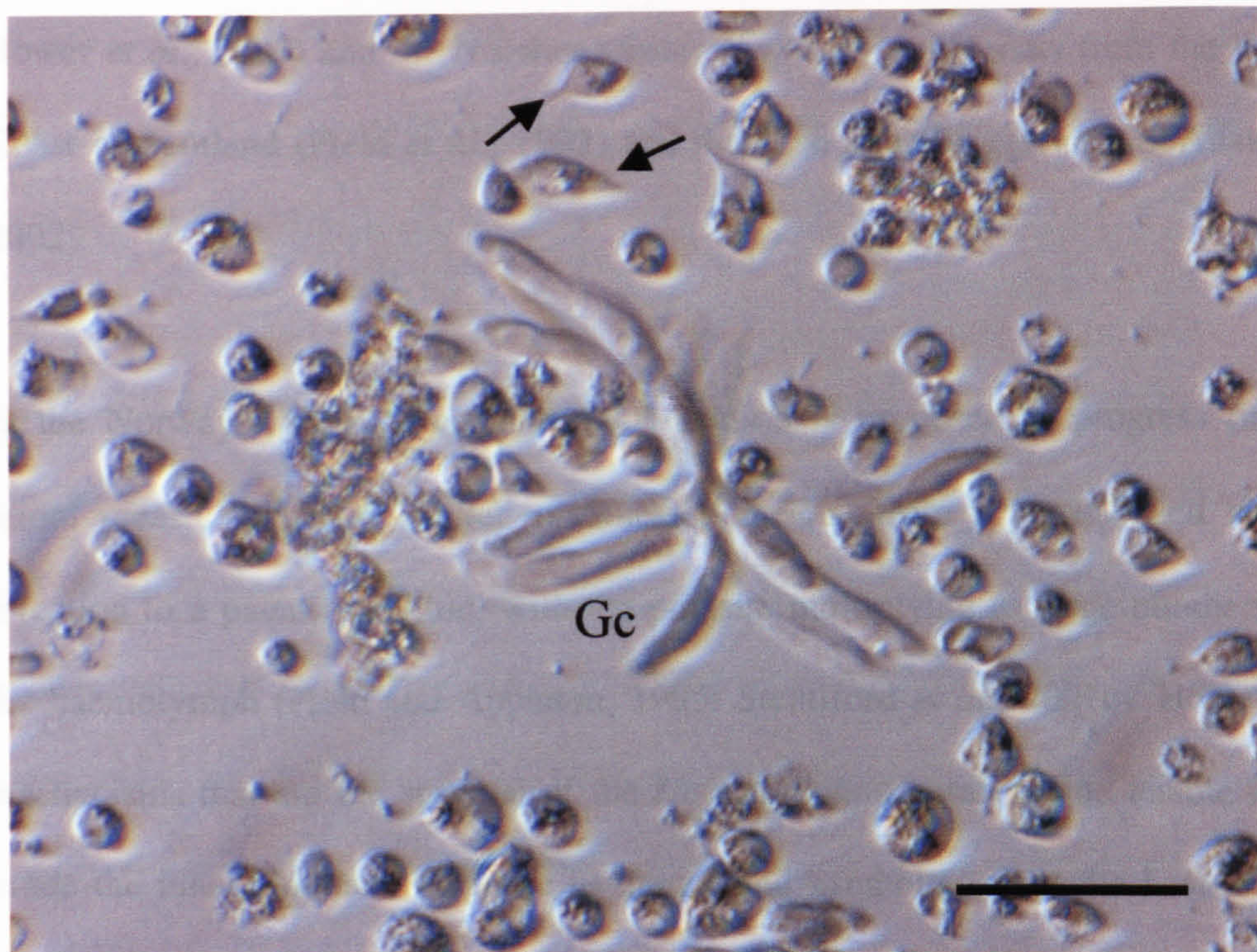


Figure 5.11 Filamentous trophonts (Ft) of *H. perezi*, from an 11-day culture. Scale bar = 100 μm.

Figure 5.12 A gorgonlock colony (Gc) of *H. perezi* formed from the aggregation of several filamentous trophonts after 11 days in culture. Note that several cells are irregular in shape (arrows) and beginning to elongate and differentiate, possibly into filamentous trophonts. Scale bar = 100 μm.


Ft


Gc

Chapter 6

Possible virulence factors produced by *Hematodinium* species.

6.1 Introduction

Parasitic dinoflagellates of the genus *Hematodinium* have been recorded infecting several species of decapod crustaceans such as the harbour crab *Liocarcinus depurator* (Chatton and Poisson, 1931), the sand crab *Portunus pelagicus* (Hudson and Shields, 1994), the blue crab *Callinectes sapidus* (Newman and Johnson, 1975), the edible crab *Cancer pagurus* (Latrouite *et al.*, 1988), several crabs of the genus *Chionoecetes* (Meyers *et al.*, 1987; Taylor and Khan, 1995; Bower *et al.*, 2003) and the Norway lobster *Nephrops norvegicus* from the West Coast of Scotland (Field *et al.*, 1992) and the Irish Sea (Briggs and McAliskey, 2002).

In the Norway lobster, *Hematodinium* infection is proposed to progress (after initial parasite entry by as yet unidentified means) from a tissue-based latent infection to a patent infection, with the parasite multiplying to large numbers in the haemolymph (Field and Appleton, 1995; Stentiford *et al.*, 2001c). However, mechanisms that aid the survival of the *Hematodinium* parasite, and enable it to evade the innate immune response in tissues and haemolymph of the host are at present unknown.

Several pathogens have been shown to possess virulence factors that assist invasion and colonisation of the host, evasion of host defences and the spread of infection (Moss *et al.*, 1995). Mechanisms include antiphagocytic capsules, selective and specific adherence factors, extracellular enzymes, endo- and exo-toxins, and substances that alter phagocyte functions (Roth *et al.*, 1995). Acid phosphatases (AP) have recently been identified as having an important role in microbial pathogenesis. Studies of membrane-bound and secreted AP from several intracellular parasites including, *Leishmania* spp. (Gottlieb, 1980; Gottlieb and Dwyer, 1981a), *Trypanosoma cruzi* (Pereira *et al.*, 1985), *Toxoplasma gondii* (Vivier and Petiprez, 1972) and *Coxiella burnetii* (Baca *et al.*, 1993) suggest that they play a crucial role in their survival within host phagocytic cells. Similarly, the acetosporan oyster parasite *Bonamia ostreae* has AP levels equivalent to those found in several *Leishmania* species (Hervio *et al.*, 1991). Another apicomplexan oyster parasite, *Perkinsus marinus*, has also been shown to contain and secrete AP (Volety and Chu, 1997).

Extracellular parasites such as *Entamoeba histolytica* (Talamás-Rohana *et al.*, 1999) and *L. mexicana* promastigotes (Lovelace *et al.*, 1986) have been found to secrete AP into their surrounding environment. It has been hypothesised that these parasite-derived acid phosphatases inhibit the production of host-derived superoxide ions (Remaley *et al.*, 1984) and can act as virulence markers between isolates (Lovelace and Gottlieb, 1986; Katakura and Kobayashi, 1988; Singla *et al.*, 1992).

Shields *et al.* (2003) demonstrated that the enzymatic profile of haemolymph from blue crabs infected with *H. perezi* differs from that of uninfected haemolymph, with AP, napthol AS-BI phosphohydrolase and β-galactosidase all elevated in infected sera. While high AP activity was detected in infected whole haemolymph, cell-free serum had no detectable activity, indicating that AP activity is located intracellularly in the parasite.

This chapter provides information on the enzymatic profiles of *Hematodinium* spp. from *N. norvegicus* and *C. sapidus*, and in particular on the identification, characterization and ultrastructural location of AP activity in the *Hematodinium* sp. infecting *N. norvegicus*.

6.2 Materials and Methods

6.2.1 Collection and maintenance of experimental animals

Norway lobsters (*Nephrops norvegicus*) were caught by otter bottom trawl (70 mm mesh size) at locations south of Little Cumbrae in the Clyde Sea Area (55.41° N, 4.56° W). The lobsters were transported in a cool, damp environment after capture, then maintained in a closed seawater system at 10 °C and 33 ppt salinity prior to use in experimental work. Blue crabs (*Callinectes sapidus*) were obtained form Virginia, USA, as described in section 5.2.1.

6.2.2 Diagnosis of *Hematodinium* infection and parasite isolation

Haemolymph samples were withdrawn from the base of the fifth pereiopod using a disposable 1 ml syringe and 25-gauge needle following sterilisation of the cuticle with 70% (v/v) ethanol. Norway lobsters with advanced infections were identified by the gross body colour method of Field *et al.* (1992). To confirm infection status, 100 µl samples of haemolymph from lobsters were subjected to ELISA, as described by Small *et al.* (2002) and in Chapter 2. Serum samples from infected and uninfected Norway lobsters were prepared by immediately centrifuging withdrawn haemolymph samples at 400 x g for 10 min at 4 °C. The cell free serum was removed, filtered (0.2 µm) and frozen at –80 °C prior to experimental analysis. Infected blue crabs were identified by examining individual crab haemolymph smears for the uptake of neutral red solution by parasite cells, as described in section 5.2.2.

Parasites were isolated from the haemolymph of blue crabs as described in section 5.2.3. Parasites were isolated from the haemolymph of Norway lobsters by a similar technique: 0.25 ml of infected haemolymph was added to 10 ml of isolation medium in sterile 25 cm^2 tissue culture flasks, which were then incubated for 30 min at 8 °C. For initial parasite isolation, a minimum of 4 flasks were prepared from each lobster. The isolation medium consisted of autoclaved balanced *Nephrops* saline (Appleton and Vickerman, 1998, containing NaCl, 27.99 g l^{-1}; KCl 0.95 g l^{-1}; $CaCl_2$ 2.014 g l^{-1}; $MgSO_4$ 2.465 g l^{-1}; Na_2SO_4 0.554 g l^{-}

^{1}; HEPES 1.92 g l^{-1}) adjusted to pH 7.8, with penicillin G (10 U ml^{-1}) and streptomycin (10 µg ml^{-1}) added to inhibit bacterial contamination. The medium was then filter sterilised (0.2 µm). After initial incubation for 30 min, parasite suspensions were transferred into new sterile 25 cm^2 culture flasks and incubated at 8 °C for a further 30 min. Isolates were then pooled and centrifuged at 400 x g for 10 min at 4 °C; the resulting parasite suspension was then washed three times with isolation medium.

6.2.3 Preparation of parasite lysates

After resuspending the purified parasites in isolation medium, their cell density was assessed using a haemocytometer and was then adjusted to give a range of cell densities (1-10 x 10^6 cells ml^{-1}). Crude cell lysates were prepared by freezing and thawing the sample twice. The cell free lysate was prepared by freezing and thawing the sample twice, followed by centrifugation (10,000 x g for 10 min at 4 °C). The resulting supernatant fraction represented the cell-free parasite lysate used in this study. This was filtered through a 0.2 µm filter and stored frozen at – 80 °C.

To investigate AP activity in cell membrane and soluble components of crude cell lysates, aliquots of crude lysates (10 x 10^6 cells ml^{-1}) were centrifuged at 10,000 x g for 10 min at 4 °C. The resulting supernatant (soluble fraction of the crude lysate) was removed, and filtered (0.2 µm) prior to experimental use. The

remaining cell debris pellet was washed three times with isolation medium, and was then resuspended in the original cell sample volume of isolation medium (to give the all membrane fraction). All three samples (crude lysate, soluble fraction and cell membrane fraction) were assayed for AP activity as described in section 6.2.6.

6.2.4 Parasite culture

Isolated *Hematodinium* sporoblast parasites (section 6.2.2) were resuspended in isolation medium supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS). Cell density was assessed using a haemocytometer and adjusted to give 1 x 10^6 or 2 x 10^6 cells ml^{-1}. Cell suspensions (2 ml) were incubated in triplicate in 12 well culture plates at 8 °C for 7 days (for *Hematodinium* cells derived from the Norway lobster), or 23 °C for 6 days (for *Hematodinium* cells derived from blue crabs). Cultures were checked for contamination daily. On day 6/7, the cell suspensions in each well were collected, and cell viability was assessed using trypan blue (0.25% w/v in isolation medium) for *Hematodinium* cells derived from the Norway lobster, and both trypan blue and neutral red solution (0.25% w/v in isolation medium) for *Hematodinium* cells derived from blue crabs. Parasites were separated from the cell culture medium by centrifugation at 800 x g for 10 min. The cell-free culture medium was removed, passed through a 0.2 μm filter and frozen at -80 °C. Culture medium in which no *Hematodinium* cells had been incubated was used as a control.

6.2.5 API ZYM enzyme analysis

Parasite cell lysates, the isolation medium and culture media used were assayed in duplicate (from each of 3 cultures/media samples) using the manufacturer's (BioMérieux) protocol for the following enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, napthol-AS-BI-phosphohydrolase, α-galactosidase, β- galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase. Briefly, 65 µl of the sample was added to each cupule and the test strips were incubated for 4 h at 37 °C. Following incubation, 1 drop of ZYM A (API; tris-hydroxymethyl-aminomethane, hydrochloric acid, sodium lauryl sulphate, H_2O) and ZYM B (API; fast blue BB, 2-methoxyethanol) was added to each cupule and the reaction was allowed to proceed for 5 min. The test strips were then read and the results scored using the following scale: 0, negative reaction; 1, weak positive; 2-3, positive reaction; 4-5, strong positive reaction.

6.2.6 AP assay

AP activity in *Hematodinium* cell lysates, culture medium, and serum from infected Norway lobsters was assayed using the colorimetric method of Bodley *et al.* (1995), with minor modifications. The assay is based on the release of p-nitrophenol (PNP) and inorganic phosphate (IP) from p-nitrophenylphosphate

(PNPP) by the AP enzyme. The substrate, p-nitrophenylphosphate, was prepared at a concentration of 40 mg ml^{-1} in 1 M sodium acetate at pH 5.5. The assay was initiated by the addition of 100 µl cell lysate/cell culture medium, or 50 µl lobster serum, to 10 µl substrate solution. Samples were incubated for 2-8 h at 37 °C. The resulting yellow-coloured reaction product was measured at 405 nm on a microtiter plate reader (Titerkek Multiscan MCC/340).

Controls comprised isolation and cell culture media without the addition of *Hematodinium* cells, and uninfected Norway lobster serum. The activity of AP enzyme was determined using an extinction coefficient of 1.83×10^4 M^{-1} cm^{-1} and a path length of 0.4 cm on the plate reader. Activities were expressed as the amount (mol) of PNP formed per hour by the volume of sample assayed. Statistical analysis of the enzymatic activities in the culture media with different densities of *Hematodinium* cells, and the lobster serum samples was performed using the Mann-Whitney U test. Significance was considered to be at $p < 0.05$.

6.2.7 AP histochemistry

Haemolymph smears from a *Hematodinium*-infected lobster were air-dried and stained using the simultaneous azo dye coupling method for AP (Burstone, 1958) as detailed in Bancroft (1967). The recommended substrate, naphthol AS-BI phosphate was used along with hexazonium pararosanilin as the diazonium salt. The combination of hexazonium pararosanilin and naphthol AS-BI phosphate

allows the accurate localization of AP as a bright red reaction product at the sites of enzyme activity. Following the localization of AP, the smears were lightly stained with haematoxylin (Carazzi, 1911) as a nuclear counterstain.

6.3 Results

6.3.1 *Hematodinium* cell culture

Hematodinium sporoblast cells were observed to aggregate into clumps in the bottom of cell culture wells. This clumping of cells prevented cell density measurements. Measurement of lactate dehydrogenase (as a marker of cell integrity), by the method of Denton (1996) was attempted, but the cell culture media used gave a strong positive result, eliminating this as a viability indicator. However, cell viability was consistently over 99 % based on trypan blue/neutral red staining.

6.3.2 API ZYM enzyme profiles of *Hematodinium* cell lysates and cell culture media

The results of enzymatic activities of *Hematodinium* (from the Norway lobster) and *H. perezi* (from the blue crab) cell lysates are shown in Tables 6.1 and 6.2. Cell lysates of *Hematodinium* were strongly positive for acid phosphatase, and were positive for napthol-AS-BI-phosphohydrolase and α-fucosidase. Weak

positive reactions were observed for esterase (C4), leucine arylamidase, β-glucuronidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase. Negative reactions were obtained for all other enzymes of the API ZYM system. Cell lysates of *H. perezi* had a similar profile, with strong positive activity observed for acid phosphatase, and weak positive activity for esterase (C4), napthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase. In contrast to the lysates of parasites from the Norway lobster, strong positive reactions were observed for leucine arylamidase. Negative reactions were observed for all other enzymes tested. Negative reactions for all enzyme reactions were obtained from both of the cell isolation media used in this study.

Enzymatic activities of *Hematodinium* from the Norway lobster and *H. perezi* cell culture media are shown in Tables 6.3 and 6.4. Strong positive reactions for several enzymes were observed for the control *Hematodinium*-free culture medium, which prevented them being assayed in the *Hematodinium* cell culture mediums. Measurable increases in enzyme activity between control culture media and *Hematodinium* cell culture media were observed with acid phosphatase (weak positive to strong positive) and napthol-AS-BI-phosphohydrolase (weak positive to positive). *H. perezi* cell culture media were observed to have only one enzyme, leucine arylamidase, with a measurable difference in activity between control and cell culture media (positive to strong positive).

6.3.3 AP activity in *Hematodinium* cell lysates

No AP activity was detected in the isolation medium that was used to make *Hematodinium* cell suspensions for lysate experiments. AP activity, directly proportional to cell density was observed for crude cell lysates (Fig. 6.1).

Fractionation of crude *Hematodinium* cell lysates into soluble and membrane components allowed the distribution of intracellular AP activity to be examined (Fig. 6.2). The soluble and membrane components accounted for 69.5 ± 1.4 % and 33.0 ± 1.7 %, respectively, compared to the total crude lysate activity.

6.3.4 AP activity in *Hematodinium* cell culture medium

A low level of AP activity was detected in the control, *Hematodinium*-free, cell culture media (0.17 ± 0.02 nmol h^{-1} 100 µl $sample^{-1}$) and was attributed to enzymatic activity remaining in the FCS present in the medium. However, a cell density-dependent enzyme activity was observed in *Hematodinium* culture media after 7 days incubation (Fig. 6.3). Levels of AP activity in culture media seeded with 1 x 10^6 cells ml^{-1} (0.38 ± 0.02 nmol h^{-1} 100 µl $sample^{-1}$) and 2 x 10^6 cells ml^{-1} (0.50 ± 0.03 nmol h^{-1} 100 µl $sample^{-1}$) were both significantly higher than in the control media (Mann-Whitney U test: $p = 0.01$).

6.3.5 AP activity in *Hematodinium*-infected *N. norvegicus* serum

Cell-free serum from patently *Hematodinium*-infected Norway lobsters had significantly higher AP activity (4.00 ± nmol h^{-1} 50 µl $sample^{-1}$) than was present in cell-free haemolymph from uninfected lobsters (as confirmed by PCR, see Ch 3) (0.33 ± 0.03 nmol h^{-1} 50 µl $sample^{-1}$) (Fig. 6.4) (Mann-Whitney U test: $p = 0.001$).

6.3.6 Localisation of AP activity in *Hematodinium* cells

Light microscopic examination of *Hematodinium* cell smears stained for AP activity using the described procedure indicated that the enzyme is localised to cytoplasmic granules and to the membrane surrounding the cell nucleus (Fig. 6.5). No deposition of reaction product was detectable in the nucleus, or associated with the outer membrane or any other cellular structures.

6.4 Discussion

In the present study, enzyme profiles of two species of the parasitic dinoflagellate *Hematodinium* from *N. norvegicus* and *C. sapidus* have been characterized. In particular, intracellular and extracellular AP activity in *Hematodinium* cells from *N. norvegicus* have been analysed.

The API ZYM system has been used successfully to identify and type a number of medically important bacterial pathogens (Poh and Loh, 1985; Poh and Loh, 1988; Hermosa de Mendoza *et al.*, 1993; Heroldova *et al.*, 2001), fungal pathogens (Youngchim *et al.*, 1999; Malinowski *et al.*, 2001), and yeasts (García-Martos *et al.*, 2000). Thus, as well as providing information on the cellular metabolism of this parasite, the pattern of enzyme activities may be useful in helping to differentiate different species of *Hematodinium.*

Cell lysates of *Hematodinium* from the Norway lobster and the blue crab showed different patterns of enzymes present within the parasite cells, with those of *H. perezi* having high levels of both AP and leucine arylamidase activity, while *Hematodinium* from *N. norvegicus* has high levels of AP, but only very low levels of leucine arylamidase. Differences also exist in the secretion of enzymes, as reflected in the composition of the culture media used. Cell culture medium from blue crab *H. perezi* had no AP activity but high levels of leucine arylamidase activity, while that from Norway lobster *Hematodinium* had high AP activity but no detectable leucine arylamidase activity. This finding indicates that AP is located intracellularly and is not secreted by *H. perezi.* This is consistent with the result reported by Shields *et al.* (2003), that this enzyme is detectable in whole haemolymph but not in the cell-free serum from an infected crab. However, measurements of AP activity in the cell-free serum were obtained from only one infected individual, and needs to be repeated in order to confirm the absence of AP secretion by *H. perezi in vivo.* The reasons for *H. perezi* not secreting AP are

unclear at present, but the results suggests that this species may have other mechanisms for evading the host immune reaction, as indicated by leucine arylamidase secretion (see below).

Cell culture medium from blue crab *H. perezi* was found to have high levels of leucine arylamidase activity. The fact that AP activity was high in the cell lysates but absent in culture medium indicates that there was no significant cell death in the media. Such cell death, if it had occurred, may have given rise to false positive measures of enzyme secretion. This strongly suggests that the extra-cellular leucine arylamidase activity detected in the medium is the result of genuine secretion, rather than cell death. Extracellular N-terminal proteolytic activity by leucine arylamidase has been observed in several pathogens (Grehn *et al.*, 1991; Dettori *et al.*, 1995; Farto *et al.*, 1998), has been used to identify *Clostridium difficile* (Kudhair *et al.*, 1986), and has been implicated as a virulence factor in a *Vibrio* strain infecting turbot (Farto *et al.*, 1998). This may also be the case for *H. perezi*, since Shields and Squyars (2000) have shown that experimentally-infected blue crabs (1 x 10^5 *H. perezi* cells/crab) die on average between 14 and 40 days post-inoculation, and have a mortality rate of 86 %. Eaton *et al.* (1991) also reported experimental infection of tanner crabs following injection of naturally-generated dinospores of their *Hematodinium* sp. In contrast, experimental infection of Norway lobsters with its associated *Hematodinium sp.* has never been successful (Vickerman, 1994; Appleton and Vickerman, 1998). Thus, the discovery that *H. perezi* cells secrete an aminopeptidase may be linked with its

increased virulence when compared to the *Hematodinium* sp. from *N. norvegicus*. This warrants further investigation.

In this study, both *Hematodinium* species have been shown to possess high intracellular AP activity, but only one species, that from the Norway lobster, has been found to secrete AP during *in vitro* culture (Fig. 6.3). This finding is substantiated by the elevated levels of the enzyme measured in cell-free serum from infected lobsters (Fig 6.4).

In many microorganisms, AP activity is associated with pathogenic mechanisms and virulence. Studies on AP from *Leishmania donovani* (Remaley *et al.*, 1984), *Entamoeba histolytica* (Talamás-Rohana *et al.*, 1999), *Coxiella burnetii* (Baca *et al.*, 1993), and *Legionella micdadei* (Saha *et al.*, 1985) suggest that they may play an important role in the modulation of the host immune response, while De Jonckheere and Dierickx (1982) have shown increased AP levels in pathogenic species of *Naegleria fowleri* compared to non-pathogenic species. *Leishmania* AP has been shown to inhibit superoxide ion production by human neutrophils (Remaley *et al.*, 1984). Likewise, *B. ostreae* (Hervio *et al.*, 1988), *P. marinus* (Volety and Chu, 1995), and rickettisiales-like organisms (Le Gall *et al.*, 1991), all of which contain or secrete AP, have been observed to interfere with the production of superoxide radicals (O_2^-) by host bivalve haemocytes *in vitro*.

Reactive oxygen species (ROS) are produced by stimulated haemocytes from many aquatic organisms, including fish (Higson *et al.*, 1984; Secombes *et al.*, 1988), molluscs (Dikkeboom *et al.*, 1987; Pipe, 1992) and crustaceans (Bell and Smith, 1993, 1994) during the respiratory burst. Hence the secretion of AP by *Hematodinium* cells may cause suppression of ROS production by lobster haemocytes by dephosphorylating the enzymes involved in manufacture of ROS, such as protein kinase C. Studies involving the measurement of possible inhibition of ROS by *Hematodinium* AP are needed to clarify this potential pathogenic role.

Although AP is classically considered to be a lysosomal marker, it has an extra-lysosomal distribution in several organisms. The enzyme has been found on the surface membrane of *Leishmania donovani* promastigotes (Gottlieb and Dwyer, 1981b), in the dense granules and rhoptries of *Toxoplasma gondii* (Metsis *et al.*, 1995), in the dense bodies of *Bonamia ostrea* (Hervio *et al.*, 1991), in the periplasmic space of *C. burnetii* (Baca *et al.*, 1993), and in the vacuoles and at the amoeba-host cell interface of *E. histolytica* (Ventura-Juárez *et al.*, 2000). Localisation studies on *Hematodinium* cells indicate that AP is associated with cytoplasmic vesicles (Fig. 6.5). Fractionation studies support this, as they indicate that enzyme activity is predominantly within the soluble fraction, with approximately 30 % being membrane-associated (Fig. 6.2). Such vesicles might represent lysosomes as *Hematodinium* cells have previously been shown to possess micropores (Appleton and Vickerman, 1996) that may permit endocytosis. Alternatively, such vesicles may contain soluble enzyme destined for

exocytosis. Characterisation of secreted, soluble, and membrane associated AP forms is needed to clarify its role in *Hematodinium.*

It is worth considering that many important cellular processes are controlled by phosphorylation and dephosphorylation of proteins. Phosphatases are involved in DNA synthesis (Brautigan, 1992), cell cycle regulation (Freeman and Donoghue, 1991) and signal transduction (Walton and Dixon, 1993). Consequently *Hematodinium* AP may be involved in the sequence of events that lead to cell division, although the enzymes would function internally and should not be detectable in culture medium or in the host serum.

It is also possible that the enzymatic profiles of the *Hematodinium* species investigated may be influenced by life cycle stage and culture conditions. Although the *in vitro* cultures of both parasites consisted of morphologically similar sporoblast cells, the *in vitro* life cycle of *H. perezi* has not been fully documented and may differ from that of *Hematodinium* sp. from *N. norvegicus* (Appleton and Vickerman, 1998). *H. perezi* cells were cultured at 23 °C while those of the *Hematodinium* sp. from *N. norvegicus* was cultured at 8 °C. Although the two culture media had the same basic constituents, some were at different concentrations, and the *H. perezi* medium contained glucose and a different source of FCS. Thus differences in salt concentration, glucose and FCS may have influenced the catalytic activities of the enzymes assayed.

Table 6.1 Enzymatic activities of Norway lobster *Hematodinium* cell lysates (1×10^6 ml^{-1}) detected by the API ZYM system. ++, strong positive; +, positive; ± weak positive; -, negative.

Table 6.2 Enzymatic activities of blue crab *Hematodinium perezi* cell lysates (1×10^6 ml^{-1}) detected by the API ZYM system. ++, strong positive; +, positive; ± weak positive; -, negative.

Enzyme	Isolation medium	Cell lysate ($1x10^6$ ml^{-1})
Alkaline phosphatase	-	-
Esterase (C4)	-	±
Esterase lipase (C8)	-	-
Lipase (C14)	-	-
Leucine arylamidase	-	±
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α-chymotrypsin	-	-
Acid phosphatase	-	++
Napthol-AS-BI-phosphohydrolase	-	+
α-galactosidase	-	-
β- galactosidase	-	-
β-glucuronidase	-	±
α-glucosidase	-	±
β-glucosidase	-	±
N-acetyl-β-glucosaminidase	-	±
α-mannosidase	-	-
α-fucosidase	-	+

Enzyme	Isolation medium	Cell lysate ($1x10^6$ ml^{-1})
Alkaline phosphatase	-	-
Esterase (C4)	-	±
Esterase lipase (C8)	-	-
Lipase (C14)	-	-
Leucine arylamidase	-	++
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α-chymotrypsin	-	-
Acid phosphatase	-	++
Napthol-AS-BI-phosphohydrolase	-	±
α-galactosidase	-	-
β- galactosidase	-	-
β-glucuronidase	-	-
α-glucosidase	-	-
β-glucosidase	-	-
N-acetyl-β-glucosaminidase	-	±
α-mannosidase	-	-
α-fucosidase	-	-

Table 6.3 Enzymatic activities of Norway lobster *Hematodinium* cell culture media (1×10^6 ml^{-1}) after 7-day incubation, detected by the API ZYM system. ++, strong positive; +, positive; ± weak positive; -, negative.

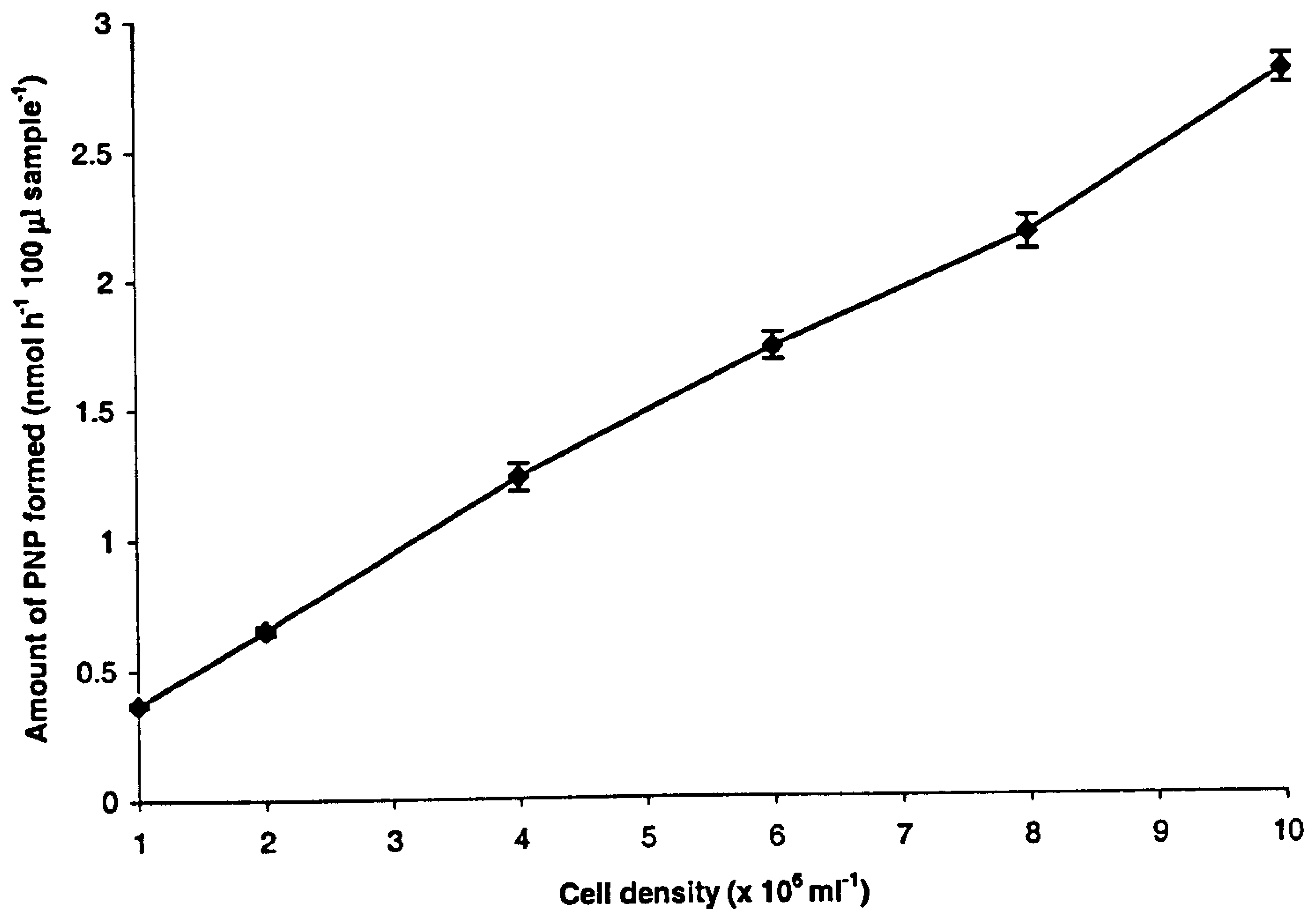
Table 6.4 Enzymatic activities of *H. perezi* cell culture media (1×10^6 ml^{-1}) after 6-day incubation, detected by the API ZYM system. ++, strong positive; +, positive; ± weak positive; -, negative.

Enzyme	Control culture medium	Cell culture medium ($1x10^6$ ml^{-1})
Alkaline phosphatase	++	++
Esterase (C4)	+	+
Esterase lipase (C8)	+	+
Lipase (C14)	-	-
Leucine arylamidase	++	++
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α-chymotrypsin	-	-
Acid phosphatase	±	++
Napthol-AS-BI-phosphohydrolase	±	+
α-galactosidase	-	-
β- galactosidase	±	±
β-glucuronidase	-	-
α-glucosidase	±	±
β-glucosidase	±	±
N-acetyl-β-glucosaminidase	±	±
α-mannosidase	±	±
α-fucosidase	-	-

Enzyme	Control culture medium	Cell culture medium ($1x10^6$ ml^{-1})
Alkaline phosphatase	±	±
Esterase (C4)	+	+
Esterase lipase (C8)	+	+
Lipase (C14)	-	-
Leucine arylamidase	+	++
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α-chymotrypsin	-	-
Acid phosphatase	±	±
Napthol-AS-BI-phosphohydrolase	±	±
α-galactosidase	-	-
β- galactosidase	-	-
β-glucuronidase	-	-
α-glucosidase	-	-
β-glucosidase	-	-
N-acetyl-β-glucosaminidase	±	±
α-mannosidase	-	-
α-fucosidase	-	-

Figure 6.1 AP activity of crude *Hematodinium* cell lysates. Mean ± SD, N=5. Assay incubation time: 6 h.

Figure 6.2 Determination of AP activity in *Hematodinium* cell lysates (10×10^6 cells ml^{-1}). Data are represented as percentage of activity associated with each fraction, where activity of 100% (crude lysate) is equivalent to 3.02 ± 0.023 nmol h^{-1} 100 μl^{-1} sample PNP formed during the 6 h assay incubation time. Mean ± SD, N=5.


Amount of PNP formed (nmol h-1 100 µl sample-1)
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
Cell density (x 10^6 ml-1)

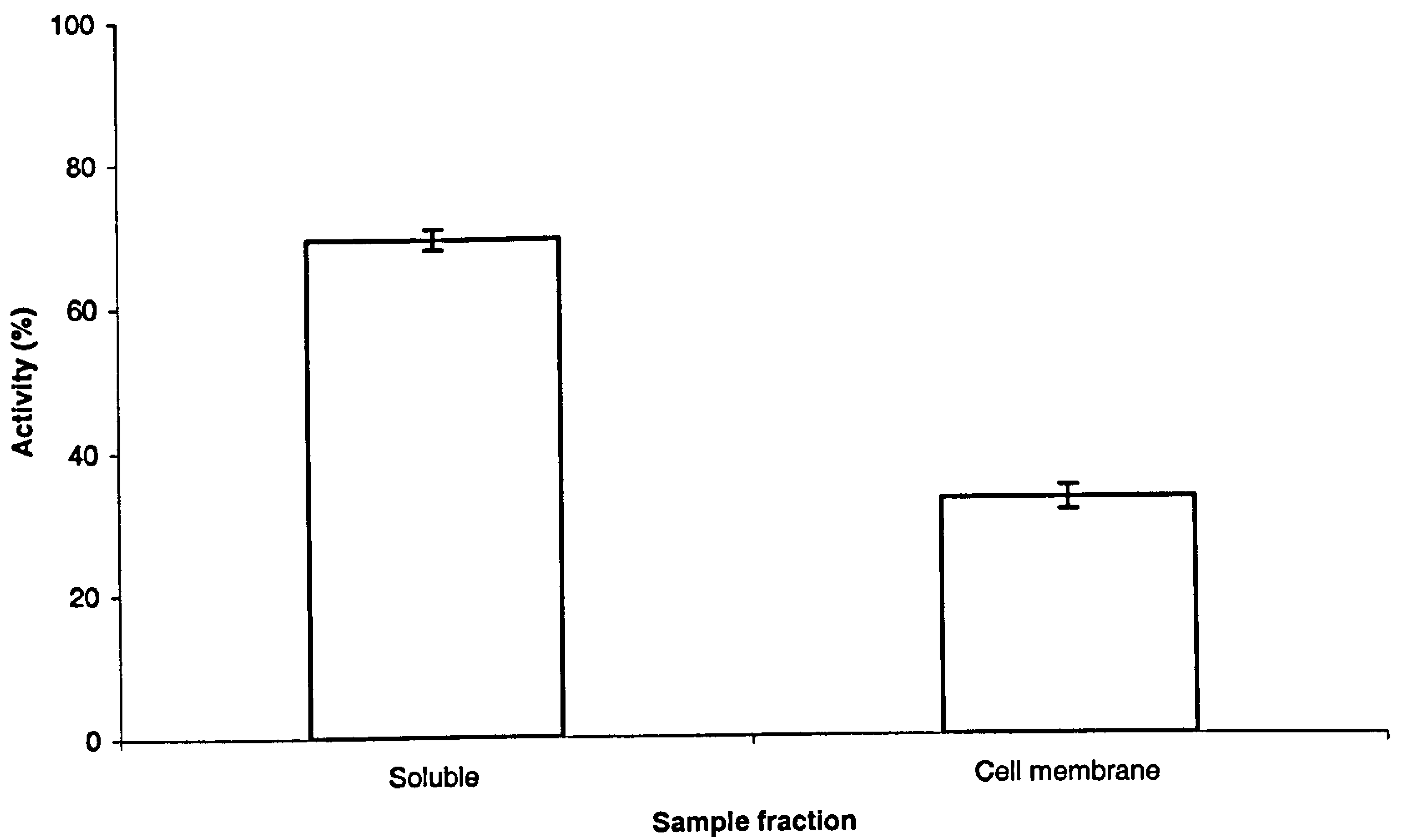
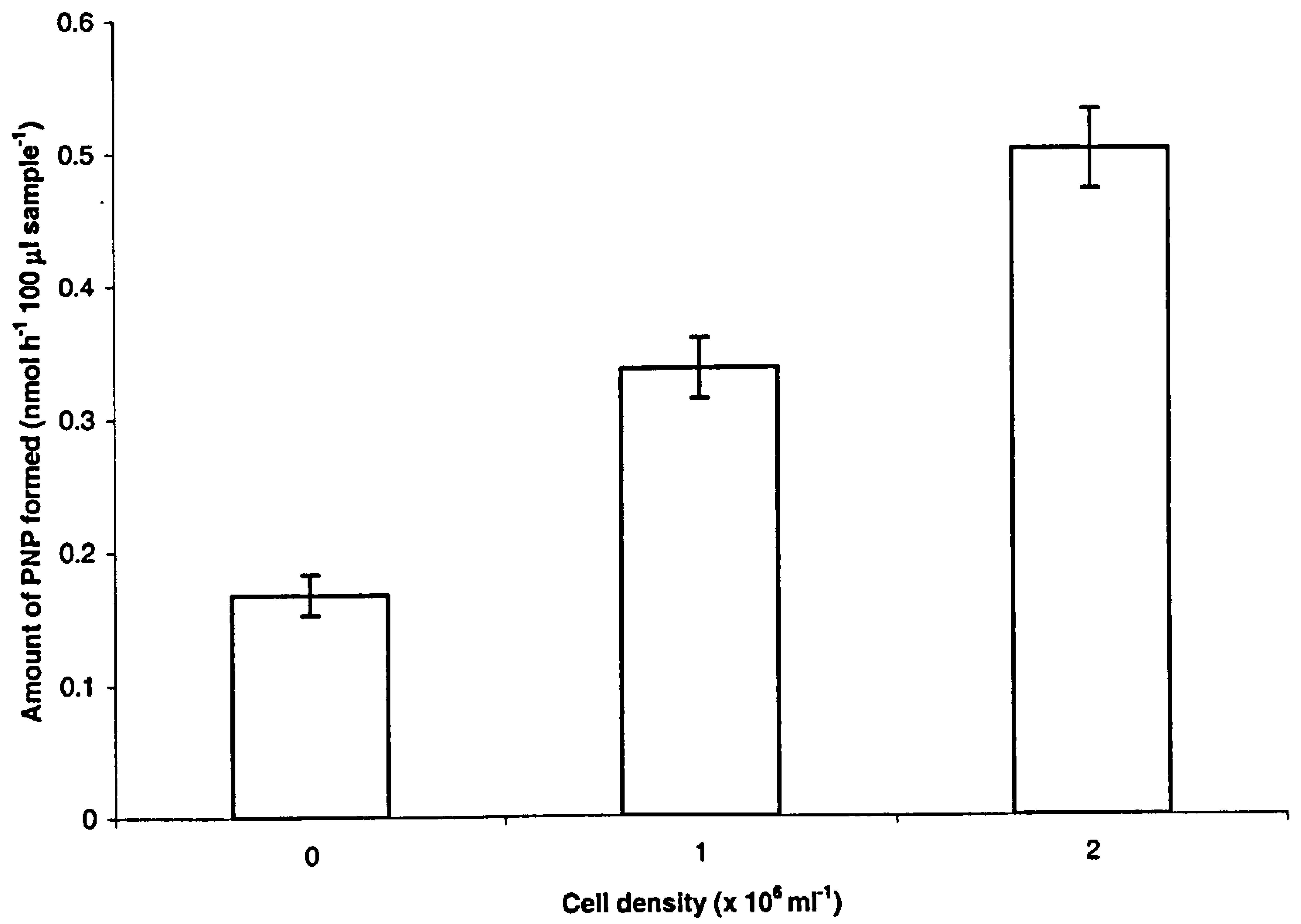

Activity (%)
100
80
60
40
20
0
Soluble
Cell membrane
Sample fraction

Figure 6.3 AP activity of *Hematodinium* culture media with initial densities of 0, 1 and 2 x 10^6 cells ml^{-1} after 7-day incubation. Mean ± SD, N=5. Assay incubation time: 8 h. Data are representative of three separate experiments.

Figure 6.4 AP activity of serum samples from 6 *Hematodinium*-infected and uninfected *N. norvegicus*. Mean ± SD, N=24. Assay incubation time: 3 h.


Amount of PNP formed (nmol h-1 100 µl sample-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
Cell density (x 10⁶ ml-1)

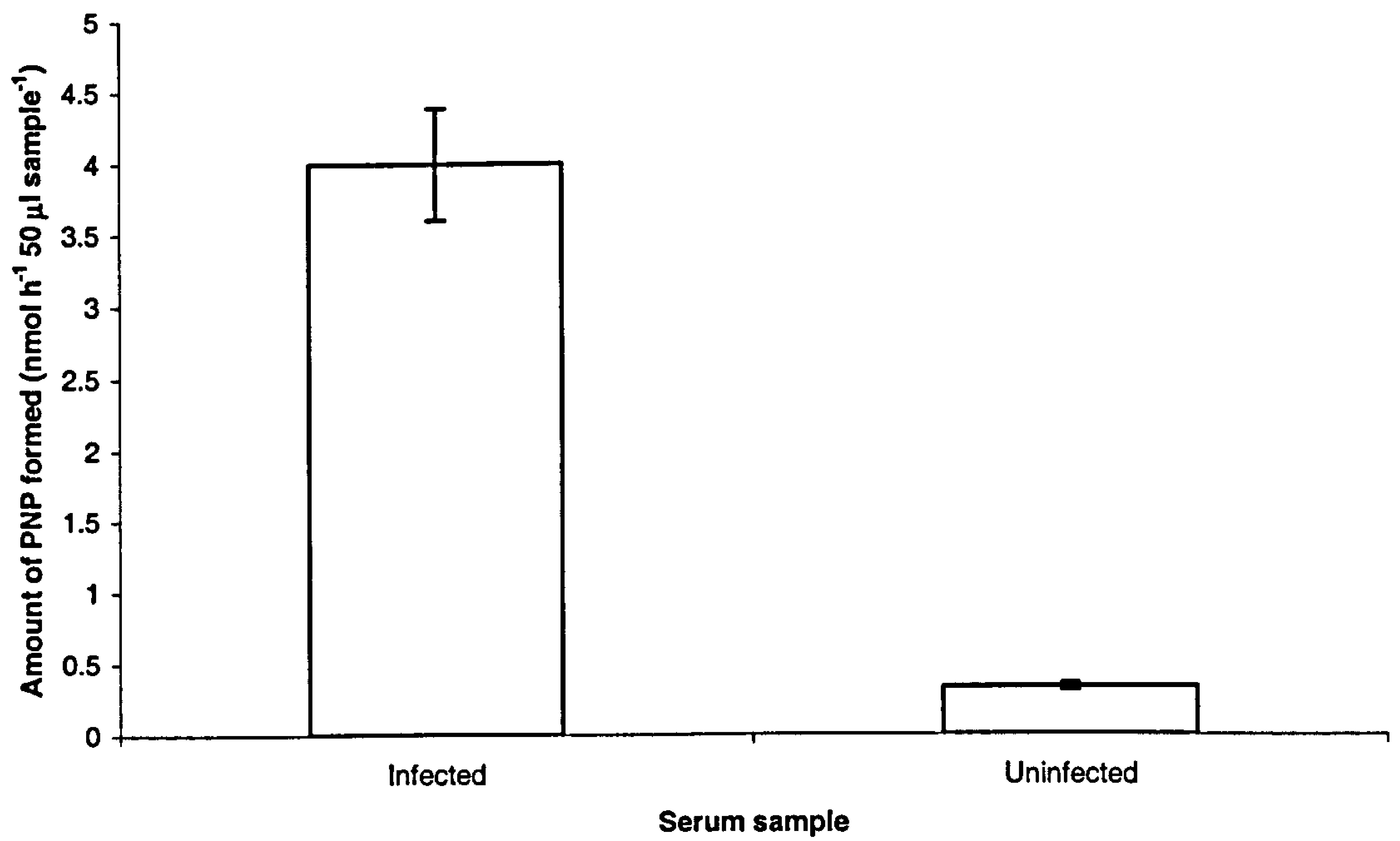
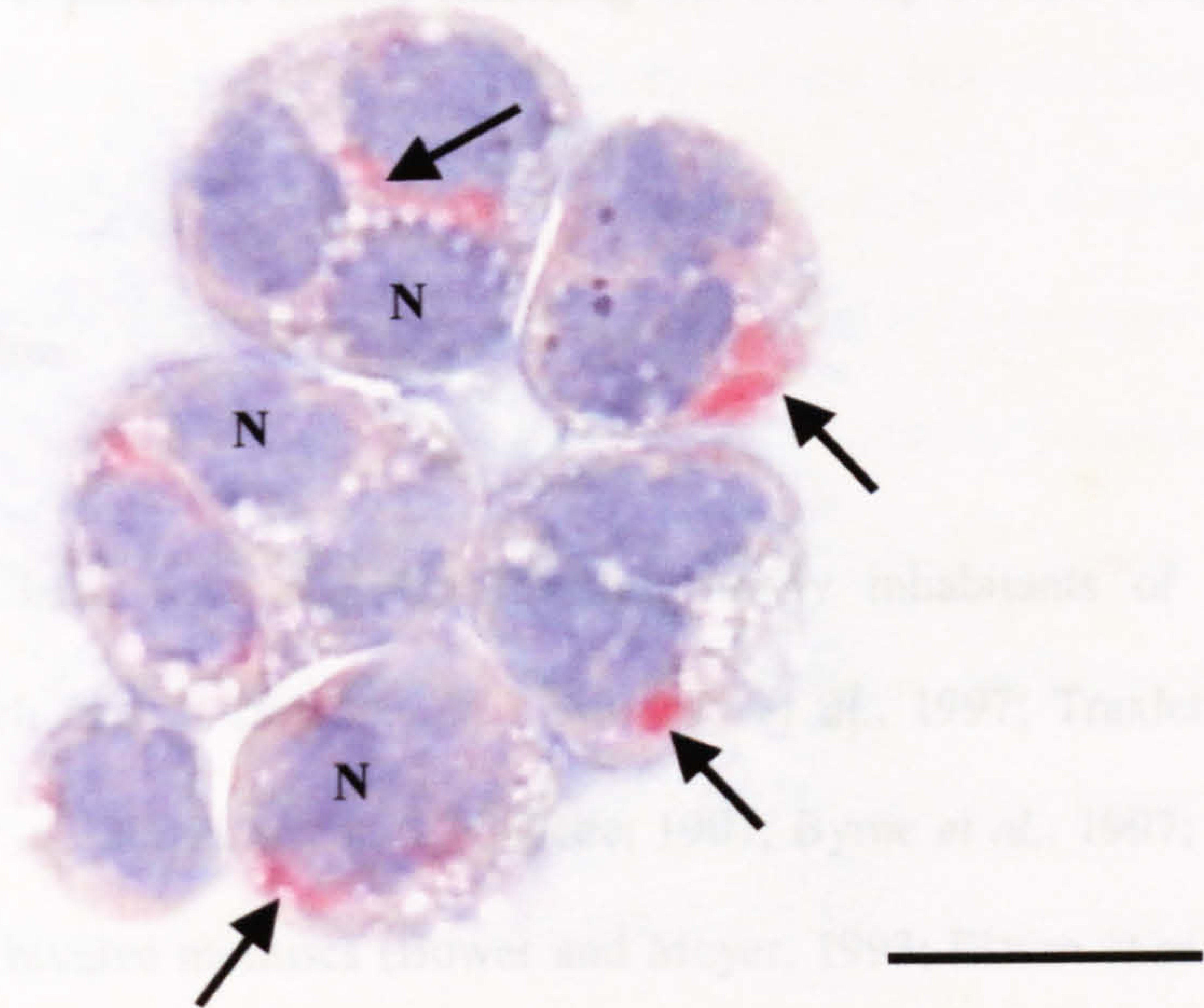

Amount of PNP formed (nmol h-1 50 µl sample-1)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Infected
Uninfected
Serum sample

Figure 6.5 Light micrograph of *Hematodinium* cells showing AP activity as a red reaction product (arrows) localised in single, bi- and tri-nucleated parasites. N= nucleus. Scale bar = 10 μm.


N
N
N

Chapter 7

Identification of a parasitic ciliate infecting the Norway lobster (*Nephrops norvegicus*).

7.1 Introduction

Parasitic ciliates have been reported infecting many inhabitants of marine environments, such as fish (Jessop, 1995; Munday *et al.*, 1997; Traxler *et al.*, 1998; Inglesias *et al.*, 2001), sea stars (Cépède, 1907; Byrne *et al.*, 1997; Stickle *et al.*, 2001), and bivalve molluscs (Bower and Meyer, 1993; Elston *et al.*, 1999; Karatayev *et al.*, 2002). In contrast, reports of systemic infections of Crustacea by ciliated protozoa are relatively rare, but have received recent attention due to their detrimental impact on several ecologically and economically important crustacean species (Morado and Small, 1995). Several species of the marine scuticociliate *Mesanophrys* (synonyms *Anophrys*, *Paranophrys*) have been described from a variety of crabs (Cattaneo, 1888; Poisson, 1930; Bang *et al.*, 1972; Armstrong *et al.*, 1981; Sparks *et al.*, 1982; Morado and Small, 1994; Messick and Small, 1996), as well as isopods (Hibbits and Sparks, 1983; Wiąchowski *et al.*, 1999), and in the American lobster *A. haemophilia* (Aiken *et al.*, 1973; Cawthorn *et al.*, 1996). Systemic ciliate infections of freshwater crayfish by *Tetrahymena pyriformis* (Edgerton *et al.*, 1996), of prawns by *Parauronema* sp. (Couch, 1978) and of krill by *Collinia* sp. (Gómez-Gutiérrez *et al.*, 2003) have also been

observed. In addition, a number of ciliates have been observed in association with aquatic hosts, predominantly as ectocommensal organisms (Corliss, 1979).

The occurrence of ciliated protozoa in Norway lobsters has previously been observed in association with an infection by the dinoflagellate *Hematodinium* (Field *et al.*, 1992; Field and Appleton, 1995; Appleton, 1996), but these ciliates were never described in detail. In the present study, a systemic parasitic ciliate infection was discovered in two individual Norway lobsters during routine investigations into the seasonal prevalence of *Hematodinium* infection; the first soon after capture from the Clyde Sea Area, and the second after being held in captivity for 14 days (after capture from the Clyde Sea Area) in a running seawater system at the University of Glasgow. These findings provided an opportunity to compile the first complete report on the morphology, associated histopathology and ribosomal DNA (rDNA) sequence data for a parasitic ciliate found in the haemolymph and tissues of Norway lobsters.

7.2 Materials and Methods

7.2.1 Sample collection and infection monitoring

Norway lobsters were caught as described in section 2.2.1. Following sterilisation of the cuticle with 70 % (v/v) ethanol, haemolymph samples were withdrawn from the base of the fifth pereiopod using a disposable 1 ml syringe and 25-gauge

needle. A drop of haemolymph was then smeared onto a poly-L-lysine-coated slide and viewed under light microscopy for the presence of ciliate parasites. Bi-monthly samples of haemolymph from 50 freshly caught lobsters from August 2002 until August 2003 were analysed for the presence of the ciliate after its initial discovery in two lobsters in November 2001.

7.2.2 Histology

Samples of heart, gill, hepatopancreas and tail muscle tissue were removed from the two infected lobsters. Tissues were preserved in 10 % (v/v) formol saline, followed by standard dehydration through a graded alcohol series and embedding in paraffin wax. Sections of 6 μm thickness obtained from each tissue were stained in haematoxylin and eosin (H & E) prior to examination.

7.2.3 Ciliate culture

Following sterilization of the cuticle with 70 % (v/v) ethanol, haemolymph samples were withdrawn aseptically from the base of the fifth pereiopod using a 1 ml disposable syringe and 25-gauge needle. The parasites were isolated in 3.5 cm well plates with 0.2 ml infected haemolymph added to 5 ml culture medium in each well.

The culture medium consisted of 10 % (v/v) foetal calf serum, heat inactivated (FCS) in autoclaved balanced *N. norvegicus* saline (Appleton and Vickerman, 1998, containing NaCl, 27.99 g l^{-1}; KCl 0.95 g l^{-1}; $CaCl_2$ 2.014 g l^{-1}; $MgSO_4$ 2.465 g l^{-1}; Na_2SO_4 0.554 g l^{-1}; HEPES 1.92 g l^{-1}) adjusted to pH 7.8, with penicillin G (10 U ml^{-1}) and streptomycin (10 μg ml^{-1}) added to inhibit bacterial contamination. The medium was then filter sterilised (0.2 μm). Cultures were incubated at 8°C.

7.2.4 Pyridine silver carbonate staining

Ciliates cultured in *N. norvegicus* saline supplemented with 10 % (v/v) FCS and antibiotics were used for ammonical silver carbonate staining as described by Fernández-Galiano (1994) with slight modifications. Briefly, 2 ml of ciliate culture (5 x 10^4 cells ml^{-1}) was added to 0.5 ml formaldehyde, 7.5 ml of double distilled H_2O (ddH_2O) was then added and the ciliates were pelleted by centrifugation at 400 x g for 5 min. The supernatant was discarded and the remaining cell pellet was resuspended in 10 ml of ddH_2O and centrifuged at 400 x g for 5 min. This washing step was repeated four times to remove any salts from the culture medium. To the fixed ciliates in 0.5 ml ddH_2O in a 40 ml beaker, the following were added in strict order; 3 drops formalin (40 % w/v), 5 ml ddH_2O, 20 drops bacteriological peptone solution (5 g bactopeptone dissolved in 100 ml ddH_2O, with the addition of 25 drops formalin (40 % w/v)), 10 drops pyridine, 2 ml ammoniacal silver carbonate solution (see Fernández-Galiano, 1994), 10 ml

ddH_2O. The suspension was mixed and the beaker was placed in a water bath at 65 °C for approximately 15 min until the solution darkened to a brown black colour. The impregnated ciliate cell suspension was then centrifuged at 100 x g for 2 min, the supernatant was removed and the residual ciliate suspension was placed on slides and viewed under a microscope. Morphometric measurements of stained ciliates were taken using the computer package NIH Image (Scion Corporation).

7.2.5 Electron microscopy (carried out by Kelly Bateman, EM Unit, The Centre for Environment, Fisheries and Aquaculture Science (CEFAS) Weymouth Lab, Weymouth, Dorset DT4 8UB)

Ciliate cells were fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and 1.75 % (w/v) sodium chloride for 2 h at room temperature. Fixed ciliates were washed in 0.1 M sodium cacodylate buffer (pH 7.4) before being post-fixed in 1 % (w/v) osmium tetroxide in sodium cacodylate buffer (pH 7.4) for 1 h. Samples were rinsed in buffer and then dehydrated through a graded acetone series. SEM samples were critical point dried and sputter coated in a layer of gold approx 5 nm thick. TEM samples were infiltrated with Epon premix resin 812 and polymerised in an oven overnight at 60 °C. Semi-thin sections (1-2 μm) were stained with toluidine blue, and ultrathin sections (70-90 nm) were collected on copper grids and stained using uranyl acetate and Reynolds lead citrate.

Preparations were examined using a JEOL 1210 transmission electron microscope and a JEOL 5200 scanning electron microscope.

7.2.6 rDNA amplification and sequencing

Ciliate genomic DNA was extracted from an *in vitro* culture according to standard procedures (Sambrook *et al.*, 1989). Briefly, 1 x 10^5 cells were centrifuged at 1,000 x g for 4 min at 4 °C. The resulting cell pellet was resuspended in 250 µl extraction buffer (50 mM Tris, 5 mM EDTA, 100 mM NaCl, pH 8), 100 µl of 10 % (w/v) SDS and Proteinase-K (0.28 ng μl^{-1}) and incubated at 56 °C for 18-24 h. DNA was purified by a single step standard phenol/chloroform (1:1) extraction, precipitated in 550 µl 100% ethanol using 20 µl 5M NaCl, and resuspended in 100 µl sterile deionised water. DNA concentrations and purity were estimated by measuring the 260/280 optical density ratios using a spectrophotometer and adjusted accordingly. The first internal transcribed spacer (ITS1), 5.8S gene and second internal transcribed spacer (ITS2) of the ribosomal gene complex were amplified using oligonucleotides previously described to amplify rDNA from scuticociliates (Goggin and Murphy, 2000). The amplification reaction mixtures contained 100 ng genomic DNA, 26.6 mM Tris-HCl, pH 8.8, 13.3 mM KCl, 13.3 mM $(NH_4)_2SO_4$, 2.6 mM $MgSO_4$, 2 µg BSA, 0.13 % (v/v) Triton X-100, 100 µM dNTPs, 7.5 pmol each primer, 1 unit of Pfu DNA polymerase (Promega), and sterile deionised water to a final volume of 15 µl. Reactions were overlaid with 10 µl of mineral oil. Thermal cycling conditions were as follows: denaturation at 94

°C for 1 min; primer annealing at 55 °C for 1 min; chain extension at 72 °C for 5 min; repeated for 30 cycles, with a final cycle incorporating a 7 min extension. Amplification products were run on 1.5 % (w/v) agarose gels, stained with ethidium bromide and viewed under UV illumination and images obtained using a gel documentation system (Appligene). Each amplification product of approximately 750 bp was excised from the agarose gel and purified by the use of a QIA-quick gel extraction kit (Qiagen).

Purified amplification products were ligated into the pGEMT-Easy plasmid vector (Promega), and used to transform *Escherichia coli* (strain JM 109) as described in section 3.2.3. Sequencing of selected plasmids was performed by MWG-AG Biotech (Germany). Three independent PCR, cloning and sequencing reactions were performed for the ciliate DNA isolate. The consensus sequence was compared to known sequences stored in GenBank using the Basic Local Alignment Search Tool (BLAST) routine (Altschul *et al.*, 1990) available through the National Center for Biotechnology Information (NCBI). Multiple sequence alignments were constructed with similar sequences using the software programs ClustalX 1.81 (Thompson *et al.*, 1994) and BoxShade 3.21 (http://www.ch.emb net.org /software/BOX_form.html).

7.3 Results

7.3.1 Prevalence of ciliates in *N. norvegicus*

The ciliate infection of lobsters was originally discovered in 2 lobsters in November 2001. Bi-monthly sampling and analysis of haemolymph smears from 50 lobsters from August 2002 until August 2003 failed to identify any further lobsters that were infected with ciliates.

7.3.2 Histopathology

Hemocytopenia characteristic of crustacean ciliate infections was indicated by the complete lack of haemocytes in haemolymph samples removed to initiate *in vitro* cultures. Examination of H & E stained tissue sections revealed a systemic infection of both lobsters, with ciliates present in several tissues. The heart had numerous ciliates present in the lumen and connective tissues of the myocardium tissues, but not in the epicardium (Fig. 7.1) Myocardial muscle and connective tissues were greatly reduced (when compared with uninfected animals, see Field 1992), possibly indicating tissue lysis or consumption by the ciliates. Ciliate cells were found in large numbers in the haemal spaces of gill filaments, leading to disruption of internal gill structures (Fig. 7.2). In the hepatopancreatic tissues, ciliate cells were observed in the haemal spaces between the tubules (Fig. 7.3), where few if any host haemocytes were present. The abdominal muscle tissues

were necrotic, with many ciliate cells invading the tissue and with areas of tissue lysis. This was apparent as an increase in the separation of fibre bundles adjacent to the ciliates (Fig. 7.4).

7.3.3 Morphology and cell structure of the ciliate

The ciliates were uniformly fusiform in shape, having a tapering anterior end and a rounded posterior end. Live ciliates were very active and flexible, continually moving forward and stopping to change direction. Ciliates were observed to have a contractile vacuole (Fig. 7.5) and a caudal cilium (Fig. 7.6) at the posterior end. Ten equally spaced somatic kineties spiralled round the surface of the cell (Fig 7.7). Silver impregnated ciliate cells were between 39-48 µm long, and between 12-24 µm wide, with one macronucleus and one micronucleus (Fig. 7.8, Table 7.1). The oral polykinetids (OPK1, OPK2, OPK3) and oral dikinetids (ODKb and ODKc) of the ciliate were highly ordered ciliary fields positioned at the anterior end between kineties 1-10 (Fig. 7.8). Oral polykinetid 1 (OPK1) was the most anterior of the oral structures, with a mean length of 4.4 µm. Fragmentation of OPK1 was not observed. Oral polykinetid 2 (OPK2) was slightly smaller in length (3.5 µm). Oral polykinetid 3 (OPK3) was notably smaller in length (1.9 µm) with several pairs of kinetosomes aligned perpendicular to the long axis of the body. Oral dikinetid structures were composed of ODKb (7.2 µm) and ODKc, however only ODKb was routinely observed by this staining technique. The ODKb structure originated immediately posterior from OPK2, and proceeded in a curve

posteriorly around the cytostome to the ODKc segment. The ODKc segment was rarely stained, consisting of several pairs of kinetosomes aligned in a "Y" configuration at the posterior end of ODKb.

Several mitochondrial structures were observed between the somatic kineties surrounding the ciliate cell (Fig. 7.9). Many vesicles were observed within the cytoplasm of the cells (Fig. 7.10), and an irregular distribution of possible mucocysts was also observed.

7.3.4 rDNA sequence comparison

The three independent PCR, cloning and sequencing steps resulted in identical rDNA sequences. BLAST searches of GenBank indicated that the ciliate consensus sequence was identical to *Orchitophrya stellarum* (GenBank accession numbers AF107773, AF107774, AF107775 and AF107776), and very similar to *Mesanophrys pugettensis* (AF107777) and *M. chesapeakensis* (AF107778). Multiple sequence alignments revealed that the ITS1, 5.8S and ITS2 rDNA sequences of the ciliate under study were the same as those of 4 isolates of *Orchitophrya stellarum*. Both *M. pugettensis* and *M. chesapeakensis* were also identical over ITS1, 5.8S and ITS2 regions. Because of this, *Orchitophrya stellarum* (AF107773) and *M. pugettensis* (AF107777) were used in the multiple alignment generated to represent the identical sequences identified (Fig. 7.11). The rDNA sequence descriptions by Goggin and Murphy (2000) allowed the ITS1

(140 bp), 5.8S (119 bp) and ITS2 (236 bp) domain lengths to be calculated for the ciliate. The consensus sequence of the experimental ciliate differed by 9.2 % in the ITS1 (8 transversions, 2 transitions, 2 additions, 1 deletion) and 5.0 % in the ITS2 (3 transversions, 6 transitions, 3 additions) from that of the *Mesanophrys* sequences held in GenBank. The 5.8S gene was completely conserved in all sequences.

7.4 Discussion

The morphological characteristics of live and silver-stained specimens of the ciliate found infecting Norway lobsters are consistent with those of the genus *Mesanophrys* (Small and Lynn, 1985). Moreover, comparison of this ciliate with other species of *Mesanophrys* that have been stained with silver impregnation techniques (Small and Lynn, 1985; Morado and Small, 1994; Messick and Small, 1996; Wiąchowski *et al.*, 1999), indicate that all have similar structural features and are closely related.

The ciliate has a mean body length of 48.2 ± 4.6 µm and width of 17.5 ± 2.9 µm (N = 47) when stained by the pyridine-silver-carbonate method. The smallest (39.2 µm length, 12.5 µ width) and largest (57.9 µm length, 24.5 µm width) morphometric cell measurements from this study are of comparable length and width to *M. chesapeakensis* (37.6 µm, 13.4 µm), *M. pugettensis* (59.0 µm, 17.8

μm), and *M. carcini* (44 μm, 13 μm), but more closely resemble *M. maggii* (47.8 μm, 18.6 μm) Cattaneo (1888).

The ciliate detected in the Norway lobster has a single macronucleus, a single micronucleus, and 10 somatic kineties, similar to ciliates within the genus *Mesanophrys*. Its OPK1 and OPK2 are of approximate equal length to each other, with OPKI slightly larger (4.4 μm, 3.3 μm respectively, Table 7.1). OPK3 is the shortest oral structure (1.9 μm) in length, but is the widest. The biometric measurements made are slightly smaller than those of pyridine-silver-stained *M. pugettensis*, which are OPK1 4.8 μm, OPK2 3.9 μm, and OPK3 1.5 μm (Morado and Small, 1994). Those authors also noted that in early stomatogenesis of *M. pugettensis* a fourth transitional ciliary field occurs, suggesting that the first oral polykinetid (OPK1) is composed of two functionally related segments. As a result, *M. pugettensis* was described as a new species. Wiąchowski *et al.* (1999) also observed OPK1 segmentation in *Mesanophrys* sp. from the isopod *Saduria entomon*, but interpreted it as a general character linked with stomatogenesis, suggesting that it should not be used as a specific character. As only the silver carbonate staining technique was employed during this study, and protargol staining was not attempted, the fine detail of the kinetosomal structure of the oral apparatus was not observed and cannot be commented upon.

Relatively few ciliate infections of wild crustacean populations have been documented (Cattaneo, 1888; Poisson, 1930; Morado *et al.*, 1999; Lavallée *et al.*,

2001; Gómez-Gutiérrez *et al.*, 2003). Of those that have been, very small numbers of infected individuals have been recorded. Poisson (1930) found only 0.2 % (7 of 3000) of shore crabs *Carcinus maenas*, with histophagous ciliates, while Lavallée *et al.* (2001) recorded a prevalence of 0.39 % for *Anophryoides haemophila* in the American lobster *Homarus americanus.* However, Hibbits and Sparks (1983) reported a higher prevalence of 14 % (5/37) for *Paranophrys* infection of the isopod *Gnorimosphaeroma oregonensis.* A *Paranophrys*-like ciliate had previously been observed in aquaria-held, *Hematodinium*-infected Norway lobsters (Field *et al.*, 1992; Field and Appleton, 1996) and on only one other occasion was it identified from a freshly caught lobster (Appleton, 1996). The present study failed to identify any further infected Norway lobsters within wild stocks, indicating that the affected individuals came from a localised short-term infection event. Thus it is concluded that the incidence of ciliate infection is very low and that probably the host lobsters were compromised in some way and so were susceptible to infection.

The available data suggest that ciliates of the genus *Mesanophrys* are considered to be facultative histophages, with infection of crustaceans being opportunistic in nature. The route of entry for ciliates into lobsters is not known but Morado *et al.* (1999) suggest that shell condition and size are important factors for *M. pugettensis* infection of dungeness crabs. Unfortunately, the condition of the infected Norway lobsters was not noted at the time of ciliate infection diagnosis. Experimental infection studies are needed to establish the route of infection.

Expansive host tissue penetration and destruction are common observations in infections by *Mesanophrys* (Armstrong *et al.*, 1981; Sparks *et al.*, 1982; Hibbits and Sparks, 1983; Messick and Small, 1996), *Tetrahymena* (Edgerton *et al.*, 1996), and *Collinia* (Gómez-Gutiérrez *et al.*, 2003) infections of crustaceans. The ciliate infecting Norway lobsters was found in several tissues causing extensive damage, particularly in the myocardial regions of the heart. The normal architecture of musculature had completely degenerated and tissue necrosis was apparent. Armstrong *et al.* (1981) suggested that extracellular lysosomes provide chemical means for disrupting tissues and cells during *Mesanophrys* infection of dungeness crabs. The ciliate from the Norway lobster had several mucocysts, the contents of which are released by exocytosis. The possible secretion of lysosomal enzymes that facilitate tissue destruction and consumption is addressed in Chapter 8.

Ribosomal DNA sequences from the ciliate infecting Norway lobsters were found to be identical over the ITS1, 5.8S and ITS2 regions to *Orchitophrya stellarum* sequences held in Genbank (Fig. 7.11). *O. stellarum* is a scuticociliate parasitic castrator of male sea stars of the family Asteriidae from the North Atlantic and Pacific (Cépède 1907; Leighton *et al.*, 1991; Claereboudt and Bouland, 1994; Byrne *et al.*, 1997). It was first described by Cépède (1907) infecting the sea star *Asteracanthion rubens* from France. The ciliate was very rare and only 3 out of the several thousand sea stars sampled were infected. The ciliate has a pointed anterior and a rounded posterior, with a granular structure of the cytoplasm and

one macronucleus and one micronucleus. Its length is between 35-65 μm, and it has a width of 12-26 μm. Its oral structures are at the anterior end and consist of 3 oral polykinetids and an oral dikinetid. The ciliate infecting Norway lobsters appears morphologically similar to this description, but importantly, however, *O. stellarum* is reported to have between 18-20 somatic kineties (Cépède 1907; Claereboudt and Bouland, 1994; Stickle *et al.*, 2001), whereas the ciliate studied here has only 10 (Figs. 7.7 and 7.9).

Differences in the ITS regions between strains and species of ciliates have previously been observed. Diggles and Adlard (1997) reported that 4 wild isolates of the parasitic ciliate of fish, *Cryptocaryon irritans*, differed by up to 4 % in ITS1. Additionally, two species of *Tetrahymena* differed by 3 % in the ITS1 and 10 % in the ITS2 (Engberg *et al.*, 1990). ITS1 sequences from *C. irritans* have also been observed to alter rapidly with passage in the laboratory, and differed by 5.9 % over the period of 1 year from initial isolation (Diggles and Adlard, 1997). However, Goggin and Murphy (2000) noted that *M. chesapeakensis* isolated from *Callinectes sapidus* from the Atlantic Ocean, and *M. pugettensis* from *Cancer magister* from the Pacific Ocean had no differences in both ITS and the 5.8S ribosomal regions, yet both ciliates were held in culture for 18 months.

In summary, morphologically, the ciliate parasite of the Norway lobster is very similar to members of the genus *Mesanophrys*, but rDNA sequences suggest a puzzling affinity with *O. stellarum*. The ciliate sequences identified in Genbank

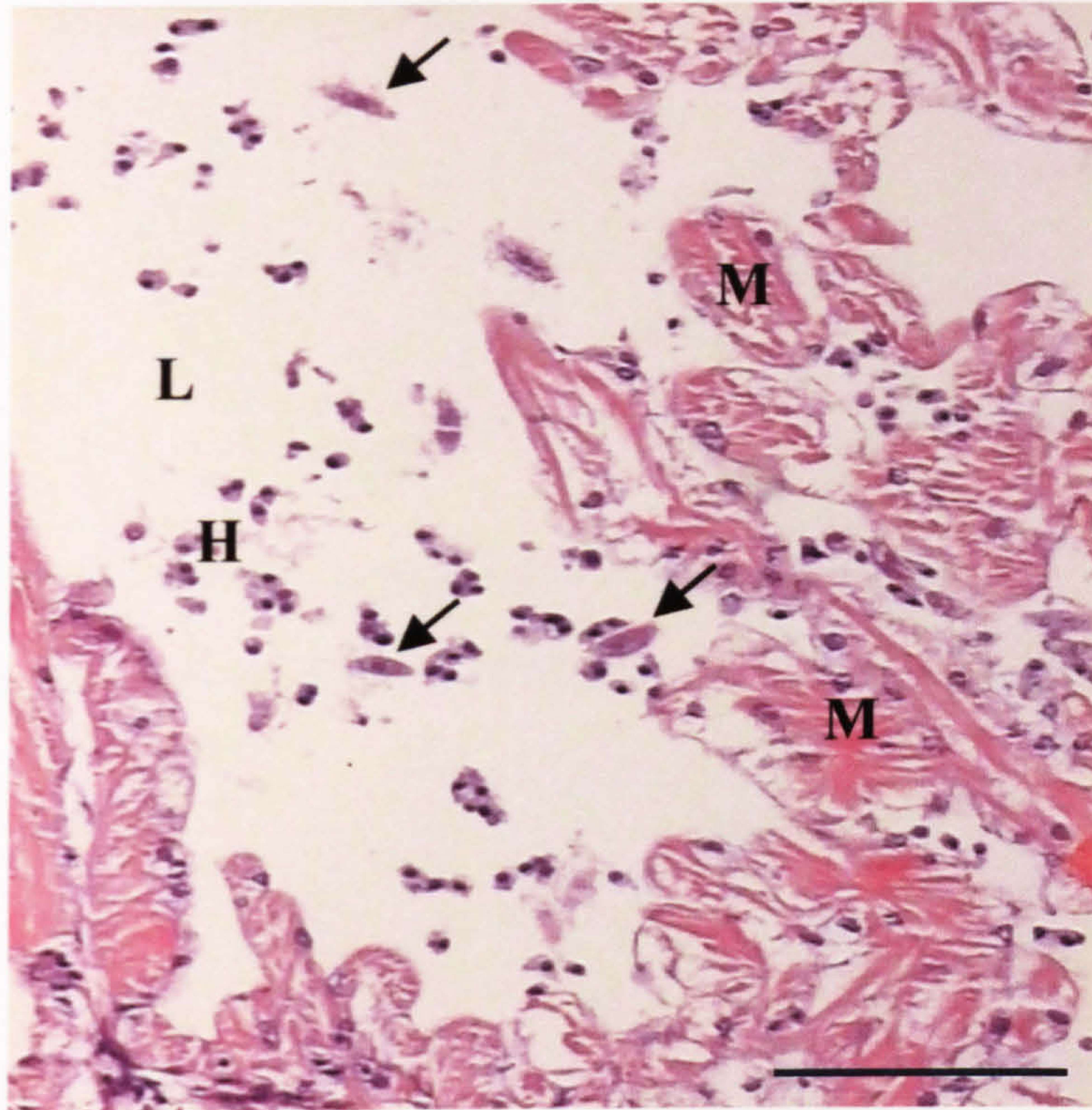
and used in the multiple alignments were obtained by the same researcher, and there is the possibility that samples were mislabelled resulting in the incorrect cataloguing within GenBank, which would explain the present inconsistency. Alternatively, there may be different rDNA sequences in the macro and micronucleus of the ciliate resulting in preferential amplification; however identical triplicate sequencing reactions obtained in this study and by Goggin and Murphy (2000) indicate that this is not the case. The data also suggests that the number of somatic kineties that a ciliate possesses may not be a robust morphological feature when identifying closely related species. Sequencing of the ITS regions of all nominal *Mesanophrys* and *O. stellarum* species in conjunction with silver impregnation staining techniques will be essential in order to resolve this issue.

Table 7.1 Biometric characteristics for pyridine-silver-carbonate impregnated ciliates from an *in vitro* culture. All dimensions are in μm.

Character	Mean	SD	Min	Max	N
Body length	48.2	4.6	39.2	57.9	47
Body width	17.5	2.9	12.5	24.5	47
No. somatic kineties	10	0	0	0	47
Micronucleus length	2.6	0.4	1.5	3.5	47
Micronucleus width	1.9	0.4	1.3	2.9	47
Macronucleus length	10.6	0.4	8.0	15.4	47
Macronucleus width	8.6	1.2	6.1	11.4	47
OPK1 length	4.4	0.5	3.0	5.5	41
OPK2 length	3.5	0.3	2.8	4.2	41
OPK3 length	1.9	0.2	1.3	2.3	41
ODKb length	7.2	1.2	5.2	10.7	41
Distance from OPK1-OPK3	10.3	0.9	8.0	11.9	41
Distance from OPK1-OPK2	8.1	0.8	6.3	9.9	41
Distance from OPK2-OPK3	5.6	0.4	4.5	6.8	41
Distance anterior end-OPK3	12.5	1.4	8.4	15.6	41
Distance anterior end-ODK	16.1	1.7	13.3	19.4	41

Figure 7.1 Ciliates (arrows) present in the haemal spaces and lumen of myocardial heart tissue. The myocardial muscle and connective tissues are greatly reduced when compared to heat tissues from healthy individuals (see Field, 1992). H and E staining. L = lumen; H = haemocytes in lumen; M = myocardial muscle. Scale bar = 100 μm.

Figure 7.2 Large number of ciliates (arrows) packing a gill filament. C = cuticle; Ct = connective tissue. H and E staining. Scale bar = 100 μm.


M
L
H
M

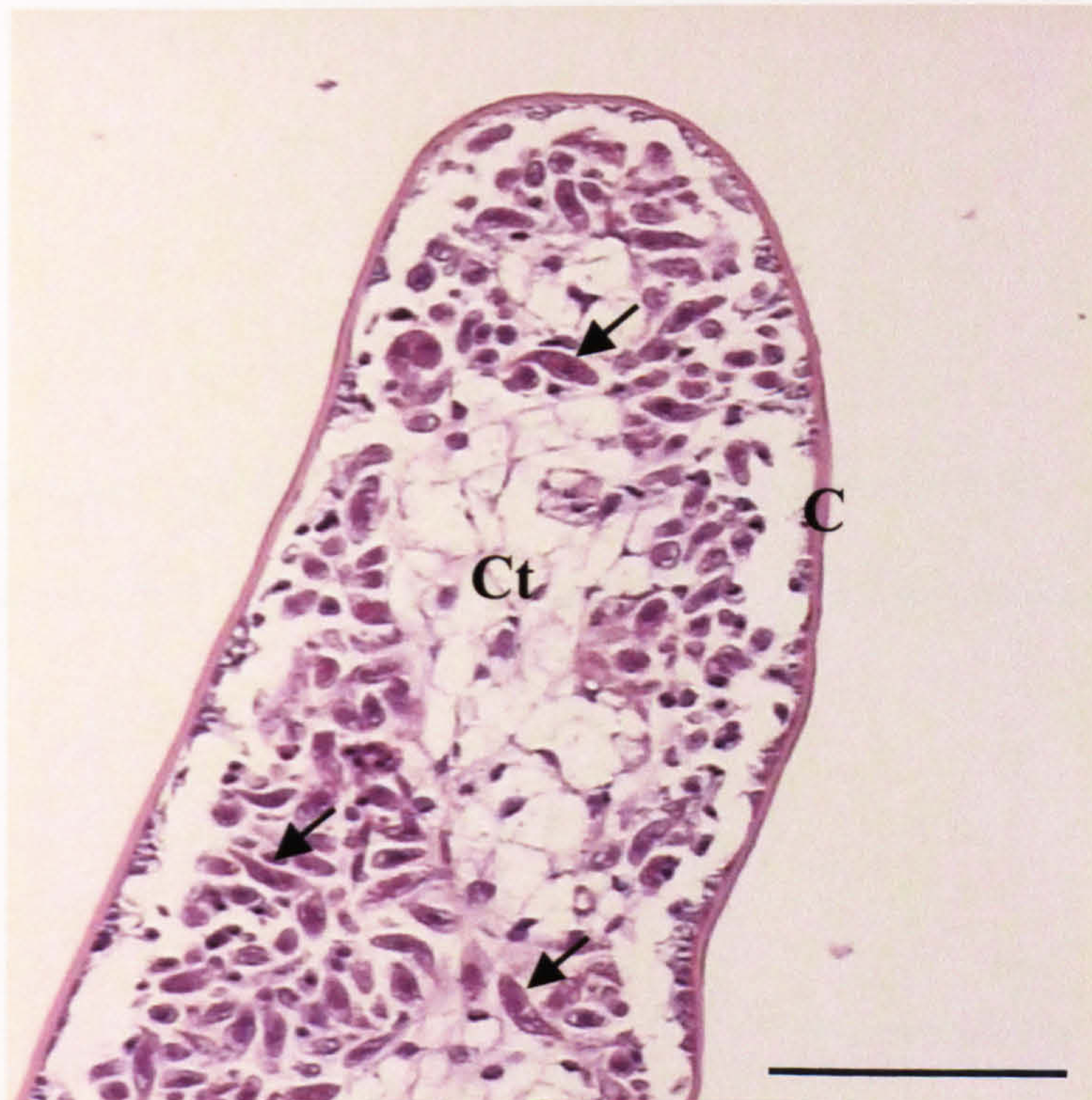
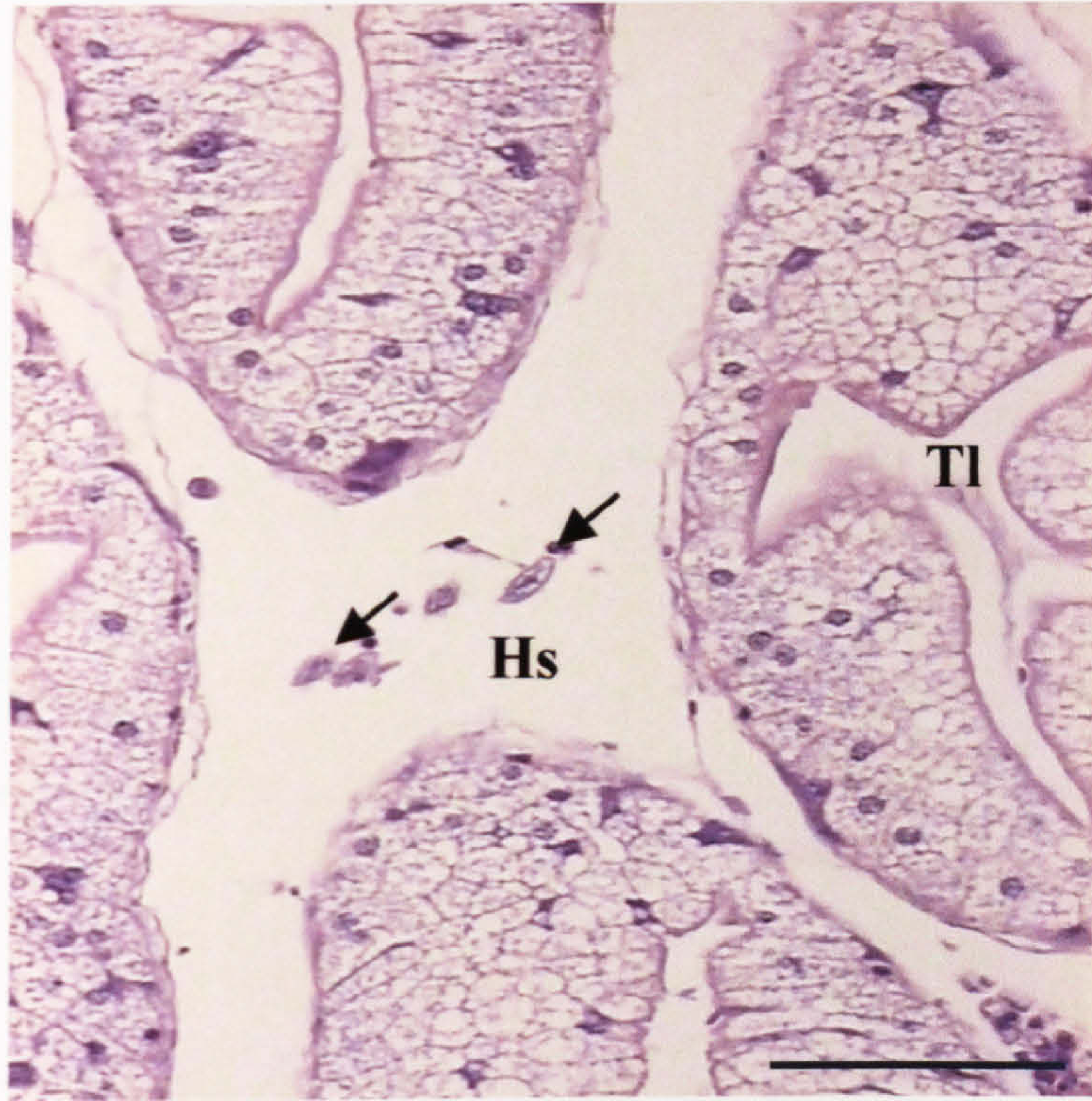

C
Ct

Figure 7.3 Ciliates (arrows) present in the haemal spaces between the tubules in the hepatopancreas. Hs = haemal space; Tl = tubule lumen. H and E staining. Scale bar = 100 μm.

Figure 7.4 Abdominal deep flexor muscle with ciliates (arrows) invading spaces between muscle bundles. L= area of lysis. H and E staining. Scale bar = 100 μm.


Tl
Hs

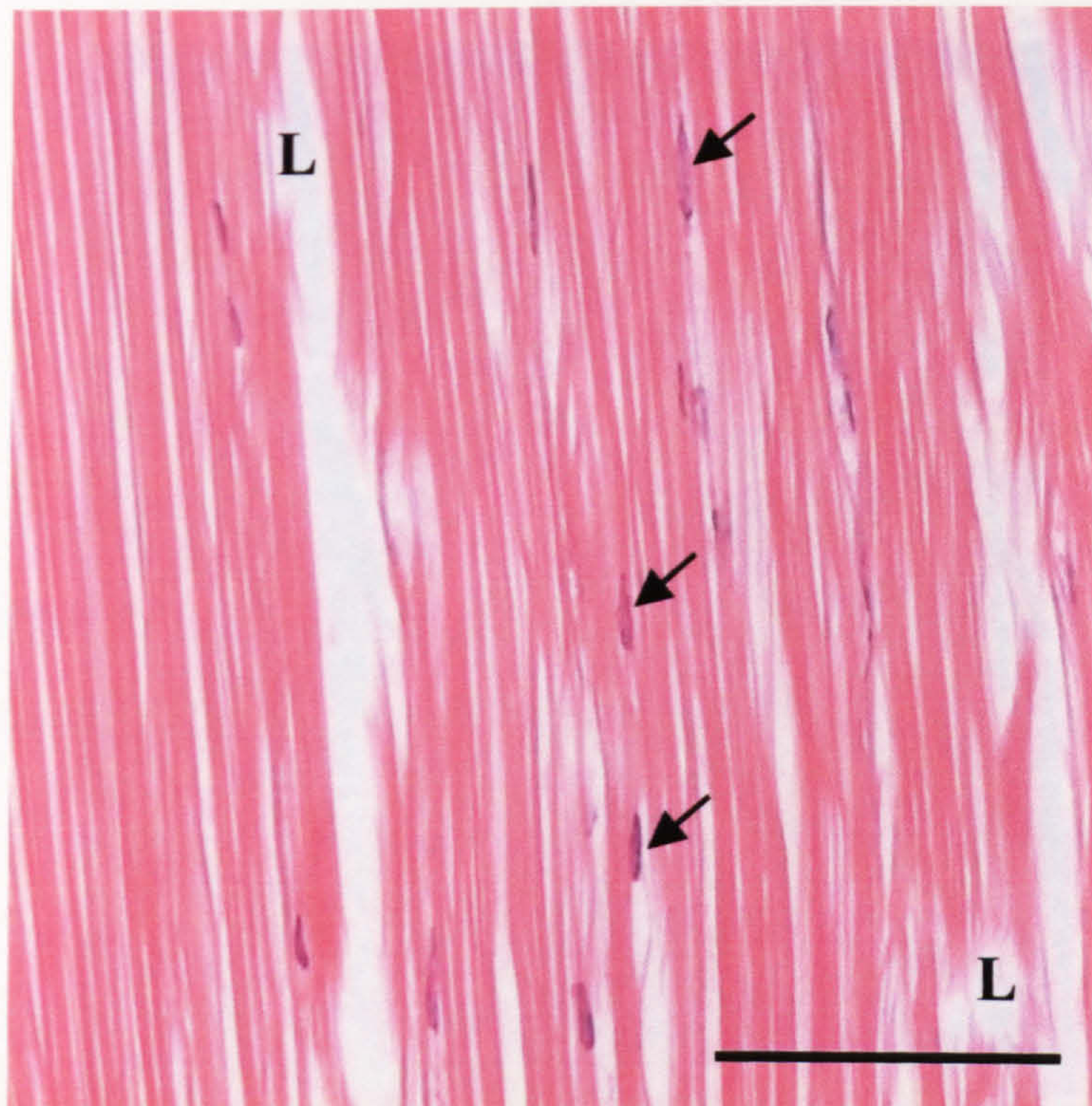
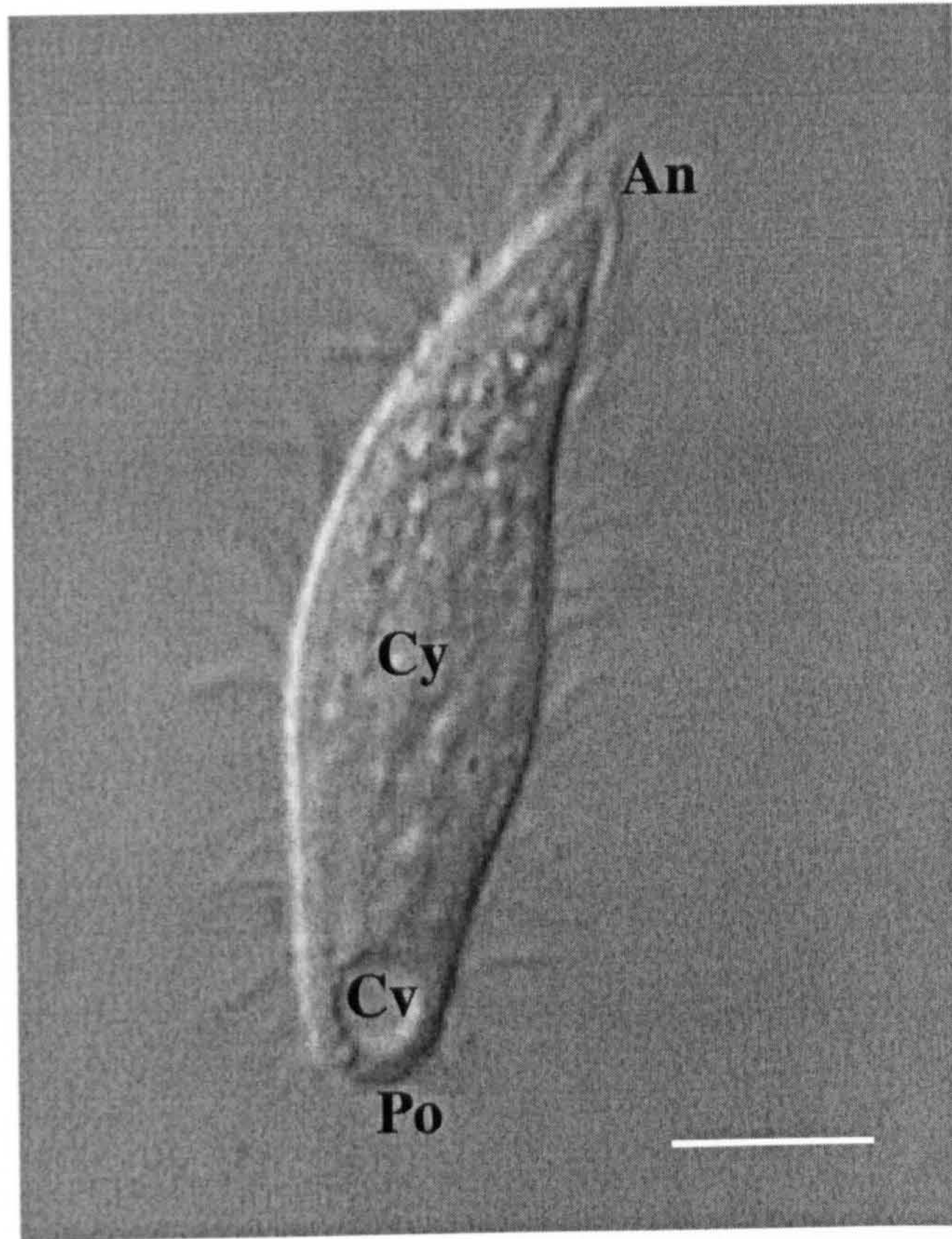

L
L

Figure 7.5 Live specimen of a ciliate from *in vitro* culture. Note the contractile vacuole (Cv) and the granular appearance of the cytoplasm (Cy). An = anterior end; Po = posterior end. Differential interference contrast. Scale bar = 5 μm.

Figure 7.6 Scanning electron micrograph of a ciliate from *in vitro* culture. Note the oral apparatus (arrow) at the anterior end. Cc = caudal cilium at the posterior end. Scale bar = 5 μm.


An
Cy
Cv
Po

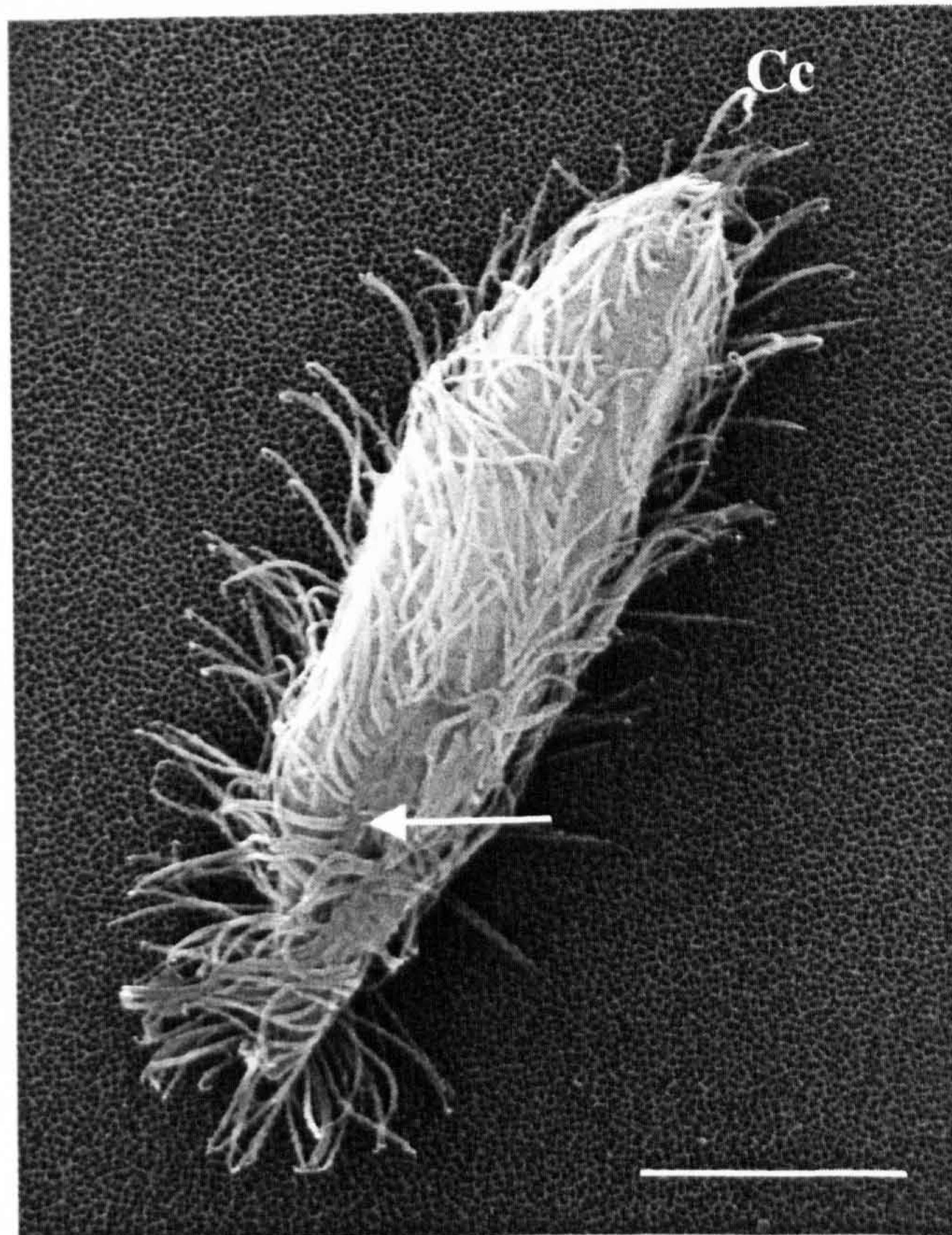
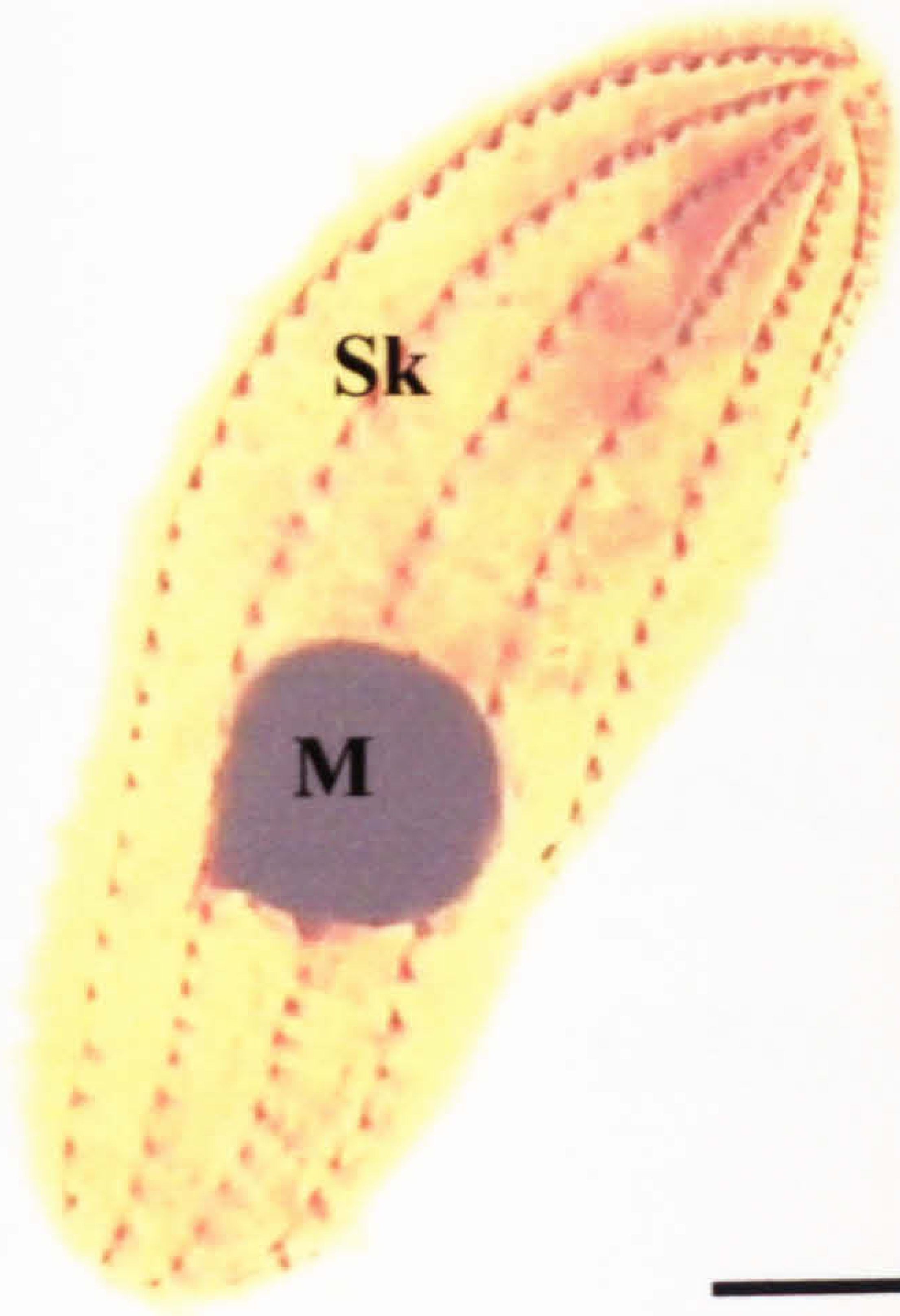
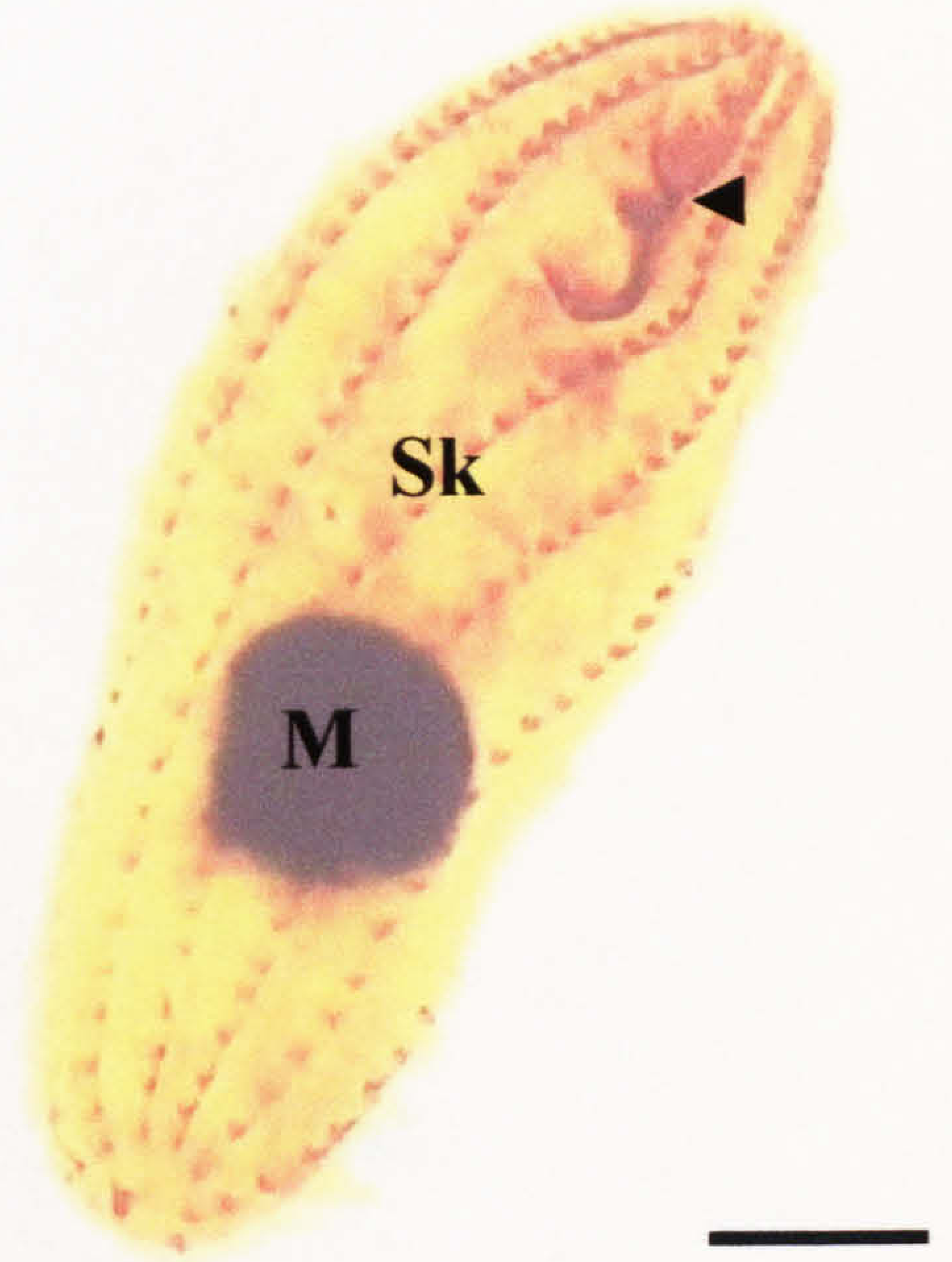

Cc

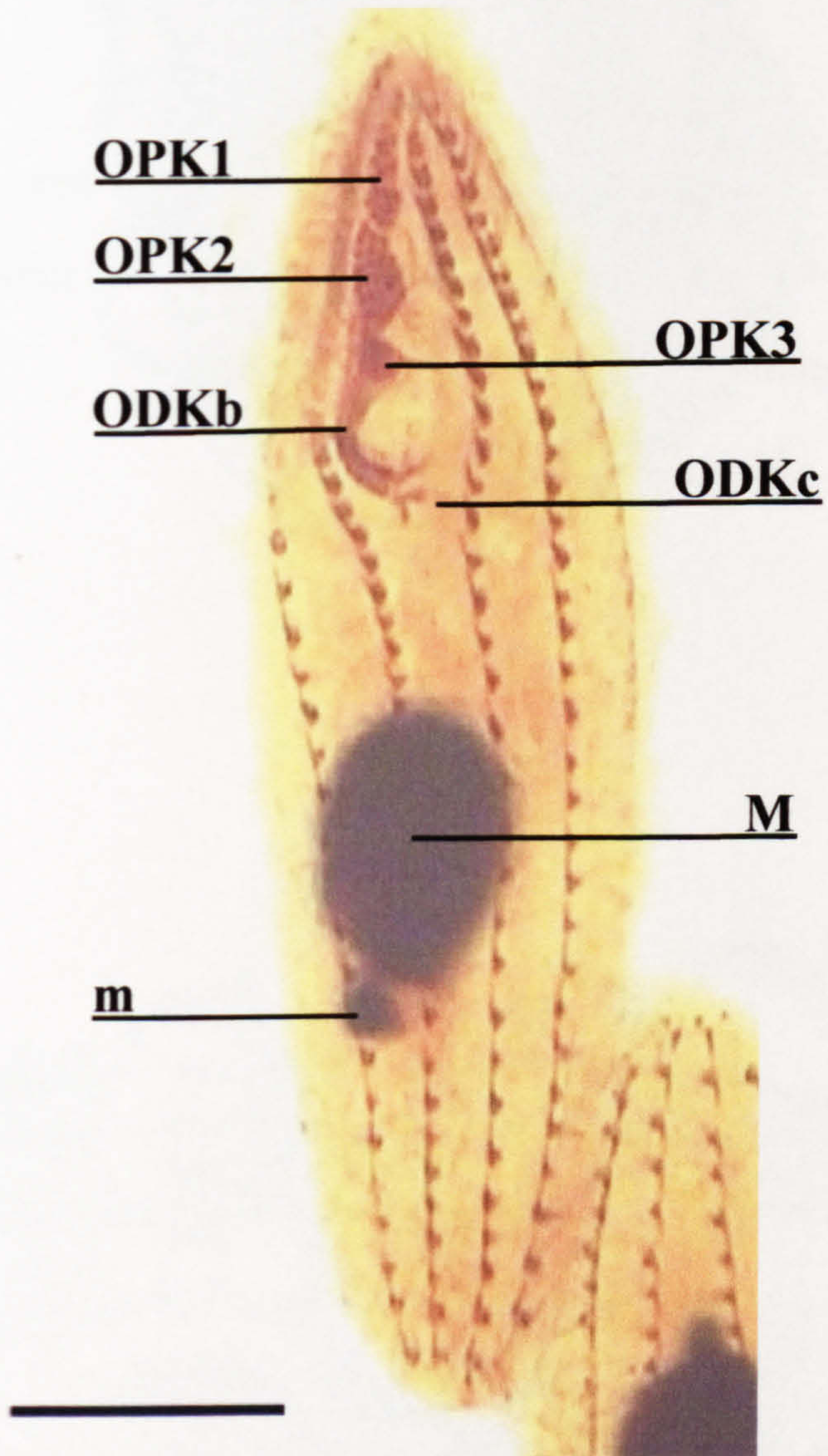
Figure 7.7 Pyridine-silver-carbonate impregnated ciliates from *in vitro* culture. Upper (A), lower (B), and anterior end (C) of ciliates showing 10 somatic kineties (Sk) on outer surface of cell. Arrowhead = oral polykinetid structures; arrow = first oral polykinetid; M = macronucleus. Scale bar = 10 μm.


A
Sk
M


B
Sk
M

C
Sk

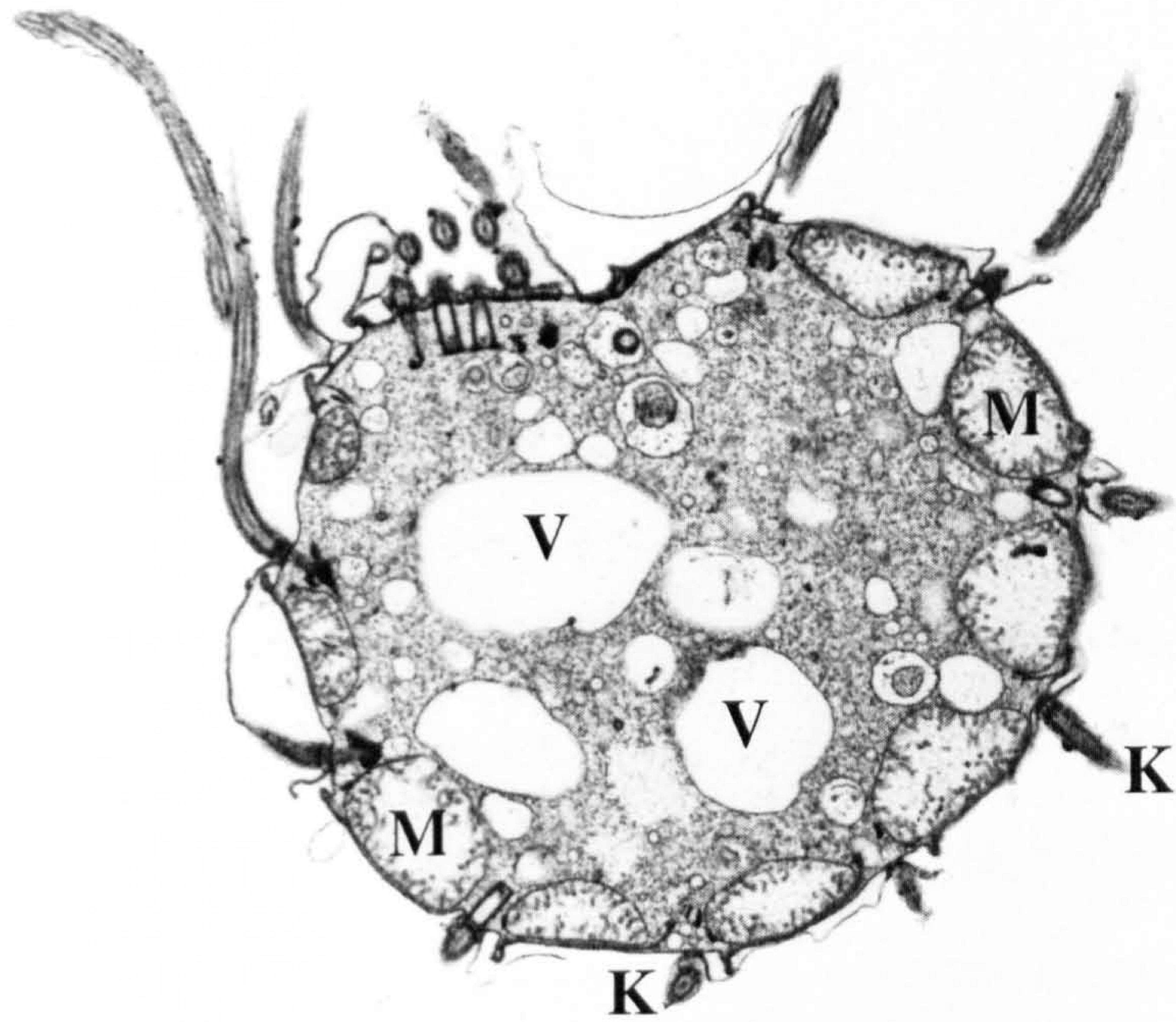
Figure 7.8 Pyridine-silver-carbonate impregnated ciliate from *in vitro* culture showing oral apparatus. M = macronucleus; m = micronucleus; OPK1 = first oral polykinetid; OPK2 = second oral polykinetid; OPK3 = third oral polykinetid; ODKb = oral dikinetid b; ODKc = oral dikinetid c. Scale bar = 10 μm.



OPK1
OPK2
OPK3
ODKb
ODKc
M
m

Figure 7.9 Transmission electron micrograph of a ciliate from *in vitro* culture. M = mitochondrial structures adjacent to the inner cell surface in-between kineties; K = kineties; V = vesicles. Scale bar = 5 μm.

Figure 7.10 Transmission electron micrograph of a ciliate from *in vitro* culture. K = kinetie; V = vesicles; arrow = possible mucocysts and associated vesicles. Scale bar = 5 μm.


M
V
V
K
M
K

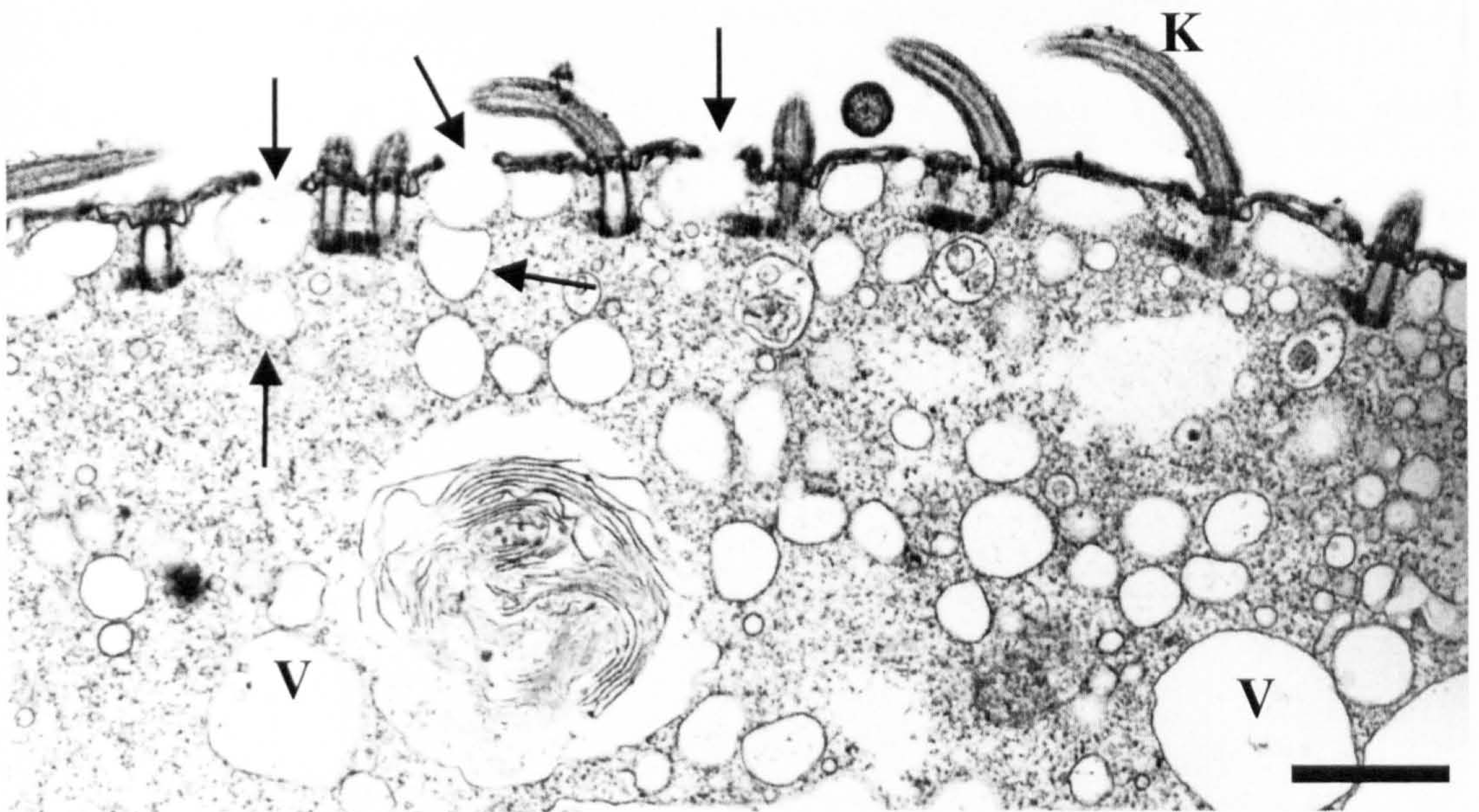

K
V
V

Figure 7.11 Multiple alignment of rDNA sequences from the first internal transcribed spacer (ITS1), 5.8S and second internal transcribed spacer (ITS2) of the ciliate under investigation (Exp. ciliate), *Orchitophrya stellarum* (GenBank accession number AF107773) and *Mesanophrys pugettensis* (AF107777). Note that *O. stellarum* (AF107773) from a seastar *Asterias amurensis* in Japan, is identical to rDNA sequences for *O. stellarum* from *Asterias vulgaris* from Prince Edward Island, Canada (AF107774); *Pisaster ochraceus* from British Columbia, Canada (AF107775); and *Asterias rubens* from The Netherlands (AF107776). Also note that *M. pugettensis* (AF107777) is identical to *M. chesapeakensis* (AF107778). Dots indicate conserved nucleotides; dashes indicated missing nucleotides; letters indicate substitutions. Box indicates conserved 5.8S gene sequence.

```
Exp. ciliate       1 ACACATTCAATAATGAAACACCTTAAC-TTAAGTTCTTGAAGGCGTGTTTTGAAGTAATT
O. stellarum       1 ...........................-................................
M. pugettensis     1 ...........................C................T.....-...A..T..   ITS1

Exp. ciliate      60 TATTATGGAAAAACCGCTTTCATTTCTTAAACAAACTTTTAATAAAAACAACATAACCAA
O. stellarum      60 ............................................................
M. pugettensis    60 ACG...T..G..-.A.......................................C.....

Exp. ciliate     120 AATAAAATCTAAACAAAAATTAAAAATTTTCAACGGAGGATATCTTGGTTCCCATATCGA
O. stellarum     120 ............................................................
M. pugettensis   119 ............................................................   5.8S

Exp. ciliate     180 TGAAGAACGCAGCCAAATGCGATACGCAATGCGAATTGCAGAATTCCGCGAGTCATCAGA
O. stellarum     180 ............................................................
M. pugettensis   179 ............................................................

Exp. ciliate     240 TCTTTGAACGCAAGTGGCGCTGGGATAAACAATACCCCAGCATGTTTGTTTCAGTGTGTT
O. stellarum     240 ............................................................
M. pugettensis   239 ............................................................

Exp. ciliate     300 AGGAATCATATATCTTAATGCGATTGAGAAGTCTAACTTTTCTCTCGTTAAATATGAAAG
O. stellarum     300 ............................................................
M. pugettensis   299 ............................GGA.....T.C.....................   ITS2

Exp. ciliate     360 CGCTGAATCGTTCAGTGCCGATCGAAGTAGTCACTACTCGCTAGTGATCTCGATTGTGCT
O. stellarum     360 ............................................................
M. pugettensis   359 ............................................................

Exp. ciliate     420 ATACTGAGGATTCACTACAGCGACTTTTTTTAAAATTAAATATCTCCTCTCAACACCTGA
O. stellarum     420 ............................................................
M. pugettensis   419 ............................ACA...C.---.....................

Exp. ciliate     480 AATCAAGCAAGAACAC
O. stellarum     480 ................
M. pugettensis   476 ................
```

Chapter 8

Characterisation of proteases secreted by a ciliate parasite of the Norway lobster.

8.1 Introduction

Histophagous ciliates of the genus *Mesanophrys* have been described parasitizing several crustaceans, including the shore crab *Carcinus maenas* (Cattaneo, 1888), the edible crab *Cancer pagurus* (Grolièr and Leglise, 1977), the dungeness crab *Cancer magister* (Morado and Small, 1994) and the blue crab *Callinectes sapidus* (Messick and Small, 1996). A histophagous ciliate identified to be of the genus *Mesanophrys* has now been found infecting Norway lobsters (*Nephrops norvegicus*) from Clyde Sea Area in Scotland (see Chapter 7).

It is important to understand how such histophagous ciliates establish themselves within their crustacean hosts. One proposal is that they enter via existing lesions of the epidermal surface of the host, and once inside they spread through the body and multiply to high densities in tissues and haemolymph. Morado *et al.* (1999) report an example of this for *Mesanophrys pugettensis* infecting the dungeness crab, where the ciliate enters via lesions associated with the moult cycle.

In several parasitic infections, proteins released by the pathogen are found to play multiple roles in the establishment and progression of the disease. For example, parasite proteases have been found to degrade host extracellular matrix proteins, thus facilitating spread of the parasite within host tissues (Schulte and Scholze, 1989). Many bacterial, fungal and protozoan marine pathogens are known to secrete proteases during *in vitro* culture; examples include *Aeromonas salmonicida*, the causative agent of fish furunculosis in salmon (Rockey *et al.*, 1988), the crayfish pathogen *Aphanomyces astaci* (Söderhäll and Unestam, 1975), and the oyster parasite *Perkinsus marinus* (La Peyre and Faisal, 1995; La Peyre *et al.*, 1995). Parasitic scuticociliates of many fish species have also been reported to secrete proteases (Lee *et al.*, 2003).

Proteases secreted by these pathogens are also thought to be directly responsible for many of the clinical signs associated with the diseases, such as tissue destruction and necrotic lesions. Several reports of *Mesanophrys* infections in crustaceans have indicated that the ciliate infiltrates and consumes host tissues and haemocytes (Poisson, 1930; Bang *et al.*, 1972; Messick and Small, 1996).

The discovery of a parasitic ciliate infecting Norway lobsters, and isolation of the ciliate *in vitro*, has allowed the proteolytic factors that may play a role in its establishment and progression to be investigated.

8.2 Materials and Methods

8.2.1 Isolation and culture of parasitic ciliate

Routine screening of *N. norvegicus* from the Clyde Sea Area for the parasitic dinoflagellate *Hematodinium* led to the discovery and identification of a ciliate infection in a number of lobsters (see Chapter 7). Haemolymph samples were withdrawn aseptically from the base of the fifth pereiopod using a 1 ml disposable syringe and 25-gauge needle following sterilization of the cuticle with 70 % (v/v) ethanol. Infections with ciliates were identified by viewing haemolymph smears under low power light microscopy. Parasitic ciliates were isolated in 3.5 cm well plates with 0.2 ml infected haemolymph added to 5 ml culture medium in each well. The culture medium consisted of 10 % (v/v) heat inactivated foetal calf serum in autoclaved balanced *Nephrops* saline (Appleton and Vickerman, 1998, containing NaCl, 27.99 g l^{-1}; KCl 0.95 g l^{-1}; $CaCl_2$ 2.014 g l^{-1}; $MgSO_4$ 2.465 g l^{-1}; Na_2SO_4 0.554 g l^{-1}; HEPES 1.92 g l^{-1}) adjusted to pH 7.8, with penicillin G (10 U ml^{-1}) and streptomycin (10 µg ml^{-1}) added to inhibit bacterial contamination. The medium was then filter-sterilised (0.2 µm) after addition of all constituents. Cultures were incubated at 8 °C. Serial sub-culturing gave rise, in time, to axenic cultures.

Experimental sub-cultures for protease analysis were initiated with the addition of 1×10^5 ciliates to 5 ml culture medium in 3.5 cm well plates, in quadruplicate, and

were maintained at 8 °C. Cultures were checked for contamination daily. Cell viability was assessed by viewing cultures under phase contrast. The total volume of medium in each well was collected on days one, three, five and seven. The aliquots were centrifuged at 400 x g for 10 minutes and 4.9 ml supernatant was collected, passed through a 0.2 µm filter and frozen at – 80 °C. The remaining cell pellet was resuspended in 100 µl culture medium and 10 µl of this was mixed with 10 µl 1% (w/v) formaldehyde (in 1 x PBS) to immobilise the ciliates. Ciliates were counted using an Improved Neubauer counting chamber and the numbers per ml were estimated using standard procedures.

8.2.2 Preparation of ciliate lysates

Ciliates from an *in vitro* culture were collected, and the cell density was estimated as described above. The resulting ciliate suspension (5 x 10^4 cells 100 μl^{-1}) was washed three times with *Nephrops* saline before being resuspended in 100 µl *Nephrops* saline. Cell lysates were prepared by freezing and thawing the sample three times, followed by centrifugation (10,000 x g for 10 min at 4 °C). The supernatant fraction of the cell lysate was carefully removed, filtered through a 0.2 µm filter and stored frozen at –80 °C.

8.2.3 Spectrophotometric assay for protease activity

Protease activities in ciliate culture medium samples were assayed by the method of Sarath *et al.* (1989). Azocasein substrate (3 % w/v) was prepared by dissolving azocasein (Sigma) in 0.1 M Tris-HCl, pH 8.0 followed by centrifugation at 10,000 x g for 10 min. The supernatant (substrate) was removed and stored on ice prior to use. The assay was initiated by the addition of 75 µl of ciliate culture medium sample to 125 µl substrate solution. Samples were incubated for 18 h at 37 °C. The reaction was then terminated by the addition of 600 µl ice cold 10 % (w/v) trichloroacetic acid (TCA), after which the mixtures were kept at 4 °C for 30 min. The samples were centrifuged at 9,000 x g for 5 min to pellet the precipitated proteins (including undigested azocasein). 0.6 ml of the supernatant was removed and mixed with 0.7 ml 1.0 M NaOH, and the absorbance of the mixture read at 440 nm on a spectrophotometer. One unit of protease activity was defined as the enzyme activity resulting in an absorbance of 1.0 in a 1 cm cuvette, under the conditions of the assay. Controls comprised cell culture media without the addition of ciliates. Each sample was assayed in triplicate.

8.2.4 Substrate–impregnated SDS-PAGE

The proteases in the ciliate culture medium and in ciliate lysates were separated using non-denaturing substrate gel electrophoresis according to the method of La Peyre *et al.* (1995), with minor modifications. Gelatin was added to the 8 % (w/v)

acrylamide resolving gel at a final concentration of 0.2 % protein (w/v). Ciliate culture medium/lysate samples (5 μl) were mixed with 15 μl of electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, containing SDS (2 % w/v), β-mercaptoethanol (5 % w/v), glycerol (10 % w/v) and 0.004 % pyronin Y) before loading onto gels containing co-polymerised gelatin. Electrophoresis was performed using a BioRad mini-Protean system at 30 mA constant current, containing the Tris-glycine buffer, for 90 min at 4 °C. After electrophoresis, the resolved gels were incubated with shaking in Triton X-100 (2.5 % v/v in distilled H_2O) for 30 min at 4 °C to remove SDS and reactivate the resolved proteases. To detect proteolytic activity, the gels were incubated in 0.1 M Tris-HCl pH 8.0 for 4-12 h at 37 °C. The gels were fixed and stained with Coomassie blue, then destained until clear bands were apparent where hydrolysis of embedded substrate had occurred. Apparent molecular weights of the proteases were determined from their mobility relative to known protein standards.

8.2.5 Effect of inhibitors on protease activity

Stock solutions of phenylmethylsulphonyl fluoride (PMSF, 200 mM), pepstatin A (1 mM), and 1,10-phenanthroline (200 mM) were prepared in 100 % methanol. A stock solution of N-(N-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl)-agmatine (E-64, 10 mM) was prepared in 100 % ethanol. Stock solutions of leupeptin (10 mM) and ethylenediaminetetraacetic acid (EDTA, 500 mM) were prepared in 0.1 M Tris-HCl pH 8.0. Stock solutions of all the inhibitors were diluted in 0.1 M Tris-

HCl pH 8.0 to the appropriate working concentration as listed in Table 8.1. Ciliate culture medium with the maximum protease activity (after 7 days of culture) was used in the inhibitor studies. To quantify the effects of the inhibitors, each of the appropriately diluted inhibitors was incubated for 1 h at 37 °C with 100 µl of ciliate culture medium. To this was added 125 µl of azocasein substrate solution, and the samples incubated for 18 h at 37 °C. The proteolytic activity was measured spectrophotometrically as described in section 8.2.3. The samples were compared with assays run in the absence of inhibitors. The effect of each inhibitor was assayed in triplicate.

To investigate the possible differences in inhibitor susceptibility between proteolytic bands, ciliate culture medium samples were resolved by gelatin-SDS-PAGE as previously described in section 8.2.4, with minor alterations. The supernatant-sample buffer mixture was loaded across all wells of the gelatin-SDS-PAGE mini-gel. After electrophoresis and Triton X-100 treatment, the gel was sectioned into six 1.5 cm vertical strips, and the strips were incubated individually in 5 ml of 0.1 M Tris-HCl, pH 8.0, containing the appropriate inhibitor for 6 h at 37 °C. The gels were fixed and stained with Coomassie blue, then destained. Inhibitor concentrations were as detailed in Table 8.1.

8.2.6 Effect of temperature and pH on protease activity

The effect of temperature on protease activity in ciliate cell culture medium samples was examined spectrophotometrically as described in section 8.2.3. The procedure was identical, apart from incubating the substrate-sample mixtures at 8, 25 and 37 °C for 18 h. For each temperature, samples were assayed in triplicate.

The effect of pH on protease activity in ciliate culture medium samples was examined spectrophotometrically and by substrate–impregnated SDS-PAGE. For the spectrophotometric assay, azocasein substrate (3 % w/v) was dissolved in 0.1 M Tris-HCl pH 7.0, followed by centrifugation at 10,000 x g for 10 min. The supernatant was removed and the pH increased by the addition of 10 M NaOH. Samples of substrate at pH 7.0 and 8.0 were removed and stored on ice prior to use. Ciliate culture medium samples (75 μl) were mixed with 125 μl substrate solution at the different pH values, and incubated for 18 h at 37 °C. Protease activity was measured as described in section 8.2.3. Controls comprised culture media without the addition of ciliates. Each substrate sample was assayed in triplicate. Ciliate culture medium samples were subjected to gelatin SDS-PAGE, and washed in Triton X-100 as described in section 8.2.4. Each gel was sectioned into respective sample lanes and incubated individually in 5 ml of 0.1 M Tris-HCl at pH 7.0 and 8.0 for 6 h at 37 °C. The gels were then stained and destained as described in section 8.2.4.

8.2.7 Effect of proteases on host muscle proteins

The effect of proteases present in ciliate culture medium, on host tissue proteins was investigated using a sample of abdominal flexor muscle from the Norway lobster. Briefly, 100 mg of abdominal flexor muscle was homogenised in 500 µl 0.1 M Tris-HCl pH 8.0, followed by centrifugation at 3,000 x g for 10 min. The muscle protein supernatant was removed and placed on ice prior to use. Samples of protein supernatant (20 µl) were mixed with 20 µl ciliate culture medium (day 7) and 20 µl 0.1 M Tris-HCl pH 8.0. Reaction mixtures were incubated at 8 °C for 15 h. Controls included the incubation of 0.1 M Tris-HCl pH 8.0, with protein supernatant, ciliate culture medium, and un-inoculated culture medium under the same conditions. Protein degradation was evaluated by standard SDS-PAGE (Sambrook *et al.*, 1989).

8.3 Results

8.3.1 Protease activity and ciliate growth *in vitro*

Ciliate density increased approximately 10-fold over 7 days in culture. Ciliates were always observed to be mobile and viable when cultures were examined daily under phase contrast. Measurement of lactate dehydrogenase (as a marker of cell integrity), by the method of Denton (1996), was attempted, but the culture media used gave a strong positive result, eliminating this as an additional indicator of

viability. Protease activity in ciliate culture medium samples was detected as early as 1 day post-inoculation, and increased over days 3, 5, and 7. This increase in activity correlated closely with the increase in ciliate density (Fig. 8.1). No proteolytic activity could be detected in un-inoculated culture medium.

8.3.2 Detection of proteases in ciliate culture medium and lysates by gelatin SDS-PAGE

The presence of multiple protease activities in ciliate culture medium and lysate samples was confirmed by gelatin SDS-PAGE. Twelve zones of hydrolysis with estimated molecular masses ranging from 20 to 220 kDa were detected in the culture medium samples, while only 8 areas of distinct hydrolysis were evident in lysate samples, with estimated molecular masses ranging from 20 to 220 kDa (Fig. 8.2). The profile of protease activities in the ciliate lysate differed not only in a reduction of bands of gelatin hydrolysis (notably the absence of the 50 kDa band (band 7) observed in ciliate culture medium samples), but also in intensity of gelatin digestion. The low molecular mass bands (20-50 kDa) appeared faint, while the high molecular mass bands (70-220 kDa) were very intense.

8.3.3 Effect of inhibitors on proteases present in the ciliate culture medium

Pre-incubation of ciliate culture medium samples with protease inhibitors before measuring the effect by the spectrophotometric assay, revealed marked inhibition

of proteolytic activity when samples were incubated in the presence of 10 mM 1,10-phenanthroline (2.5 ± 8.8 % activity) (Table 8.1). Partial inhibition was observed with the addition of EDTA (42.9 ± 3.3 % activity, 10 mM). However, addition of the inhibitors PMSF, Pepstatin A, E-64 and Leupeptin had no effect on the proteolytic activity of culture medium samples. The activities of all proteases separated by gelatin SDS-PAGE were completely inhibited when the post-electrophoresis activation buffer contained 10 mM 1,10-phenanthroline (Table 8.1). The inclusion of 10 mM EDTA in the incubation buffer inhibited totally the majority of proteases apart from the 3 largest proteases, which were partially inhibited in apparent activity. Addition of the inhibitors PMSF, Pepstatin A, E-64 and Leupeptin did not detectably inhibit hydrolysis of gelatin.

8.3.4 Effect of temperature and pH on protease activity in ciliate culture medium

In order to investigate the effect of temperature on enzyme activity, protease activity in ciliate culture medium samples was assayed by the spectrophotometric assay at different temperatures. Protease activity could be detected at 8 °C (2.17 ± 0.48 U ml^{-1}), and increased 4.5 fold to 9.77 ± 0.19 U ml^{-1} when the assay was performed at 37 °C (Table 8.2). Spectrophotometric measurements of protease activity indicated that the hydrolysis of azocasein was highest at pH 8.0 (7.42 ± 0.22 U ml^{-1}) when using 0.1 M Tris-HCl as the buffer. Decreasing the pH to 7.0 resulted in a reduction of protease activity (6.45 ± 0.23 U ml^{-1}). Gelatin SDS-

PAGE of ciliate culture medium samples revealed 12 distinct zones of hydrolysis using 0.1 M Tris-HCl at pH 7.0 and 8.0 (data not shown). However, the hydrolysis was more intense at pH 8.0.

8.3.5 Host protein degradation by proteases present in ciliate culture medium

The multiple proteases present in the ciliate culture medium samples were shown to have a selective effect on several abdominal flexor muscle proteins (lane C, Fig. 8.3) In particular, the myosin heavy chain and one of the troponin I family of proteins were completely degraded. There was also degradation of several unidentified muscle proteins with masses in the range of 100-120 kDa. However, the paramyosin, actin and other muscle proteins remained unaffected. Degradation of the FCS protein component of the culture medium secreted proteases, was apparent in ciliate culture medium samples (lane D, Fig. 8.3) but not in non-inoculated control culture media (lane E, Fig. 8.3).

8.4 Discussion

Parasitic proteolytic enzymes are proposed to be involved in parasite nutrition, anti-coagulation and evasion of the host immune system (McKerrow, 1989). They are also thought to play a key role in the processes of penetration and migration through host tissues (Klemba and Goldberg, 2002). The extensive tissue

infiltration and damage observed during *Mesanophrys* infections in crustaceans (Armstrong *et al.*, 1981; Sparks *et al.*, 1982; Hibbits and Sparks, 1983; Messick and Small, 1996) also indicates that proteases may be involved in such processes.

The results of this study show that the *Mesanophrys* sp. ciliate multiplied during *in vitro* culture, with density increasing 10-fold over the 7-day period of the experiment. Comparable increases in ciliate numbers have been observed in experimental infections of *Cancer pagurus* and *Cancer magister* with *Mesanophrys* sp. (Bang *et al.*, 1972; Cain and Morado, 2001). A number of other marine ciliates have been cultured under axenic conditions (Hanna and Lilly, 1971; Nerad and Daggett, 1992), including several ciliate parasites of aquatic hosts (Ekless and Matthews, 1993; Morado, 1993; Sterud, 1998; Inglesias *et al.*, 2003). Ciliate densities of the scuticociliate fish pathogen *Philasterides dicentrarchi* have been observed to multiply 5-fold over 7 days (reaching a plateau phase after 3 days) during *in vitro* culture using a similar seeding density to that used in the present study (Inglesias *et al.*, 2003). However the culture volumes were only 0.5 ml, and this may have limited further growth due to limited nutrients and the build-up of waste products.

Associated with the increase in ciliate growth *in vitro* was the release of extracellular proteins that possess proteolytic activity. As the *Mesanophrys* ciliates in this study appeared viable at all inspections, and in exponential growth when the experiment was terminated (day 7), the extracellular proteases detected

in the culture medium were interpreted as having been secreted. The linear relationship of protease secretion with time suggests that the secreted proteases did not accumulate in the medium, possibly indicating that the enzymes were degraded, or had an autocrine function.

Inhibitory studies using both spectroscopic and substrate impregnated SDS-PAGE methods (Table 8.1) indicate that the proteases secreted by the ciliate are of the metallo class, with complete and partial inhibition of activity observed using the inhibitors 1,10-phenanthroline and EDTA, respectively. Metalloproteases produced by several protozoan parasites are thought to contribute to the invasion and degradation of host tissues (McKerrow *et al.,* 1993), and thus play an important role in the progress of the disease. Metalloproteases have been documented from a number of marine pathogens during *in vitro* culture, including the piscine haemoflagellate *Cryptobia salmositica* (Zuo and Woo, 1998), the bivalve pathogen *Vibrio alginolyticus* (Nottage and Birkbeck, 1987), the fish pathogen *Aeromonas salmonicida* (Arnesen *et al.,* 1995), and, significantly, the scuticociliate parasite of fish *Uronema marinum* (Lee *et al.*, 2003). In the study of Lee *et al.*, metalloprotease secretion was detected as early as 10 min in ciliate cell suspensions by fluorescence polarization, and they suggest that metalloproteases are involved in the pathogenesis of ciliate infection in fish. Other species of ciliates such as *Tetrahymena pyriformis* and *T. thermophilia* have previously been shown to contain and secrete proteases (Banno *et al.*, 1983; Straus *et al.*, 1992). In these examples, it is proposed that the proteases are involved in both intracellular

and extracellular digestion. Furthermore, lysosomal-released enzymes from *T. thermophilia* have been implicated in the process of extracellular digestion and parasite nutrition, with proteolytic enzymes facilitating the release of host particulate and soluble nutrients (Florin-Christensen *et al.*, 1985; Tiedtke and Rasmussen, 1988). Therefore, the enzymes released by this *Mesanophrys* sp. may facilitate nutrient uptake, both *in vitro* and *in vivo.*

Many parasitic protozoa contain or secrete multiple proteases, examples being the clam parasite *Pseudoperkinsus tapetis* (Ordás *et al.*, 2001), and the oyster parasite *P. marinus* (La Peyre *et al.*, 1995). In the present study, the proteases observed in the ciliate culture medium migrated as 12 distinct proteolytic bands on gelatin SDS-PAGE gels having molecular masses of between 20 to 200 kDa. Substrate gel electrophoresis depends upon the regeneration of proteolytic activities following electrophoresis, however it is possible that some proteases may be inactivated by electrophoresis and are therefore undetected. The estimated molecular weights of the proteolytic enzymes resolved are an approximation due to the electrophoretic technique, and purification of the proteases from the culture medium is required for accurate determination of molecular weights.

The substrate specificities of individual enzymes were not examined but warrants further investigation as the proteases secreted have been shown to act in a selective manner on host tissue proteins. Proteases present in the culture medium were observed to be active at 8 °C, and had maximum activity at pH 8.0 but also

had significant activity at pH 7.0 (87.3 %), indicating that at the physiological temperature and pH range of lobster tissues and haemolymph the proteolytic enzymes would be active.

During *Mesanophrys* infections, expansive host tissue penetration and destruction are common observations (Armstrong *et al.*, 1981; Sparks *et al.*, 1982; Hibbits and Sparks, 1983; Messick and Small, 1996). The present study has shown that proteases present in ciliate culture medium selectively degrade the myosin heavy chain along with one of the troponin I family of proteins from *N. norvegicus* abdominal flexor muscle *in vitro*. Several unidentified muscle proteins with masses in the range of 100-120 kDa were also degraded, but the paramyosin, actin and other muscle proteins remained unaffected by the proteases. It has been suggested that the multiple proteases secreted by *Perkinsus marinus* degrade gut epithelium and basement membranes of the eastern oyster, *Crassostrea virginica*, favouring parasitic invasion and spread in oyster tissues (La Peyre *et al.*, 1996). The myosin heavy chain proteins are present in all muscle groups and also other tissues within the lobster host, and thus proteases secreted by the ciliate *in vivo* will degrade these tissues, facilitating tissue penetration and consumption.

Hemocytopenia is a classic symptom of advanced ciliate infections in crustaceans (Cattaneo, 1888; Armstrong *et al.*, 1981; Sparks *et al.*, 1982; Cain and Morado, 2001). A recent study has shown that experimental infection of dungeness crabs with *Mesanophrys pugettensis* resulted in a marked reduction in haemocyte

numbers 6 days post-inoculation, with differential cell counts indicating that intermediate granulocytes were more rapidly depleted than other cell types (Cain and Morado, 2001). It is known that extracellular proteins can modify the immune system of the host. An example of this is the 64 kDa protease and extracellular proteins secreted by *A. salmonicida*, which suppresses the humoral immune response of Atlantic salmon, *Salmo salar*, resulting in leucopenia and allowing the bacteria to spread via the circulatory system and invade other tissues (Ellis *et al.*, 1981; Hussain *et al.*, 2000). Extracellular proteins from *A. salmonicida* are also reported to induce degranulation of eosinophilic granular cells, releasing vasoactive compounds leading to acute shock in Atlantic salmon (Ellis *et al.*, 1981). Similarly, the pathogenic haemoflagellate *Cryptobia salmositica* secretes a metalloprotease which can directly lyse fish erythrocytes causing anaemia (Zuo and Woo, 2000), while the proteases secreted by the apicomplexan oyster parasite *P. marinus* compromise oyster host defence mechanisms by inhibiting haemocyte motility and degrading plasma lysozyme and hemagglutinins (Garreis *et al.*, 1996). Therefore the depletion of haemocytes, and in particular granulocytes, during *Mesanophrys* infections of crustaceans, attributed to consumption by ciliates, may also be due in part to the effect of secreted proteases and this possibility warrants further study.

Table 8.1 Effect of inhibitors on the activity of proteases present in ciliate culture medium. Activity of 100% is equivalent to 7.08 ± 0.33 U ml^{-1}. Mean ± SD, N=3.

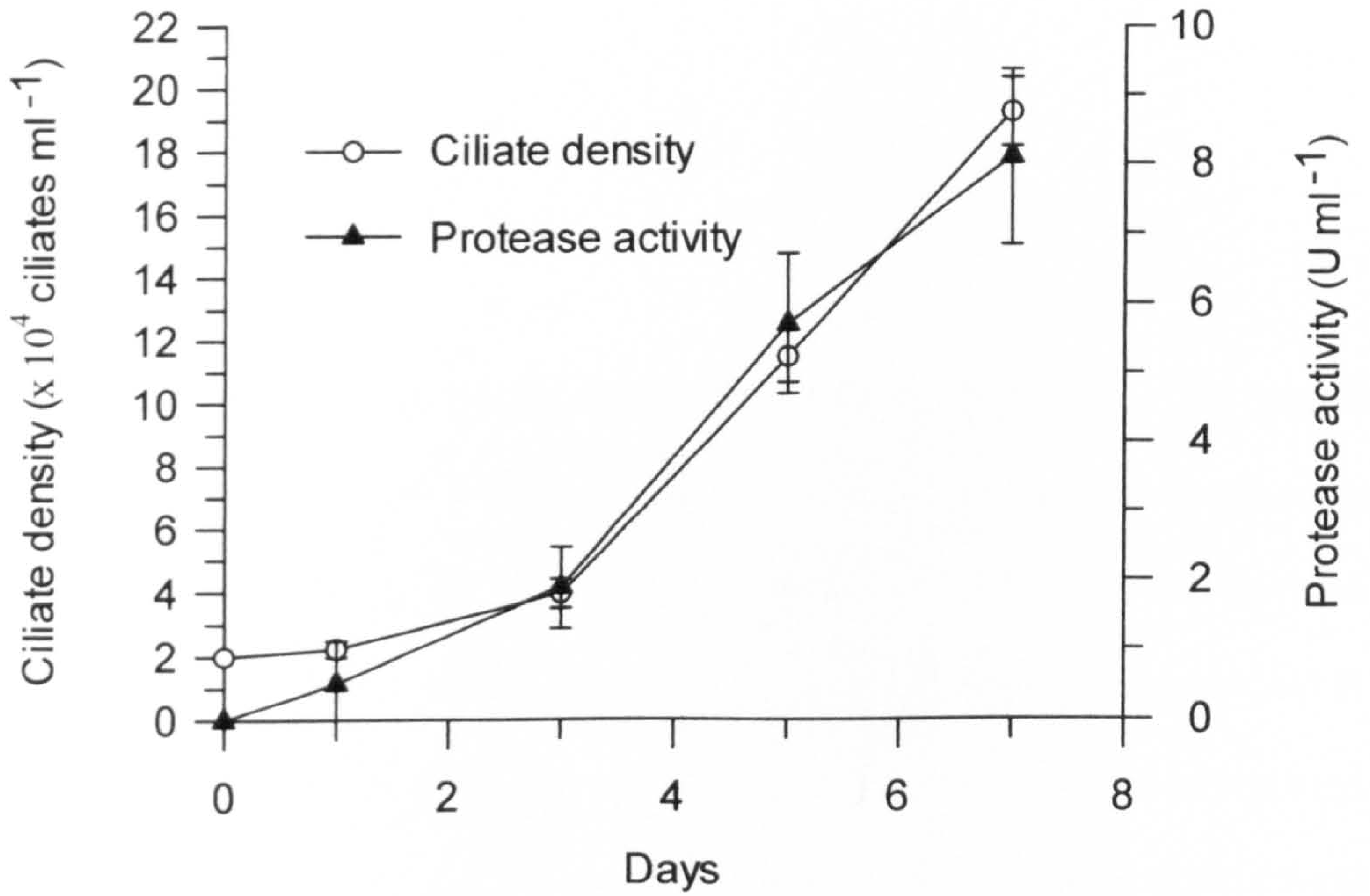
Table 8.2 Effect of temperature on the activity of proteases present in ciliate culture medium. Mean ± SD, N=3.

Inhibitor	Concentration	Protease activity (% Control)	Inhibition of gelatin SDS-PAGE digestion
Control (0.1M Tris-HCl) pH 8.0		100 ± 4.6	None
Control (Ethanol)		94.5 ± 6.1	None
Control (Methanol)		95.9 ± 2.9	None
Serine protease/Cysteine protease inhibitors:			
PMSF	1 mM	94.6 ± 2.4	
	10 mM	92.0 ± 2.8	None
Leupeptin	1 μM	94.4 ± 2.9	
	10 μM	91.1 ± 3.7	None
Cysteine protease inhibitor:			
E-64	1 μM	97.5 ± 5.2	
	10 μM	94.2 ± 2.5	None
Aspartic protease inhibitor:			
Pepstatin A	1 μM	92.5 ± 4.4	None
Metallo-protease inhibitors:			
1,10 phenanthroline	1 mM	41.4 ± 3.8	
	10 mM	2.5 ± 8.8	Complete
EDTA	1 mM	87.0 ± 3.6	
	10 mM	42.9 ± 3.3	Partial

Assay temperature (°C)	Protease activity (U ml^{-1})
8	2.17 ± 0.48
25	6.23 ± 0.31
37	9.77 ± 0.19

Figure 8.1 Changes in ciliate density and protease activity in culture medium during the experimental time period of 7 days. Mean ± SD, N=3.

Figure 8.2 Detection of proteases in ciliate culture medium (A), and ciliate crude lysates (B), by gelatin-SDS-PAGE. The molecular mass (MW) standards are indicated on the left in kilodaltons (kDa), arrows indicate areas of hydrolysis.


Ciliate density
Protease activity
Ciliate density (x 10^4 ciliates ml^{-1})
Protease activity (U ml^{-1})
Days
0
2
4
6
8
10
12
14
16
18
20
22

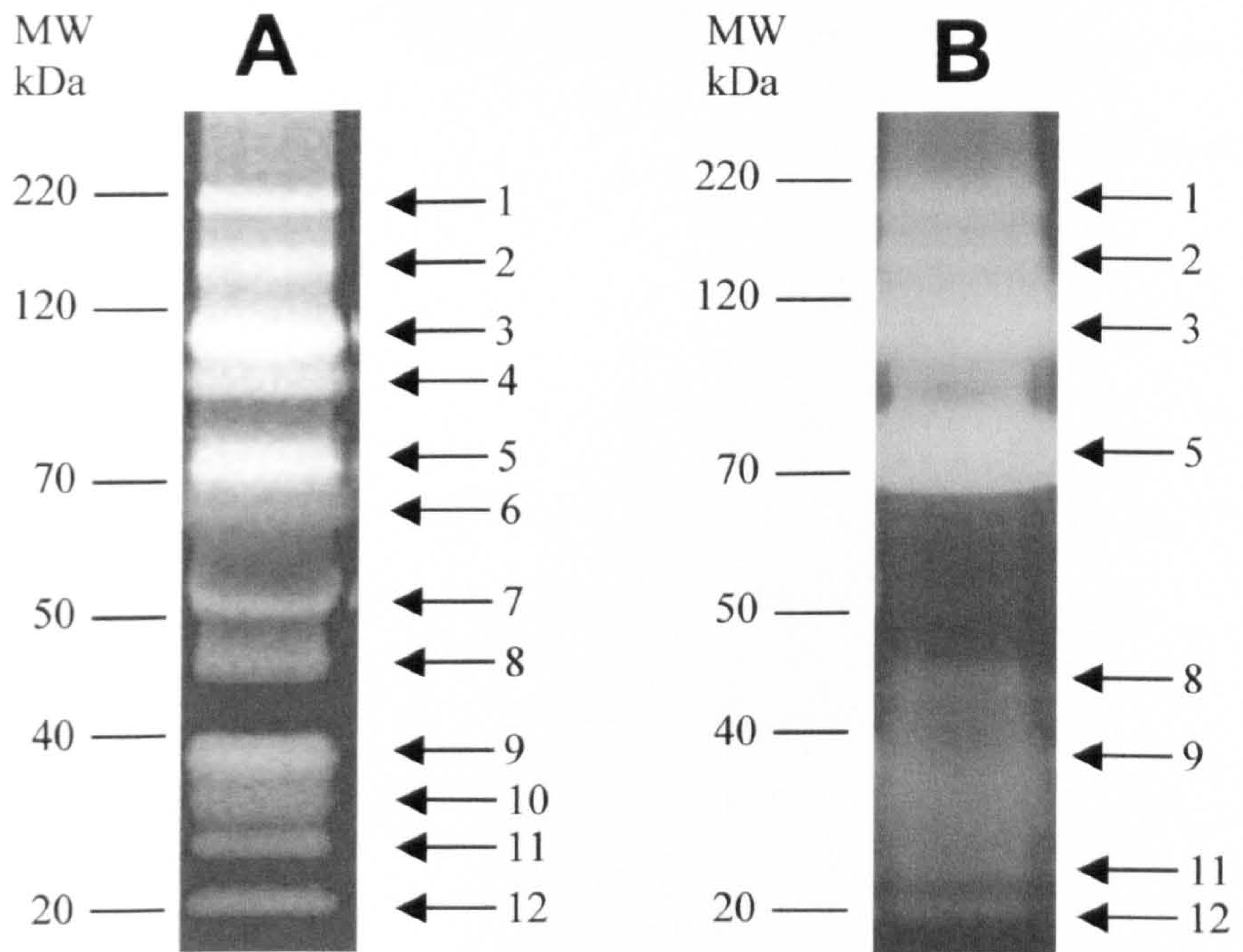
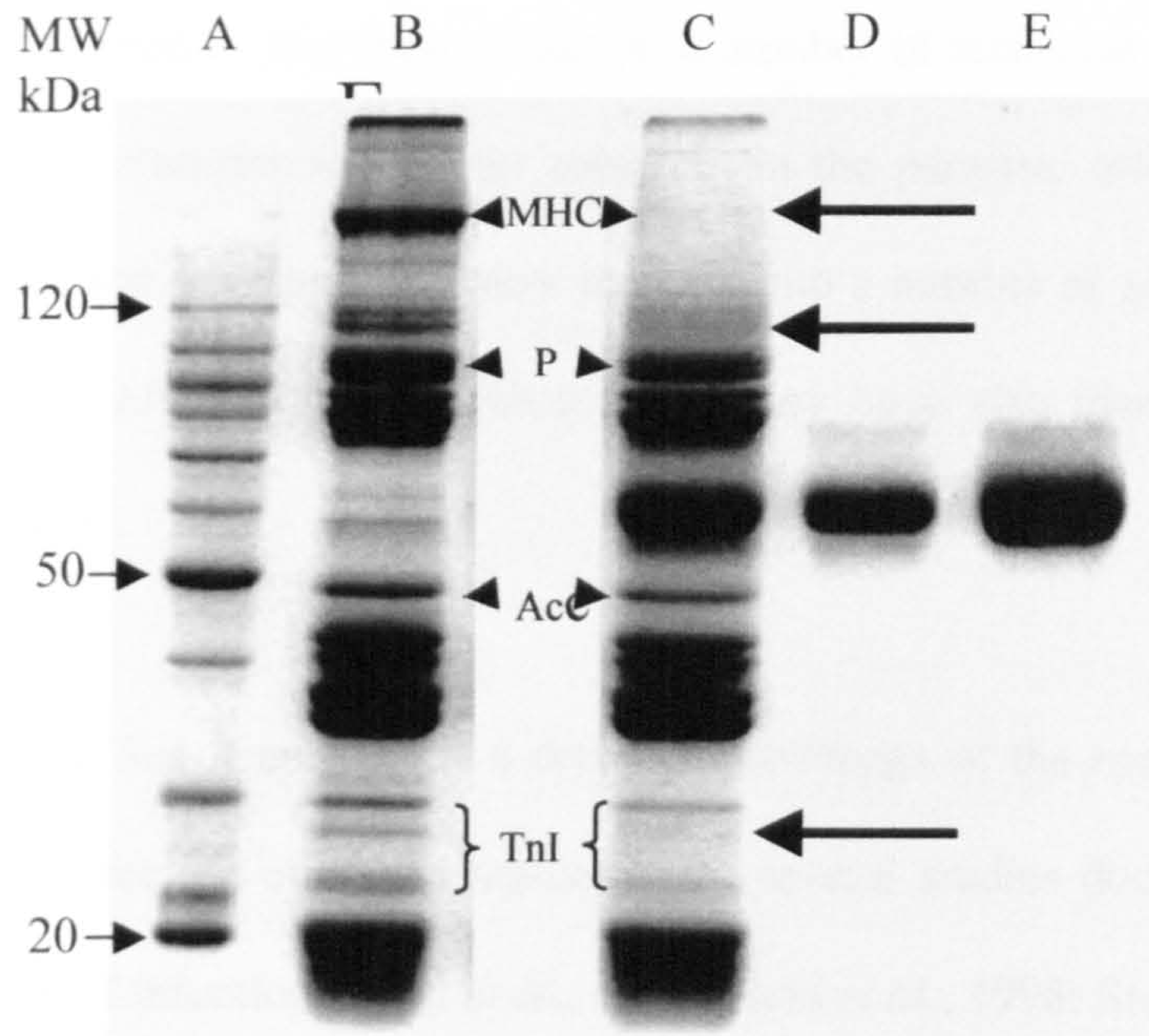

MW kDa
A
B
220
120
70
50
40
20
1
2
3
4
5
6
7
8
9
10
11
12

Figure 8.3 Digestion of deep abdominal flexor (DAF) muscle proteins by proteases present in ciliate culture medium. Lane A, molecular mass standards, sizes are indicated on the left in kilodaltons (kDa); lane B, DAF protein sample incubated in the absence of ciliate culture medium; lane C, DAF protein sample incubated in the presence of ciliate culture medium; lane D, ciliate culture medium sample; lane E, un-inoculated (control) culture medium sample. Note the degradation of DAF muscle proteins in lane C (arrows). Also note the reduced intensity (digestion) of FCS proteins in the ciliate culture medium sample (lane D) compared to the control medium sample (lane E). MHC, myosin heavy chain; P, paramyosin; Ac, actin; TnI, Troponin I family.


MW
kDa
A
B
C
D
E
120
50
20
MHC
P
Acc
TnI

Chapter 9

General Discussion

The results presented in this thesis describe a number of technical developments that provide opportunities for further research on the parasitic infections of the Norway lobster and have provided new insights into a number of aspects of their genetic identity, infectivity and virulence, but they have also identified several unresolved issues.

Within the Clyde Sea Area there is a detailed knowledge of the epidemiology of *Hematodinium* infection of *N. norvegicus*, with several studies documenting the seasonal pattern of infection (Field *et al.*, 1992; Field *et al.*, 1998; Stentiford *et al.*, 2001c), and the effects on host tissue function and physiology (Field *et al.*, 1995; Taylor *et al.*, 1996; Stentiford *et al.*, 2000b, 2001a), and behaviour (Stentiford *et al.*, 2000a, 2001d). In contrast to the detailed knowledge for this *N. norvegicus* fishery, the information available for other *N. norvegicus* stocks around the UK coast is limited to basic prevalence studies, e.g. for the Irish Sea (Briggs and McAliskey, 2002). In part, this is due to the fact that a simple diagnostic assay has not been available. Chapter 2 describes the development of such an assay, which uses previously obtained polyclonal anti-*Hematodinium* antiserum in an enzyme linked immunosorbent assay (ELISA). This diagnostic method can be used for detection of the parasite in the haemolymph of affected lobsters, and is

particularly useful for screening large numbers of samples. It will therefore facilitate the investigation of the prevalence of *Hematodinium* infections in *N. norvegicus* and other crustacean stocks around the UK and elsewhere.

Among the questions that may be addressed by such surveys are: does the seasonal pattern of infection of *N. norvegicus* observed in the Clyde Sea Area also occur in other locations, and if so does it show any systematic shift with latitude or with geographical area? At the only other location where the infection peak has been identified, in the Swedish Skagerrak, it occurs in the autumn (in contrast to the spring peak found in the Clyde Sea Area) (K. Frohlund, Havsfiskelaboratoriet Sweden, personal communication). Therefore, it is to be expected that differences will also be found between the various Scottish locations that support important fisheries.

Fisheries surveys of this kind, using the ELISA screening assay, can make an important contribution to the interpretation of the biology of this parasite. As an example, consider the speculated link between infection and the moulting cycle of the host. Available evidence suggests that the *N. norvegicus* populations in Northern Scotland moult at a later time in the year than the Clyde population, due to their higher latitudes (J. Atkinson, University of Glasgow, personal communication). If this proves to be so, an opportunity is provided to test the hypothesis that the infection peak is linked to the stresses of moulting. If a causal link exists, then the infection peak should also occur at a later time at higher

latitudes. Other important insights into the factors that influence the seasonality or route of transmission of *Hematodinium* may also be obtained in such ways.

The development and implementation of a PCR assay and DNA probes for the detection of *Hematodinium*, based on the amplification or recognition of DNA from the parasite (Chapter 3), provide a further level of sensitivity and specificity in the diagnosis of this parasite. These techniques offer a number of opportunities not only for investigating the epidemiology of *Hematodinium* infection in greater detail, but also for comparing the identity of parasites obtained from various host species and at different locations.

Applying molecular diagnostic methods to the study of *Hematodinium* infection in *N. norvegicus* will enhance our knowledge of the life cycle of this parasite. In particular they will facilitate low-level infection diagnosis in the lobster, which is critical for the investigation of latency and for determining the sequence of life cycle stages. Furthermore, molecular probes could help investigate the mode of *Hematodinium* transmission, which at present is unknown, although it has been suggested that dinospores are the most likely infective stage (Appleton and Vickerman, 1998). The molecular probes will also be an important tool for investigating whether other crustacean species such as amphipods act as secondary hosts in the life cycle of *Hematodinium*, and are involved in its transmission to *N. norvegicus*.

Clarifying the spatial distribution of genetic variation within *Hematodinium* sp. infecting crustaceans common to UK waters (Chapter 4) is essential for a proper interpretation of potential virulence differences. While *Hematodinium* infections have previously been recorded from a variety of crustacean species at several locations around the UK (Field *et al.*, 1992; Field *et al.*, 1998, Stentiford *et al.*, 2002), the present study found no substantial strain variation in relation to either the geographical distribution of one host (*N. norvegicus*), or to a range of different crustacean hosts. This finding has a number of consequences for interpreting the virulence of *Hematodinium* infection in different hosts. Thus the severe disruption caused by *Hematodinium* infection to *Cancer pagurus* (Stentiford *et al.*, 2002), compared to the much less distinct disruption caused to abdominal muscles of *N. norvegicus* (Stentiford *et al.*, 2000b) has been ascribed to the former harbouring a more virulent strain. However, the present study indicates that these two crustacean hosts are infected by strains of *Hematodinium* that have complete genetic identity over the first internal transcribed rDNA gene region (ITS1), and hence should probably be ascribed to a single species. Therefore differences in virulence must be due either to differences in host susceptibility, or to the expression of different virulence-related genes by the same parasite species in different hosts.

This study is the first to report and confirm *Hematodinium* infection of *N. norvegicus* from the North Minch and Fladen fishing grounds, situated on the North West and North East of Scotland, respectively (Chapters 2, 3 and 4). It has

previously been suggested that limited water mixing and flushing within confined bodies of water such as the Scottish Sea lochs, the fjords of Alaska, and the shallow lagoons on the East coast of America may be contributing factors in the increased prevalences of *Hematodinium* in *N. norvegicus*, *C. bairdi* and *C. sapidus*, respectively (Shields, 1994). Associations with changing temperatures and salinities, and also with pollution are thus implied. However, since the North Minch and, in particular the Fladen fishing grounds are offshore, and hence subject to strong tidal conditions, any association of *Hematodinium* epizootics with the conditions within confined water bodies cannot be strong, and such claims may in fact be misleading. This highlights the need to apply caution when linking infection episodes with possible degradation of the environment.

The results of attempts to initiate *in vitro* cultures of *H. perezi* from the blue crab, *Callinectes sapidus*, (Chapter 5) show that this parasite has life cycle stages that are morphologically similar to those of the *Hematodinium* sp. from *N. norvegicus* (Appleton, 1996; Appleton and Vickerman, 1998). However, in both species only a few of the stages that occur *in vitro* have also been observed *in vivo* (Newman and Johnson, 1975; Field and Appleton, 1995; Field and Appleton, 1996; Shields and Squyars, 2000). This discrepancy between growth of the parasites *in vivo* and *in vitro* hinders the comprehensive analysis of their life history progression, and further attention is required to both the composition of the culture media and the growth conditions.

Appleton (1996) suggested that the *Hematodinium* culture medium might not have essential elements needed for efficient parasite growth and differentiation, as the time period between sporogenic events was lengthy. In addition, enrichment of the culture medium with amino acids was observed to alter the size and the number of nuclei in filamentous trophonts, indicating that media constituents can alter parasite morphology. Likewise, MacIntyre *et al.* (2003) reported that *Perkinsus marinus* culture medium supplemented with oyster tissue extracts had a pronounced effect on the differentiation of parasite cells, resulting in the appearance *in vitro* of *P. marinus* cells that were morphologically comparable to those observed within host oyster tissues. Consequently, those involved in the future culture of *Hematodinium* sp. may want to replace the artificial foetal calf serum component of the medium with host haemolymph or tissue extracts, and to determine if this has an effect on parasite morphology and life cycle events.

During the course of routine examinations of *Hematodinium* infection in *N. norvegicus*, a parasitic ciliate was observed in the haemolymph of a number of lobsters (Chapter 7). Silver carbonate impregnation techniques that stain the nuclear and oral structures as well as the infraciliature, along with electron microscopy studies indicate that, on morphological criteria, the ciliate is a member of the genus *Mesanophrys*. However ribosomal DNA sequence analysis point to an affinity with *Orchitophrya stellarum*, a ciliate parasite of sea stars. Both ciliates cause extensive tissue damage to their respective hosts.

It is worth noting that within the live trade market, Norway lobsters are held in impoundments for preconditioning prior to transport abroad. In such confined situations, the likelihood of infectious disease outbreaks is high. Thus reports of parasitic ciliates involved in significant post-harvest losses of the American lobster, *Homarus americanus*, (Aiken *et al.*, 1973) are an indication that the ciliate identified in this study may pose a significant threat to Norway lobsters held in impoundments. Further prevalence studies are required to address whether this is an issue for concern.

Evidence presented in this study has suggested that certain *Hematodinium* sp. (from *C. sapidus* and *N. norvegicus*) can be distinguished from each other by internal and extracellular enzyme activity (Chapter 6). Enzymatic profiles have been used to type other pathogens (Poh and Loh, 1985; Grehn *et al.*, 1991; García-Martos *et al.*, 2000) and may be applied to type *Hematodinium* sp. However, *C. sapidus* has been shown to be molecularly (Hudson and Adlard, 1996; and Chapter 4 of this study) and pathologically (Shields and Squyars, 2000) very different from the *Hematodinium* sp. infecting *N. norvegicus*. Thus it is unsurprising that internal and external enzyme profiles of the two species are different.

The secretion of the enzymes acid phosphatase and leucine arylamidase by the *Hematodinium* sp. (Chapter 6) may aid parasite survival by mediating their nutrition and/or by allowing them to evade the host immune response. The first

suggestion is supported by the studies of Appleton and Vickerman (1996), who identified circulating sporoblasts that possessed micropores that participated in endocytosis. Thus the secreted enzymes may play a role in host tissue degradation, allowing products to be internalised through the micropores. Endosomal compartments have been observed in *H. perezi* via staining with neutral red (Chapter 5) further substantiating this as a possible method of parasite cell nutrition.

The second suggestion is that the enzymes contribute to evasion of the host immune response. Both acid phosphatase and proteases have been identified as important for parasite survival of the host immune response (discussed in Chapters 6 and 8). Crustaceans possess an innate immune system, which when triggered by microbial cell wall components, initiates the synthesis of several antibacterial peptides, the activation of the prophenoloxidase system, the release of several cell adhesion proteins from granulocytes, and phagocytosis of invading microorganisms (Thörnqvist and Söderhäll, 1997). This study (Chapter 6) has shown that the *Hematodinium* sp. infecting *N. norvegicus* secretes acid phosphatases that may be involved in inhibition of oxygen radicals produced from the oxidative burst of haemocytes. Further studies are required to establish whether *Hematodinium* sp. possess other immunomodulatory enzymes such as superoxide dismutase and catalase (both of which catalyse superoxide and hydrogen peroxide into water and oxygen). In addition, measurement of immune

system markers in *Hematodinium*-infected *N. norvegicus* may elucidate the pathogenic mechanisms of parasite development.

Evidence presented in the current study suggests that the pathogenicity of the *Hematodinium* sp. from *N. norvegicus* and *C. sapidus*, and of the *Mesanophrys* sp. ciliate isolated from *N. norvegicus* may be correlated with proteases released by the parasites. The *Hematodinium* infection in *N. norvegicus* is considered to be a gradual process spanning a period of almost one year, with tissue invasion occurring before patent haemolymph infections are detectable (Field and Appleton, 1995). Additionally, it has not been possible to infect lobsters experimentally (Vickerman, 1994). Both these features correlate with an absence of secretion of proteases by the *Hematodinium* sp. from *N. norvegicus* (Chapter 6).

In contrast, *H. perezi* from *C. sapidus*, which has been found to contain and secrete proteases, is readily transmittable to naïve blue crabs and causes mortalities in as little as 14 days after injection of infected haemolymph (Shields and Squyars, 2000). In a similar way, the *Mesanophrys* sp. of ciliate identified infecting Norway lobsters (Chapter 7) was also found to secrete a number of metalloproteases, which were selective in the degradation of several host proteins (Chapter 8). Since experimental infection of other crustaceans by *Mesanophrys* sp. is nearly always fatal, and the time to death is rapid, this reinforces a relationship between proteolytic activity and virulence.

Thus the conclusion can be drawn that the *Hematodinium* sp. from the Norway lobster is less aggressive than these other highly proteolytic species in its mode of infection and replication. Its action appears to be largely by a combination of functional starvation and hypoxia due to the large number of parasites that consume the tissues and circulatory system (Taylor *et al.* 1996; Stentiford *et al.*, 2001a). However, the possibility cannot be excluded that it produces differential effects in different tissues, perhaps by triggering the release of intrinsic proteolytic enzymes (e.g. in the claw muscles). Finally it can be envisaged that the morbidity induced by *Hematodinium* facilitates secondary infections by other pathogens, and it is the effects of these that are ultimately fatal.

The present findings therefore focus attention on these more subtle aspects of the pathogenicity of *Hematodinium* infections of *N. norvegicus,* and their effect on the host immune response. These are among the more important questions to be addressed in the next phase of research on this parasite.

Future prospects

A recent meeting on *The Future of the Clyde Fisheries* at UMBS Millport in February 2004, attended by all stakeholders from fisherman to processors and regulatory bodies, has highlighted the importance of scientific knowledge about the Norway lobster for the survival and profitability of the industry. The Clyde Sea Area is now a single-species fishery for *N. norvegicus,* which is coming under

greater pressure as EU regulations restrict the capture of white fish such as cod. At the same time increasing numbers of smaller 'prawns' are being taken, in an attempt to maintain profits. However, the scientific work on the biology of *Hematodinium* infection in *N. norvegicus,* which has identified annual peaks of infection that sometimes can exceed 50% prevalence and are greatest in smaller animals, suggests a possible scenario in which the whole stock is driven towards a catastrophic collapse.

The need for proper management of these prawn stocks in the Clyde has therefore become increasingly urgent, and in an innovative move the stakeholders have agreed to set up a local management group with the aim of exploiting the fishery in a sustainable manner. As part of their management plan, a regular scientific assessment of the stock is to be implemented, including screening for *Hematodinium* using the most sensitive methods available such as those developed in this project. This will allow decisions to be made about restricting fishing activities, by week-end bans or by closed fishing zones, in order not only to conserve stocks, but also to prevent animals of reduced quality (including those infected with *Hematodinium*) from entering the supply chain, and thus threatening consumer confidence.

These developments in responsible management may represent a model for other fisheries around the world that become threatened by over-exploitation. Since infections become increasingly important factors in causing mortality when stocks

are depleted, knowledge about them is important not only from a purely biological perspective, but also in order to inform management decisions in economically-important and heavily exploited fisheries.

References

Abelló, P., Valladares, F.J. and Castellón, A. (1988). Analysis of the structure of decapod crustacean assemblages off the Catalan coast (North-West Mediterranean). *Marine Biology* **98**, 39-49.

Aguirre Guzmán, G. and Ascencio Valle, F. (2000). Infections disease in shrimp species with aquaculture potential. Recent *Research in Developmental Microbiology* **4**, 333-348.

Agundis, C., Pereyra, A., Zenteno, R., Brassart, C., Sierra, C., Vazquez, L. and Zenteno, E. (2000). Quantification of lectin in freshwater prawn (*Macrobrachium rosenbergii)* haemolymph by ELISA. *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology* **127**, 165-172.

Aiken, D.E., Sochasky, J.B. and Wells, P.G. (1973). Ciliate infestation of the blood of the lobster, *Homarus americanus*. ICES C.M. K: 46.

Akiyama, D.M., Brock, J.A. and Haley, S.R. (1982). Idiopathic muscle necrosis in the cultured freshwater prawn (*Macrobrachium rosenbergii*). *Veterinary Medicine and Small Animal Clinician* **77**, 1119-1121.

Alderman, D.J. (1996). Geographical spread of bacterial and fungal diseases of crustaceans. *Scientific and Technical Review of the Office International des Epizooties* **15**, 603-632.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Anderson, G.R. and Barker, S.C. (1993). Species differentiation in the Didymozoidae (Digenea): restriction fragment length differences in internal transcribed spacer and 5.8S ribosomal DNA. *International Journal for Parasitology* **23**, 133-136.

Anon. 2001. Report of the Working Group on *Nephrops* stocks. ICES C.M. 2001 ACFM: 16, 302 p.

Appleton, P. L. (1996). Investigations on the cytology and life-cycle of the parasitic dinoflagellate *Hematodinium* sp. associated with mortality of *Nephrops norvegicus*. Ph.D. Thesis, University of Glasgow, Scotland. 204 p.

Appleton, P.L. and Vickerman, K. (1996). Presence of Apicomplexan-type micropores in a parasitic dinoflagellate, *Hematodinium* sp. *Parasitology Research* **82**, 279-282.

Appleton, P.L., Field, R.H., Vickerman, K., Atkinson, R.J.A., Taylor, A.C., Rogerson, A. and Neil, D.M. (1997). Mortality of *Nephrops norvegicus* on the West Coast of Scotland. Report to MAFF CSG, March 1997.

Appleton, P.L. and Vickerman, K.V. (1998). *In vitro* cultivation and development cycle in culture of a parasitic dinoflagellate (*Hematodinium* sp.) associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters. *Parasitology* **116**, 115-130.

Armstrong, D.A., Buchanan, D.V. and Caldwell, R.S. (1976). A mycosis caused by *Lagenidium* sp. in laboratory-reared larvae of the Dungeness crab, *Cancer magister*, and possible chemical treatments. *Journal of Invertebrate Pathology* **28**, 329-336.

Armstrong, D.A., Burreson, E.M. and Sparks, A.K. (1981). A ciliate infection (*Paranophrys* sp.) in laboratory-held Dungeness crabs, *Cancer magister*. *Journal of Invertebrate Pathology* **37**, 201-209.

Arnesen, J.A., Eggset, G. and Jørgensen, T.O. (1995). Partial purification and purification of extracellular metalloproteases from *Aeromonas salmonicida* ssp. *Journal of Fish Diseases* **18**, 283-295.

Atkinson, R.J.A. and Naylor, E. (1976). An endogenous rhythm and rhythmicity of catches of *Nephrops norvegicus*. *Journal of Experimental Marine Biology and Ecology* **25**, 95-108.

Atkinson, R.J.A. (1987). The burrowing megafauna of the upper arms of Loch Sween. Report to the Nature Conservancy Council, Peterborough, from the University Marine Biological Station, Millport. 65 p.

Baca, O.G., Roman, M.J., Glew, R.H., Christner, R.F., Buhler, J.E. and Aragon, A.S. (1993). Acid phosphatase activity in *Coxiella burnetii*: a possible virulence factor. *Infection and Immunity* **61**, 4232-4239.

Bailey, N., Howard, F.G., and Chapman, C.J. (1986). Clyde *Nephrops*: biology and fisheries. In: The environment of the Estuary and Firth of Clyde (Eds. Allen, J.A., Barnett, P.R.O., Boyd, J.M., Kirkwood, R.C., McKay, D.W. and Smith, J.C.). Proceedings of the Royal Society of Edinburgh 90B, p 501-518.

Bancroft, J.D. (1967). Acid phosphatase: The Napthol AS Phosphate Method. In: An Introduction to Histochemical Technique. Butterworth & Co, London. p 192-194.

Bang, F.B. (1962). Serological aspects of immunity in invertebrates. *Nature* **196**, 88-89.

Bang, F.B. (1970). Disease mechanisms in crustacean and marine arthropods. In: A symposium on diseases of fish and shellfishes. (Ed. Snieszko, S.F.). American Fisheries Society, Washington, D.C. p 383-404.

Bang, F.B., Audouin, J. and Leglise, M. (1972). Ciliate infection of the blood of the edible crab, *Cancer pagurus*, in holding tanks in Brittany, France. *Journal of Invertebrate Pathology* **20**, 226-227.

Banno, Y., Yano, K. and Nozawa, Y. (1983). Purification and characterization of a secreted proteinase from *Tetrahymena pyriformis*. *European Journal of Biochemistry* **132**, 563-568.

Baross, J.A. and Tester, P.A. (1978). Incidence, microscopy, and etiology of exoskeletal lesions in the tanner crab, *Chionoecetes tanneri*. *Journal of the Fisheries Research Board of Canada* **35**, 1141-1149.

Baticados, M.C.L. and Enriquez, G.L. (1982). Development and stage of a microsporidian parasite of the white prawn, *Penaeus merguiensis* de Man 1888. *Natural and Applied Science Bulletin (Philippines)* **34**, 255-270.

Behringer, D., Butler, M. and Shields, J. (2001). The first viral disease reported in lobsters. *The Lobster Newsletter* **14**, 1-3.

Bell, K. and Smith, V.J. (1993). *In vitro* superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.). *Developmental and Comparative Immunology* **17**, 211-219.

Bell, K. and Smith, V.J. (1994). A comparative study of the respiratory burst produced by phagocytic cells of marine invertebrates. *Annals of the New York Academy of Sciences* **712**, 330-331.

Berthe, F.C.J., Le Roux, F., Peyretaillade, E., Peyret, P., Rodriguez, D., Gouy, M. and Vivares, C.P. (2000). Phylogenetic analysis of the small subunit ribosomal RNA of *Marteilia refringens* validates the existence of Phylum Paramyxea (Desportes and Perkins, 1990). *The Journal of Eukaryotic Microbiology* **47**, 288-293.

Bodley, A.L., McGarry, M.W. and Shapiro, T.A. (1995). Drug cytotoxicity assay for African trypanosomes and *Leishmania* species. *The Journal of Infectious Diseases* **172**, 1157-1159.

Bower, S.M. and Meyer, G.R. (1993). *Stegotricha enterikos* gen. n., sp. n. (Class Phyllopharyngea, Order Rhynchodida), a parasitic ciliate in the digestive gland of Pacific oysters (*Crassostrea gigas*) and its distribution in British Columbia. *Canadian Journal of Zoology* **71**, 2005-2017.

Bower, S.M., Meyer, G.R. and Boutillier, J.A. (1993). Disease of spot prawns (*Pandalus platyceros*) caused by intracellular bacterium and a *Hematodinium*-like protozoan. *Journal of Shellfish Research* **12**, 135a.

Bower, S.M., Meyer, G.M., Phillips, A., Workman, G. and Clark, D. (2003). New host and range extension of bitter crab syndrome in *Chionoecetes* spp. caused by *Hematodinium* sp. *Bulletin of the European Association of Fish Pathologists* **23**, 86-91.

Bowser, P.R., Rosemark, R. and Reiner, C.R. (1981). A preliminary report of vibriosis in cultured American lobsters, *Homarus americanus*. *Journal of Invertebrate Pathology* **37**, 80-85.

Brautigan, D.L. (1992). Great expectations: Protein tyrosine phosphatases in cell regulation. *Biochemica et Biophysica Acta* **1114**, 63-77.

Briggs, R.P. (1997). The Dublin Bay Prawn. *Journal of the Institute of Biology* 44, 246-248.

Briggs, R.P. and McAliskey, M. (1996). The Prevalence of *Hematodinium* in Irish Sea *Nephrops*. ICES *Nephrops* SG, Lorient. 4 pp.

Briggs, R.P. and McAliskey, M. (2002). The prevalence of *Hematodinium* in *Nephrops norvegicus* from the Western Irish Sea. *Journal of the Marine Biological Association of the* UK **82**, 427-433.

Brock, J.A. and Lightner, D.V. (1990). Diseases of Crustacea – Diseases caused by microorganisms. In: Diseases of marine animals Vol. 3 (Ed. Kinne, O.). Biologische Anstalt Helgoland, Hamburg, p 245-249.

Bryant, M.S., Lee, R.P., Lester, R.J.G. and Whittington, R.J. (1999). Anti-immunoglobulin antisera used in ELISA to detect antibodies in barramundi *Lates calcarifer* to *Cryptocaryon irritans*. *Diseases of Aquatic Organisms* **36**, 21-28.

Burnstone, M.S. (1958). Histochemical demonstration of acid phosphatases with naphthol AS-phosphatases. *Journal of the National Cancer Institute* **21**, 523-259.

Bushek, D., Dungan, C.F. and Lewitus, A.J. (2002). Serological affinities of the oyster pathogen *Perkinsus marinus* (Apicomplexa) with some Dinoflagellates (Dinophyceae). *Journal of Eukaryotic Microbiology* **49**, 11-16.

Byrne, M., Cerra, A., Nishigaki, T. and Hoshi, M. (1997). Infestation of the testes of the Japanese sea star *Asterias amurensis* by the ciliate *Orchitophrya stellarum*: a caution against the use of this ciliate for biological control. *Diseases of Aquatic Organisms* **28**, 235-239.

Cachon, J. and Cachon, M. (1987). Parasitic Dinoflagellates. In: The Biology of Dinoflagellates (Ed. Taylor, F.J.R.), Blackwell Scientific, Oxford, UK, p 571-610.

Cain, T.A. and Morado, J.F. (2001). Changes in total hemocyte and differential counts in Dungeness crabs infected with *Mesanophrys pugettensis*, a marine facultative parasitic ciliate. *Journal of Aquatic Animal Health* **13**, 310-319.

Canning, E.U. (1977). Microsporidia. In: Parasitic protozoa Vol. 4 (Ed. Kreier, J.P.). Academic Press, New York, p 155-191.

Carazzi, D. (1911). Eine neue Haematoxylinloesung. *Zeitschrift fuer Wissenschaftliche Mikroskopie und fuer Mikroscopische Technik* **28**, 273.

Cattaneo, G. (1888). Su di un infusorio ciliato, parasito del sangue del *Carcinus maenes*. *Zoologischer Anzeiger* **11**, 456-459.

Cavalier-Smith, T. (1998). A revised six-kingdom system of life. *Biological Reviews* **73**, 203-266.

Cawthorn, R.J., Lynn, D., Despres, B., MacMillan, R., Maloney, R., Loughlin, M. and Bayer, R. (1996). Description of *Anophryoides haemophila* n. sp. (Scuticociliatida: Orchitophryidae), a pathogen of American lobsters *Homarus americanus*. *Diseases of Aquatic Organisms* **24**, 143-148.

Cépède, C. (1907). La castration parasitaire des étoiles de mer males par un nouvel infusoire astome: *Orchitophrya stellarum* n.g., n.sp. *Comptes Rendus of the Academy of Sciences Paris* **145**, 1305-1306.

Chang, E.S., Keller, R. and Chang, S.A. (1998). Quantification of crustacean hyperglycaemic hormone by ELISA in haemolymph of the lobster, *Homarus americanus*, following various stresses. *General and Comparative Endocrinology* **111**, 359-366.

Chapman, C.J. (1980). Ecology of juvenile and adult *Nephrops*. In: The biology and management of lobsters Vol. 2, (Eds. Cobb, J.S. and Phillips, B.F.). Academic Press. New York, p 143-175.

Chatton, É. (1920). Classe des Dinoflagellates ou Péridiniens. In: Traité de Zoologie, Vol. 1 (Ed. Grassé, P.-P.). Masson et Cie, Paris, p 309-390.

Chatton, É. and Poisson, R. (1931). Sur l'existence, dans le sand des Crabes, de Péridinien parasites: *Hematodinium perezi* n. g., n. sp. (Syndinidae). *Comptes Rendus des Seances de la Societe de Biologie Paris* **105**, 553-557.

Chatton, É. and Hovasse, H. (1938). *Actinodinium apsteini* n. g., n. sp. Péridinien parasite entérocoelomiques des *Acartia* (Copépodes). *Archives de Zoologie Experimentale and Generale* **79**, 24-27.

Claereboudt, M.R. and Bouland, C. (1994). The effect of parasitic castration by a ciliate on a population of *Asterias vulgaris*. *Journal of Invertebrate Pathology* **63**, 172-177.

Cohn, F. (1866). Neue infusorien im seeaquarium. *Zeitschrift fur Wissenschaftliche Zoologie* **16**, 253-302.

Colwell, R.R., Wicks, T.C. and Tubiash, H.S. (1975). A comparative study of the bacterial flora of the haemolymph of *Callinectes sapidus*. *Marine Fisheries Review* **37**, 29-33.

Corliss, J.O. (1979). The Ciliated Protozoa; Characterization, Classification, and Guide to the Literature, 2nd Edition. Pergamon Press, Oxford and New York, 455 p.

Cornick, J.W. and Stewart, J.E. (1968). Interaction of the pathogen *Gaffkya homari* with natural defense mechanisms of *Homarus americanus*. *Journal of the Fisheries Research Board of Canada* **25**, 697-707.

Couch, J.A. (1978). Diseases, parasites, and toxic responses of commercial penaeid shrimps of the Gulf of Mexico and South Atlantis coasts of North America. *Fishery Bulletin* **76**, 1-44.

Dennis, D.M. and Munday, B.L. (1994). Microsporidiosis of palinurid lobsters from Australian waters. *Bulletin of the European Association of Fish Pathologists* **14**, 16-18.

Denton, H. (1996). Studies on the energy metabolism of *Eimeria tenella* and other coccidian. Ph.D. Thesis, University of Glasgow. 235 p.

De Jonckheere, J.F. and Dierickx, P.J. (1982). Determination of acid phosphatase and leucine amino peptidase activity as an identification method for pathogenic *Naegleria fowleri*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **76**, 773-775.

De Puytorac, P. and Grolière, C.A. (1979). Sur le cilie *Anophryoides salmacida* (Mugard, 1949). *Protistologica* **15**, 223-230.

Dettori, G., Grillo, R., Cattani, P., Calderaro, A., Chezzi, C., Milner, J., Truelove, K. and Selllwood, R. (1995). Comparative study of the enzyme activities of *Borrelia burgdorferi* and other non-intestinal and intestinal spirochaetes. *Microbiologica* **18**, 13-26.

Diggles, B.J. and Adlard, R.D. (1997). Intraspecific variation in *Cryptocaryon irritans. Journal of Eukaryotic Microbiology* **44**, 25-32.

Dikkeboom, R., Tijangel, J.M.J.H., Mulder, E.C. and Van der Knaap, W.P.W. (1987). Hemocytes of the pond snail *Lymnaea stagnalis* generate reactive forms of oxygen. *Journal of Invertebrate Pathology* **49**, 321-331.

Drebes, G. (1984). Life cycle and host specificity of marine parasitic dinophytes. *Helgoländer Meeresunters* **37**, 603-622.

Eaton, W.D., Love, D.C., Botelho, C., Meyers, T.R., Imamura, K. and Koeneman, T. (1991). Preliminary results on the seasonality and life cycle of the parasitic dinoflagellate causing bitter crab disease in Alaskan Tanner crabs (*Chionoecetes bairdi*). *Journal of Invertebrate Pathology* **57**, 426434.

Edgerton, B., O'Donoghue, P., Wingfield, M. and Owens, L. (1996). Systemic infection of freshwater crayfish *Cherax quadricarinatus* by hymenostome ciliates of the *Tetrahymena pyriformis* complex. *Diseases of Aquatic Organisms* **27**, 123-129.

Ekless, L.M. and Matthews, R.A. (1993). *Ichthyophthirius multifiliis*: axenic isolation and short-term maintenance in selected monophasic media. *Journal of Fish Diseases* **16**, 437-444.

Ellis, A.E., Hastings, T.S. and Munro, A.L.S. (1981). The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis. *Journal of Fish Diseases* **4**, 41-51.

Elston, R.A., Cheney, D., Frelier, P. and Lynn, D. (1999). Invasive orchitophryid ciliate infections in juvenile Pacific and Kumomoto oysters, *Crassostrea gigas* and *Crassostrea sikamea. Aquaculture* **174**, 1-14.

Engberg, J., Nielson, H., Lenaers, G., Murayama, O., Fujitani, H. and Higashinakagawa, T. (1990). Comparison of primary and secondary 26s RNA structures in two *Tetrahymena* species: evidence for a strong evolutionary and structural constraint in expansion segments. *Journal of Molecular Evolution* **30**, 514-521.

Evans, L.H., Fotedar, S., Fan, A and Jones, B. (1999). Investigation of idiopathic muscle necrosis and circulating hemocytes in the freshwater crayfish *Cherax tenuimanus* exposed to acute and chronic stressors. *Freshwater Crayfish* **12**, 356-370.

Farmer, A.S.D. (1974a). Reproduction in *Nephrops norvegicus* (Decapoda: Nephropidae). *Journal of Zoology* **174**, 161-183.

Farmer, A.S.D. (1974b). Burrowing behaviour of the Norway lobster, *Nephrops norvegicus* (Decapoda: Nephropidae). *Estuarine and Coastal Marine Science* **2**, 49-58.

Farmer, A.S.D. (1975). Synopsis of biological data on the Norway lobster *Nephrops norvegicus* (Linnaeus, 1758). FAO Fisheries Synopsis 112, 97 p.

Farto, R., Montes, M.M., Pérez, M.J. and Nieto, T.P. (1998). Purification of leucine arylamidase from extracellular products of a pathogenic VAR strain. Proceedings of the Third International Symposium on Aquatic Animal Health. (Meeting abstract). Baltimore, Maryland USA.

Fensome, R.A., Saldarriaga, J.F. and Taylor, F.J.R. (1999). Dinoflagellate phylogeny revisited: reconciling morphological and molecular based phylogenies. *Grana* **38**, 66-80.

Fernández-Galiano, D. (1994). The ammoniacal silver carbonate method as a general procedure in the study of protozoa from sewage (and other) waters. *Water Research* **28**, 495-496.

Field, R.H. (1992). The control of escape behaviour in, and the histopathology of, the Norway lobster, *Nephrops norvegicus* (L.). Ph.D. Thesis, University of Glasgow, Scotland. 142 p.

Field, R.H., Chapman, C.J., Taylor, A.C., Neil, D.M. and Vickerman, K. (1992). Infection of the Norway lobster *Nephrops norvegicus* by a *Hematodinium*-like species of dinoflagellate on the West Coast of Scotland. *Diseases of Aquatic Organisms* **13**, 1-15.

Field, R.H. and Appleton, P.L. (1995). A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops norvegicus*: observations on pathology and progression of infection. *Diseases of Aquatic Organisms* **22**, 115-128.

Field, R.H. and Appleton, P.L. (1996). An indirect fluorescent antibody technique for the diagnosis of *Hematodinium* sp. infection of the Norway lobster, *Nephrops norvegicus*. *Diseases of Aquatic Organisms* **24**, 199-204.

Field, R.H., Hills, J.M., Atkinson, R.J.A., Magill, S. and Shanks, A.M. (1998). Distribution and seasonal prevalence of *Hematodinium* sp. infection of the Norway lobster (*Nephrops norvegicus*) around the West coast of Scotland. *ICES Journal of Marine Science* **55**, 846-858.

Fisher, W.S. (1983). Eggs of *Palaemon macrodactylus*: III. Infection by the fungus, *Lagenidium callinectes*. *Biological Bulletin* **164**, 214-226.

Florin-Christensen, J., Florin-Christensen, M., Knudsen, J. and Rasmussen, L. (1985). Cytolytic activity released from *Tetrahymena*. *Journal of Protozoology* **32**, 657-660.

Freeman, R.S. and Donoghue, D.J. (1991). Protein kinases: biochemical regulators on the eukaryotic cell cycle. *Biochemistry* **30**, 2293-2302.

García-Martos, P., Marín, P., Harnández-Molina, J.M., García-Agudo, L., Aoufi, S. and Mira, J. (2000). Extracellular enzymatic activity in 11 *Cryptococcus* species. *Mycopathologia* **150**, 1-4.

Garreis, K.A., La Peyre, J.F. and Faisal, M. (1996). The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defence parameters *in vitro*. *Fish and Shellfish Immunology* **6**, 581-597.

Getchell, R.G. (1989). Bacterial shell disease in crustaceans: a review. *Journal of Shellfish Research* **8**, 1-6.

Goggin, C.L. and Barker, S.C. (1993). Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribosomal RNA. *Molecular Biochemical Parasitology* **60**, 65-70.

Goggin, C.L. (1994). Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). *Molecular Biochemical Parasitology* **65**, 179-182.

Goggin, C.L. and Murphy, N.E. (2000). Conservation of sequence in the internally transcribed spacers and the 5.8S ribosomal RNA among geographically separated isolates of parasitic scuticociliates (Ciliophora, Orchitophryidae). *Diseases of Aquatic Animals* **40**, 79-83.

Gómez-Gutiérrez, J., Peterson, W.T., De Robertis, A. and Brodeur, R.D. (2003). Mass mortality of krill caused by parasitoid ciliates. *Science* **301**, 339.

Gottlieb, M. (1980). Partial characterization of a secretory acid phosphatase from growth medium of *Leishmania donovani* promastigotes. Journal of *Eukaryotic Microbiology* **27**, 41a.

Gottlieb, M. and Dwyer, D.M. (1981a). *Leishmania donovani*: surface membrane acid phosphatase activity of promastigotes. *Experimental Parasitology* **52**, 117-128.

Gottlieb, M. and Dwyer, D.M. (1981b). Protozoan parasite of humans: surface membrane with externally disposed acid phosphatase. *Science* **212**, 939-941.

Grehn, M., Müller, F. and Hugelshofer, R. (1991). The API ZYM system as a tool for typing of *Pasteurella multocida* strains from humans. *Journal of Microbiological Methods* **13**, 201-206.

Grolièr, C.A. and Leglise, M. (1977). *Paranophrys carcini* n.sp., cilié Philasterina récolté dans l'hémolymphe du crabe *Cancer pagurus* Linné. *Protistologica* **13**, 503-507.

Gruebl, T., Frischer, M.E., Sheppard, M., Neumann, M., Maurer, A. and Lee, R.F. (2002). Development of an 18S rRNA gene-targeted PCR-based diagnostic for the blue crab parasite *Hematodinium* sp. *Diseases of Aquatic Organisms* **49**, 61-70.

Hanna, B.A. and Lilly, D.M. (1971). Axenic culture of *Uronema marinum*. *American zoologist* **11**, 659.

Hermosa de Mendoza, J., Arenas, A., Alonso, J.M., Rey, J., Gil, M.C., Anton, J.M. and Hermosa de Mendoza, M. (1993). Enzymatic activities of *Dermatophilus congolensis* measured by API ZYM. *Veterinary Microbiology* **37**, 175-179.

Heroldová, M., Němec, M., Hubálek, Z. (2001). Enzyme activities of *Borrelia burgdorferi* sensu lato. *Folia Microbiology* **46**, 179-182.

Hervio, D., Bachère, E., Mialhe, E. and Grizel, H. (1988). Chemiluminescent responses of *Ostrea edulis* and *Crassostrea gigas* hemocytes to *Bonamia ostreae* (Ascetospora). *Developmental and Comparative Immunology* **13**, 449.

Hervio, D., Chagot, D., Godin, P., Grizel, H. and Mialhe, E. (1991). Localization and characterization of acid phosphatase activity in *Bonamia ostreae* (Ascetospora), an intrahemocytic protozoan parasite of the flat oyster *Ostrea edulis* (Bivalvia). *Diseases of Aquatic Organisms* **12**, 67-70.

Hibbits, J., Hughes, G.C. and Sparks, A.K. (1981). *Trichomaris invadens* gen. et sp. Nov., an ascomycete parasite of the tanner crab (*Chionoecetes bairdi* Rathburn Crustacea, Brachyura.) *Canadian Journal of Botany* **59**, 2121-2128.

Hibbits, J. and Sparks, A.K. (1983). Observations on the histopathology caused by a parasitic ciliate (*Paranophrys* sp.?) in the isopod *Gnorimosphaeroma oregonensis. Journal of Invertebrate Pathology* **41**, 51-56.

Higman, W.A., Stone, D.M., Lewis, J.M. (2001). Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* (Dinophyceae) isolates from UK waters. *Phycologia* **40**, 256-262.

Higson, F.K. and Jones, O.T.G. (1984). The generation of active oxygen species by stimulated rainbow trout leucocytes in whole blood. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **77**, 583-587.

Hillis, D.M. and Dixon, M.T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly Review of Biology* **66**, 411-453.

Hsu, L.U., Wang, K.H., Yang, Y.H., Tung, M.C., Hu, C.H., Lo, C.F., Wang, C.H. and Tsu, T. (2000). Diagnosis of *Penaeus monodon*-type baculovirus by PCR and by ELISA of occlusion bodies. *Diseases of Aquatic Organisms* **40**, 93-99.

Hudson, D.A., Hudson, N.B. and Shields, J.D. (1993). Infection of *Trapezia* spp. (Decapoda: Xanthidae) by *Hematodinium* sp. (Duboscquodinida: Syndinidae): a new family record of infection. *Journal of Fish Diseases* **16**, 273-276.

Hudson, D.A. and Adlard, R.D. (1994). PCR-techniques applied to *Hematodinium* spp. and *Hematodinium*-like dinoflagellates in decapod crustaceans. *Diseases of Aquatic Organisms* **20**, 203-206.

Hudson, D.A. and Lester, R.J.G. (1994). Parasites and symbionts of wild mud crabs *Scylla serrata* (Forskal) of potential significance in aquaculture. *Aquaculture* **120**, 183-199.

Hudson, D.A. and Shields, J.D. (1994). *Hematodinium australis* n.sp., a parasitic dinoflagellate of the sand crab *Portunus pelagicus* from Moreton bay, Australia. *Diseases of Aquatic Organisms* **19**, 109-119.

Hudson, D.A. and Adlard, R.D. (1996). Nucleotide sequence determination of the partial SSU rDNA gene and ITS1 region of *Hematodinium* cf. perezi and *Hematodinium*-like dinoflagellates. *Diseases of Aquatic Organisms* **24**, 55-60.

Hussain, I., Mackie, C., Cox, D., Alderson, R. and Birkbeck, T.H. (2000). Suppression of the humoral immune response of Atlantic salmon, *Salmo salar* L. by the 64 kDa serine protease of *Aeromonas salmonicida. Fish and Shellfish Immunology* **10**, 359-373.

Iglesias, R., Paramá, A., Alvarez, M.F., Leiro, J., Fernández, J. and Sanmartín, M.L. (2001). *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida) as the causative agent of scuticociliatosis in farmed turbot *Scophthalmus maximus* in Galicia (NW Spain). *Diseases of Aquatic Organisms* **46**, 47-55.

Iglesias, R., Paramá, A., Alvarez, M.F., Leiro, J., Aja, C. and Sanmartín, M.L. (2003). *In vitro* growth requirements for the fish pathogen *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida). *Veterinary Parasitology* **111**, 19-30.

Jayasree, S., Selvam, G.S. and Thomas, S. (2000). Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for agglutinin, an antibacterial protein of the shrimp *Fenneropenaeus indicus*. *Journal of Crustacean Biology* **20**, 621-627.

Jensen, M. O. and Moestrup, O. (1997). Autecology of the toxic dinoflagellate *Alexandrium ostenfeldii*: life history and growth at different temperatures and salinities. *European Journal of Phycology* **32**, 9-18.

Jessop, B.M. (1995). *Ichthyophthirius multifiliis* in elvers and small American eels from the East River, Nova Scotia. *Journal of Aquatic Animal Health* **7**, 54-57.

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

Johnson, P.T. (1977). Paramoebiasis in the blue crab, *Callinectes sapidus*. *Journal of Invertebrate Pathology* **29**, 308-320.

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

Johnson, P.T. (1983). Pathogens: viral, rickettsial, bacterial, fungal. In: The Biology of Crustacea Vol.6, (Ed. Bliss, D.). Academic Press, New York, p 2-78.

Johnson, P.T. (1984). Viral diseases of marine invertebrates. *Helgoländer Meeresuntersuchungen* **37**, 65-98.

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

Johnson, P.T. (1988). Paramoebiasis of blue crabs. In: Disease diagnostics and control in North American marine aquaculture. Developments in aquaculture and fisheries science 17, 2nd Edition (Eds. Sinderman, C.J. and Lightner, D.V.). Elsevier, Amsterdam, p 204-207.

Kabre, G. (1992). Effects of the microsporidian *Thelohania contejeani* on the metabolism of total muscular and hepatopancreatic phospholipids in the crayfish *Austropotamobius pallipes*, Le Reboulet, 1858. *Annales des Sciences Naturelles Zoologie* **13**, 97-102.

Karatayev, A.Y., Burlakova, L.E., Molloy, D.P., Volkova, L.K. and Volosyuk, V.V. (2002). Field and laboratory studies of *Ophryoglena* sp. (Ciliata: Ophryoglenidae) infection in zebra mussels, *Dreissena polymorpha* (Bivalvia: Dreissenidae). *Journal of Invertebrate Pathology* **79**, 80-85.

Katakura, K. and Kobayashi, A. (1988). Acid phosphatase activity of virulent and avirulent clones of *Leishmania donovani* promastigotes. *Infection and Immunity* **56**, 56-60.

Kelly, J.F. (1979). Tissue specificities of *Thelohania duorara*, and *Pleistophora* sp., microsporidian parasites of the pink shrimp, *Penaeus duorarum*. *Journal of Invertebrate Pathology* **33**, 331-339.

Kirkeley, J.E. (1997). Virginia's commercial fishing industry: its economic performance and contributions. VIMS SRAMSOE #337. Virginia Sea Grant VSG-97-02.

Kleeman, S.N. and Adlard, R.D. (2000). Molecular detection of *Marteilia sydneyi*, pathogen of Sydney rock oysters. *Diseases of Aquatic Organisms* **40**, 137-146.

Kleeman, S.N., LeRoux, F., Berthe, F. and Adlard, R.D. (2002). Specificity of PCR and *in situ* hybridisation assays designed for detection of *Marteilia sydneyi* and *M. refringens*. *Parasitology*, **125**, 131-141.

Klemba, M. and Goldberg, D.E. (2002). Biological roles of proteases in parasitic protozoa. *Annual Review of Biochemistry* **71**, 275-305.

Kudhair, A., Delmée, M. and Wauters, G. (1986). Rapid identification of *Clostridium difficile* by determination of leucine arylamidase activity. European *Journal of Clinical Microbiology* **5**, 160-161.

La Peyre, J.F. and Faisal, M. (1995). *Perkinsus marinus* produces extracellular proteolytic factors *in vitro*. *Bulletin of the Association of Fish Pathologists* **15**, 28-31.

La Peyre, J.F., Schafhauser, D.Y., Rizkalla, E.H. and Faisal, M. (1995). Production of serine proteases by the oyster pathogen *Perkinsus marinus* (Apicomplexa) *in vitro*. *Journal of Eukaryotic Microbiology* **42**, 544-551.

La Peyre, J.F., Yarnall, H.A. and Faisal, M. (1996). Contribution of *Perkinsus marinus* extracellular products in the infection of Eastern oysters (*Crassostrea virginica*). *Journal of Invertebrate Pathology* **68**, 312-313.

Latrouite, D., Morizur, T., Noel, P., Chagot, D. and Wilhelm, G. (1988). Mortalite du tourteau *Cancer pagurus* provoquee par le dinoflagellate parasite: *Hematodinium* sp. Conseil International pour I'Exploration de la Mer. CM. 1988/K: 32.

Lavallée, J., Hammell, K.L., Spangler, E.S. and Cawthorn, R.J. (2001). Estimated prevalence of *Aerococcus viridans* and *Anophryoides haemophila* in American lobsters *Homarus americanus* freshly captured in the waters of Prince Edward Island, Canada. *Diseases of Aquatic Organisms* **46**, 231-236.

Lee, S.B. and Taylor, J.W. (1992). Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molecular Biology and Evolution* **9**, 636-653.

Lee, R.F. (2001). Disappearance of blue crabs from a Georgia salt marsh estuary due to a parasitic dinoflagellate: linkage to high salinities caused by drought. The Georgia Blue Crab Journal, February, p 2-3.

Lee, E.H., Kim, C.S., Cho, J.B., Ahn, K.J. and Kim, K.H. (2003). Measurement of protease activity of live *Uronema marinum* (Ciliata: Scuticociliatida) by fluorescence polarization. *Diseases of Aquatic Organisms* **54**, 85-88.

Le Gall, G., Bachère, E. and Mialhe, E. (1991). Chemiluminescence analysis of the activity of *Pecten maximus* hemocytes stimulated with zymosan and host-specific Rickettsiales-like organisms. *Diseases of Aquatic Organisms* **11**, 181-186.

Leighton, B.J., Boom, J.D.G., Bouland, C., Hartwick, E.B. and Smith, M.J. (1991). Castration and mortality in *Pisaster ochraceus* parasitized by *Orchitophrya stellarum* (Ciliophora). *Diseases of Aquatic Organisms* **10**, 71-73.

Li, W.-H., and Graur, D. (1991). Fundamentals of Molecular Evolution. Sinauer Associates, Sunderland, Massachusetts.

Lightner, D.V. and Lewis, D.H. (1975). A septicemic bacterial disease syndrome of penaeid shrimp. *Marine Fisheries Review* **37**, 25-28.

Lightner, D.V. (1977). Shrimp diseases. In: Disease diagnosis and control in North American marine aquaculture, 1st Edition (Ed. Sindermann, C.J.). Elsevier, Amsterdam, p 10-77.

Lightner, D.V. (1996). A Handbook of Shrimp Pathology and Diagnostic Procedures for Disease of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge.

Lightner, D.V. and Redman, R.M. (1998). Shrimp diseases and current diagnostic methods. *Aquaculture* **164**, 201-220.

Loo, L-O., Pihl Baden, S. and Ulmestrand, M. (1993). Suspension feeding in adult *Nephrops norvegicus* (L.) and *Homarus gammarus* (L.) (Decapoda). *Netherlands Journal of Sea Research* **31**, 291-297.

Loughlin, M.B., Bayer, R.C. and Prince, D.L. (1993). Ciliated protozoan disease in lobsters, *Homarus americanus*. Irish-American Technical Exchange on the Aquaculture of Abalone, Sea Urchins, Lobsters and Kelp. Connecticut Sea Grant College Programme. CT-SG-93-05, p 65-67.

Loughlin, M.B., Bayer, R.C. and Prince, D.L. (1994). Incidence of ciliated protozoans in feral lobsters (*Homarus americanus*). *Bulletin of the Aquaculture Association of Canada* **94**, 49-51.

Love, D.C., Rice, S.D., Moles, D.A. and Eaton, W.D. (1993). Seasonal prevalence and intensity of bitter crab dinoflagellate infection and host mortality in Alaskan Tanner crabs *Chionoecetes bairdi* from Auke Bay, Alaska, USA. *Diseases of Aquatic Organisms* **15**, 1-7.

Lovelace, J.K. and Gottlieb, M. (1986). Comparison of extracellular acid phosphatases from various isolates of *Leishmania*. *American Journal of Tropical Medicine and Hygiene* **35**, 1121-1128.

Lovelace, J.K., Dwyer, D.M. and Gottlieb, M. (1986). Purification and characterization of the extracellular acid phosphatase activity of *Leishmania donovani*. *Molecular and Biochemical Parasitology* **20**, 243-251.

MacIntyre, E.A., Earnhart, C.G. and Kaattari, S.L. (2003). Host oyster tissue extracts modulate *in vitro* protease expression and cellular differentiation in the protozoan parasite, *Perkinsus marinus*. *Parasitology* **126**, 293-302.

MacLean, S.A. and Ruddell, M.C. (1978). Three new crustacean hosts for the parasitic dinoflagellate *Hematodinium perezi* (Dinoflagellata: Syndinidae). *The Journal of Parasitology* **64**, 158-160.

Malinowski, E., Lassa, H., Kłossowska, A. and Kuźma, K. (2001). Enzymatic activity of yeast species isolated from bovine mastitis. *Bulletin of the Veterinary Institute in Pulawy* **45**, 289-295.

McKerrow, J.H. (1989). Parasite proteinases. *Experimental Parasitology* **68**, 111-115.

McKerrow, J.H., Sun, E., Rosenthal, P.J. and Bouvier, J. (1993). The proteases and pathogenicity of parasitic protozoa. *Annual Review of Microbiology* **47**, 821-853.

Merella, P., Alemany, F., Carbonell, A. and Quetglas, A. (1998). Fishery and biology of Norway Lobster *Nephrops norvegicus* (Decapoda: Nephropidae) in Mallorca (Western Mediterranean). *Journal of Natural History* **32**, 1631-1640.

Messick, G.A. (1994). *Hematodinium perezi* infections in adult and juvenile blue crabs *Callinectes sapidus* from coastal bays of Maryland and Virginia, USA. *Diseases of Aquatic Organisms* **19**, 77-82.

Messick, G.A. and Small, E.B. (1996). *Mesanophrys chesapeakensis* n.sp., a histophagous ciliate in the blue crab, *Callinectes sapidus*, and associated histopathology. *Journal of Invertebrate Biology* **115**, 1-12.

Messick, G.A. and Shields, J.D. (2000). Epizootiology of the parasitic dinoflagellate *Hematodinium* sp. in the American blue crab *Callinectes sapidus*. *Diseases of Aquatic Organisms* **43**, 139-152.

Metsis, A., Pettsersen, E. and Petersen, E. (1995). *Toxoplasma gondii*: characterization of a monoclonal antibody recognizing antigens of 36 and 38 kDa with acid phosphatase activity located in dense granules and rhoptries. *Experimental Parasitology* **81**, 472-479.

Meyers, T.R., Koeneman, T.M., Botelho, C. and Short, S. (1987). Bitter crab disease: a fatal dinoflagellate infection and marketing problem for Alaskan Tanner crabs *Chionoecetes bairdi*. *Diseases of Aquatic Organisms* **3**, 195-216.

Momoyama, K. and Matsuzato, T. (1987). Muscle necrosis of culture kuruma shrimp (*Penaeus japonicus*). *Fish Pathology* **22**, 69-75.

Moore, P.G. and Wong, Y.M. (1995). *Orchomene nanus* (Krøyer) (Amphipoda: Lysianassoidea), a selective scavenger of dead crabs: feeding preferences in the wild. *Journal of Experimental Marine Biology and Ecology* **192**, 35-45.

Morado, J.F. (1993). The morphology, morphogenesis, culture and pathogenesis of a marine facultative parasitic ciliate, *Mesanophrys* sp., of the Dungeness crab, *Cancer magister*, and its occurrence in wild crustacean populations. Ph.D. Thesis, University of Washington, Seattle. 248 p.

Morado, F.J. and Small, E.B. (1994). Morphology and stomatogenesis of *Mesanophrys pugettensis* n.sp. (Scuticociliatida: Orchitphryidae), a facultative parasitic ciliate of the Dungeness crab, *Cancer magister* (Crustacea: Decapoda). *Transactions on the American Microscopical Society* **113**, 343-364.

Morado, J.F. and Small, E.B. (1995). Ciliate parasites and related diseases of crustacea: a review. Reviews in Fisheries Science 3, 275-354.

Morado, J.F., Giesecke, R.H. and Syrjala, S.E. (1999). Molt related mortalities of the Dungeness crab *Cancer magister* caused by a marine facultative ciliate *Mesanophrys pugettensis*. *Diseases of Aquatic Organisms* **38**, 143-150.

Morris, D.J., Adams, A. and Richards, R.H. (2000). *In situ* hybridisation identifies the gill as a portal of entry for PKX (Phylum Myxozoa), the causative agent of proliferative kidney disease in salmonids. *Parasitology Research* **86**, 950-956.

Moss, J., Iglewski, B., Vaughan, M. and Tu, A.T. (1995). Handbook of Natural toxins Volume 8: Bacterial Toxins and Virulence Factors in Disease. Marcel Dekker Inc, N.Y.

Munday, B.L., O'Donoghue, P.J., Watts, M., Rough, K. and Hawkesford, T. (1997). Fatal encephalitis due to the scuticociliate *Uronema nigricans* in sea-caged, southern bluefin tuna *Thunnus maccoyii*. *Diseases of Aquatic Organisms* **30**, 17-25.

Nerad, T.A. and Daggett, P.-M. (1992). Cultivation of scuticociliates. In: Protocols in Protozoology, (Eds. Lee, J.J. and Soldo, A.T.). Allen Press, Lawrence, p A-50.1-A-50.6.

Newman, M.W. and Johnson, C.A. (1975). A disease of Blue crabs (*Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp. *The Journal of Parasitology* **63**, 554-557.

Nottage, A.S. and Birkbeck, T.H. (1987). Purification of a proteinase produced by the bivalve pathogen *Vibrio alginolyticus* NCMB 1339. *Journal of Fish Diseases* **10**, 211-220.

Ordás, M.C., Novoa, B., Faisal, M., McLaughlin, S. and Figueras, A. (2001). Proteolytic activity of cultured *Pseudoperkinsus tapetis* extracellular products. *Comparative Biochemistry and Physiology Part B* **130**, 199-206.

Pereira, N.M., Timm, S.L., Da Costa, S.C.G., Rebello, M.A. and De Souza, W. (1978). *Trypanosoma cruzi*: isolation and characterisation of membrane and flagellar fractions. *Experimental Parasitology* **46**, 225-234.

Pipe, R.K. (1992). Generation of reactive oxygen metabolites by the hemocytes of the mussel *Mytilus edulis*. *Developmental and Comparative Immunology* **16**, 111-122.

Poh, C.L. and Loh, G.K. (1985). Enzymatic profile of clinical isolates of *Acinetobacter calcoaceticus*. *Medical Microbiology and Immunology* **174**, 29-33.

Poh, C.L. and Loh, G.K. (1988). Enzymatic characterization of *Pseudomonas cepacia* by API ZYM profile. *Journal of Clinical Microbiology* **26**, 607-608.

Poisson, R. (1930). Observations sur *Anophrys sarcophaga* Cohn (= *A. maggii* Cattaneo) infusoire holotriche marin et sur son parasitisme possible chez certains crustacés. *Bulletin Biologique de la France et Belgique* **64**, 288-331.

Pouchet, G. (1884). Sur en Péridinien parasite. *Comptes Rendus de l'Académie des Sciences Paris* **98**, 1345-1346.

Poulos, B.T., Kibler, R., Bradley-Dunlop, D., Mohney, L.L. and Lightner, D.V. (1999). Production and use of antibodies for the detection of Taura syndrome virus in penaeid shrimp. *Diseases of Aquatic Organisms* **37**, 99-106.

Prince, D.L., Bayer, R.C. and Loughlin, M. (1993). Etiology and microscopy of shell diseases in impounded American lobsters, *Homarus gammarus*. *Bulletin of the Aquaculture Association of Canada* **93**, 87-89.

Reece, K.S., Bushek, D. and Graves, J.E. (1997). Molecular markers for population genetic analysis of *Perkinsus marinus*. *Molecular Marine Biology and Biotechnology* **6**, 197-206.

Reece, K.S., Burreson, E.M., Bower, S.M. and Dungan, C.F. (2000). Molecular analyses of a parasite in prawns (*Pandalus platyceros*) from British Columbia, Canada. *Journal of Shellfish Research* **19**, 647.

Reece, K.S., Bushek, D., Hudson, K.L. and Graves, J.E. (2001). Geographical distribution of *Perkinsus marinus* genetic strains along the Atlantic and Gulf coasts of the USA. *Marine Biology* **139**, 1047-1055.

Remaley, A.T., Kuhns, D.B., Basford, R.E., Glew, R.H. and Kaplan, S.S. (1984). Leishmanial phosphatase blocks neutrophil O_2-production. *Journal of Biological Chemistry* **259**, 11173-11175.

Rigdon, R.H. and Baxter, K.N. (1970). Spontaneous necroses in muscles of brown shrimp *Penaeus aztecus* Ives. *Transactions of the American Fisheries Society* **3**, 583-587.

Robledo, J.A.F., Wright, A.C., Marsh, A.G. and Vasta, G.R. (1999). Nucleotide sequence variability in the nontranscribed spacer of the rRNA locus in the oyster parasite *Perkinsus marinus*. *Journal of Parasitology* **85**, 650-656.

Rockey, D.D., Fryer, J.L. and Rohovec, J.S. (1988). Separation and *in-vivo* analysis of two extracellular proteases and the T-hemolysin from *Aeromonas salmonicida*. *Diseases of Aquatic Organisms* **5**, 197-204.

Romestand, B. and Bonami, J.-R. (2003). A sandwich enzyme linked immunosorbent assay (S-ELISA) for detection of MrNV in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Journal of Fish Diseases* **26**, 71-75.

Roth, J.A., Bolin, C.A., Brogden, K.A., Minion, F.C. and Wannemuchler, M.J. (1995). Virulence Mechanisms of Bacterial Pathogenesis. Second Edition. ASM Press, Washington, D.C.

Saha, A.K., Dowling, J.N., LaMarco, K.L., Das, S., Remaley, A.T., Olomu, N., Pope, M. and Glew, R.H. (1985). Properties of an acid phosphatase from *Legionella micdadei* which blocks superoxide anion production by human neutrophils. *Archives of Biochemistry and Biophysics* **243**, 150-160.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: a laboratory Manual, Second Edition. Cold Spring Harbour, NY.

Sarath, G., De la Motte, R.S. and Wagner, F.W. (1989). Protease assay methods. *In:* Proteolytic Enzymes: A Practical Approach, Vol. 3. (Eds. Benyon, R. J. and Bond, J. S.). IRL Press, Oxford, p 25-55.

Sawyer, T.K., Cox, R. and Higginbottom, M. (1970). Hemocyte values in healthy blue crabs *Callinectes sapidus* and crabs infected with the amoeba *Paramoeba perniciosa*. *Journal of Invertebrate Pathology* **14**, 440-446.

Sawyer, T.K. (1991). Shell disease in the Atlantic rock crab, *Cancer irroratus* Say, 1871, from the Northeastern United States. *Journal of Shellfish Research* **10**, 495-497.

Scarratt, D.J. (1965). Predation on lobsters (*Homarus americanus*) by *Anonyx* sp. (Crustacea, Amphipoda). *Journal of the Fisheries Research Board of Canada* **22**, 1103-1104.

Scholin, C.A., Villac, M.C., Buck, K.R., Krupp, J.M., Powers, D.A., Fryxell, G.A. and Chavez, F.P. (1994). Ribosomal DNA sequences discriminate among toxic and non-toxic *Pseudonitzschia* species. *Natural Toxins* **2**, 152-165.

Schulte, W. and Scholze, H. (1989). Action of major protease from *Entamoeba histolytica* on proteins of the extracellular matrix. *Journal of Protozoology* **36**, 538-543.

Secombes, C.J., Cheng, S. and Jeffries, A.H. (1988). Superoxide anion production by rainbow trout macrophages detected by the reduction of ferricytochrome c. *Developmental and Comparative Immunology* **12**, 201-206.

Shelburne, S.W. and Bean, L.L. (1991). Mortalities of impounded and feral lobsters, *Homarus americanus* H. Milne-Edwards, 1837, caused by the protozoan ciliate *Mugardia* (formerly *Anophrys* = *Paranophrys*), with initial prevalence data from ten locations along the Maine coast and one offshore area. *Journal of Shellfish Research* **10**, 315-326.

Shields, J.D. (1992). Parasites and symbionts of the crab *Portunus pelagicus* from Moreton bay, Eastern Australia. *Journal of Crustacean Biology* **12**, 94-100.

Shields, J.D. (1994). The parasitic dinoflagellates of marine crustaceans. *Annual Review of Fish Diseases* **4**, 241-271.

Shields, J.D. and Squyars, C.M. (2000). Mortality and hematology of blue crabs, *Callinectes sapidus*, experimentally infected with the parasitic dinoflagellate *Hematodinium perezi*. *Fishery Bulletin* **98**, 139-152.

Shields, J.D., Scanlon, C. and Volety, A. (2003). Aspects of the pathophysiology of blue crabs, *Callinectes sapidus*, infected with the parasitic dinoflagellate *Hematodinium perezi*. *Bulletin of Marine Science* **72**, 519-535.

Sindermann, C.J. (1990). Principal Diseases of Marine Fish and Shellfish, Second Edition. New York Academic Press.

Singla, N. Khuller, G.K. and Vinayak, V.K. (1992). Acid phosphatase activity of promastigotes of *Leishmania donovani*: a marker of virulence. *FEMS Microbiology Letters* **94**, 221-226.

Small, E.B. and Lynn, D.H. (1985). Phylum Cilophora Doflein, 1901. In: An illustrated guide to the Protozoa, (Eds. Lee, J.J., Hunter, S.H. and Bovee, E.C.). Society of Protozoologists, Lawrence, Kansas, p 393-575.

Small, H.J., Wilson, S., Neil, D.M., Hagan, P. and Coombs, G.H. (2002). Detection of the parasitic dinoflagellate *Hematodinium* in the Norway Lobster (*Nephrops norvegicus*) by ELISA. *Diseases of Aquatic Organisms* **52**, 175-177.

Söderhäll, K. and Unestam, T. (1975). Properties of extracellular enzymes from *Aphanomyces astaci* and their relevance in the penetration process of crayfish cuticle. *Physiologia Plantarum* **35**, 140-146.

Sparks, A.K. (1982). Observations on the histopathology and progression of the disease caused by *Trichomaris invadens*, an invasive ascomycete, in the Tanner crab, *Chionoecetes bairdi*. *Journal of Invertebrate Pathology* **40**, 242-254.

Sparks, A.K., Hibbits, J. and Fegley, J. (1982). Observations on the histopathology of a systemic ciliate (*Paranophrys* sp. ?) disease in the Dungeness crab, *Cancer magister*. *Journal of Invertebrate Pathology* **39**, 219-228.

Sparks, A.K. (1985). Synopsis of Invertebrate Pathology Exclusive of Insects. Elsevier, Amsterdam, p 230-234.

Stentiford, G.D. and Neil, D.M. (2000). A rapid onset, post-capture muscle necrosis in the Norway lobster, *Nephrops norvegicus* L. from the West Coast of Scotland, United Kingdom. *Journal of Fish Diseases* **23**, 251-264.

Stentiford, G.D., Neil, D.M., Atkinson, R.J.A. and Bailey, N. (2000a). An analysis of swimming performance in the Norway lobster, *Nephrops norvegicus* L. infected by a parasitic dinoflagellate of the genus *Hematodinium*. *Journal of Experimental Marine Biology and Ecology* **247**, 169-181.

Stentiford, G.D., Neil, D.M. and Coombs, G.H. (2000b). 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. Diseases of Aquatic Organisms* **42**, 133-141.

Stentiford, G.D., Chang, E.S., Chang, S.A. and Neil, D.M. (2001a). Carbohydrate dynamics and the crustacean hyperglycaemic hormone (CHH): effects of parasitic infection in Norway lobsters (*Nephrops norvegicus*). *General and Comparative Endocrinology* **121**, 13-22.

Stentiford, G.D., Neil, D.M. and Atkinson, R.J.A. (2001b). The relationship of *Hematodinium* infection prevalence in a Scottish *Nephrops norvegicus* population to seasonality, moulting and sex. *ICES Journal of Marine Science* **58**, 814-823.

Stentiford, G.D., Neil, D.M. and Coombs, G.H. (2001c). Development and application of an immunoassay diagnostic technique for studying *Hematodinium* infections in *Nephrops norvegicus* populations. *Diseases of Aquatic Organisms* **46**, 223-229.

Stentiford, G.D., Neil, D.M. and Atkinson, R.J.A. (2001d). Alteration of burrow-related behaviour of the Norway lobster, Nephrops norvegicus during infection by the parasitic dinoflagellate *Hematodinium. Marine and Freshwater Behaviour and Physiology* **34**, 139-156.

Stentiford, G.D., Green, M., Bateman, K., Small, H.J., Neil, D.M. and Feist, S.W. (2002). Infection by a *Hematodinium*-like parasitic dinoflagellate causes Pink Crab Disease (PCD) in the edible crab *Cancer pagurus. Journal of Invertebrate Pathology* **79**, 179-191.

Sterud, E. (1998). *In vitro* cultivation and temperature-dependent growth of two strains of *Spironucleus barkhanus* (Diplomonadida: Hexamitidae) from Atlantic salmon *Salmo salar* and grayling *Thymallus thymallus*. *Diseases of Aquatic Organisms* **33**, 57-61.

Stewart, J.E. (1993). Infectious Diseases of Marine Crustaceans. In: Pathobiology of Marine and Estuarine Organisms, Ch. 12 (Eds. Couch, J.A. and Fournie, J.W.) CRC Press, USA, p 319-342.

Stewart, J.E., Cornick, J.W., Spears, D.I. and McLeese, D.W. (1966). Incidence of *Gaffkya homari* in natural lobster (*Homarus americanus*) populations of the Atlantic region of Canada. *Journal of the Fisheries Research Board of Canada* **23**, 1325-1330.

Stewart, J.E. and Rabin, H. (1970). Gaffkemia, a bacterial disease of lobsters (Genus *Homarus*). *American Fisheries Society Special Publication* **5**, 431-439.

Stickle, W.B., Weidner, E.H. and Kozloff, E.N. (2001). Parasitism of *Leptasterias* spp. (Echinodermata: Asteroidea) by the ciliated protozoan *Orchitophrya stellarum* (Scuticociliata). *Invertebrate Biology* **120**, 88-95.

Stokes, N.A. and Burreson, E.M. (1995). A sensitive and specific DNA probe for the oyster pathogen *Haplosporidium nelsoni*. *Journal of Eukaryotic Microbiology* **42**, 350-357.

Straus, J.W., Migaki, G. and Finch, M.T. (1992). As assessment of proteolytic enzymes in *Tetrahymena thermophila*. *Journal of Protozoology* **39**, 655-662.

Talamás-Rohana, P., Aguirre-García, M.M., Anaya-Ruiz, M. and Rosales-Encina, J.L. (1999). *Entamoeba dispar* contains but does not secrete acid phosphatase as does *Entamoeba histolytica*. *Experimental Parasitology* **92**, 219-222.

Taylor, D.M. and Khan, R.A. (1995). Observations on the occurrence of *Hematodinium* sp. (Dinoflagellata: Syndinidae), the causative agent of bitter crab disease in Newfoundland snow crab (*Chionoecetes opilio*). *Journal of Invertebrate Pathology* **65**, 283-288.

Taylor, A.C., Field, R.H. and Parslow-Williams, P.J. (1996). The effects of *Hematodinium* sp.-infection on aspects of the respiratory physiology of the Norway lobster, *Nephrops norvegicus* (L.). *Journal of Experimental Marine Biology and Ecology* **207**, 217-228.

Templeman, W. (1954). Lobsters eaten by sea-fleas. *Progress Report of the Atlantic Coastal Station, Fisheries Research Board of Canada* **60**, 14-15.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680.

Thörnqvist, P.-O. and Söderhäll, K. (1997). Crustacean immune reactions, a short review. In: Diseases in Aquaculture III. Fish health section (Eds. Flegel, T.W. and MacRae, I.H.). Asian Fisheries Society, Manila, p 203-217.

Tiedtke, A. and Rasmussen, L. (1988). Lysosomal enzymes in extracellular digestion in the unicellular eukaryote *Tetrahymena*. *Journal of Cellular Physiology* **136**, 554-556.

Traxler, G.S., Richard, J. and McDonald, T.E. (1998). *Ichthyophthirius multifiliis* (Ich) epizootics in spawning sockeys salmon in British Columbia, Canadian *Journal of Aquatic Animal Health* **10**, 143-151.

Usup, G., Kulis, D.M. and Anderson, D.M. (1994). Growth and toxin production of the toxic dinoflagellate *Pyrodinium bahamense* var. compressum in laboratory cultures. *Natural Toxins* **2**, 254-262.

Vago, C. (1966). A virus disease in Crustacea. *Nature* **209**, 1290.

Ventura-Juárez, Aguirre-García, M.M. and Talamás-Rohana. (2000). Subcellular distribution and *in situ* localization of the acid phosphatase of *Entamoeba histolytica*. *Archives of Medical Research* **31**, 183-184.

Vickerman, K. (1994). Playing at being Pasteur. *International Journal for Parasitology* **24**, 779-786.

Vivier, E. and Petitprez, A. (1972). Données ultrastructurales complémentaires, morphologiques et cytochimiques, sur *Toxoplasma gondii*. *Protistologica* **8**, 199-221.

Vogan, C.L., Llewellyn, P.J. and Rowley, A.F. (1999). Epidemiology and dynamics of shell disease in the edible crab *Cancer pagurus*: a preliminary study of Langland Bay, Swansea, UK. *Diseases of Aquatic Organisms* **35**, 81-87.

Volety, A.K. and Chu, F-L. E. (1995). Suppression of chemiluminescence of eastern oyster (*Crassostrea virginica*) hemocytes by the protozoan parasite *Perkinsus marinus*. *Developmental and Comparative Immunology* **19**, 135-142.

Volety, A.K. and Chu, F-L. E. (1997). Acid phosphatase activity in *Perkinsus marinus*, the protistan parasite of the American oyster, *Crassostrea virginica*. *Journal of Parasitology* **83**, 1093-1098.

Walton, K.M. and Dixon, J.E. (1993). Protein tyrosine phosphatases. *Annual Review of Biochemistry* **62**, 135-142.

Wiąckowski, K., Hryniewiecka-Szyfter, Z. and Babula, A. (1999). How many species are in the genus *Mesanophrys* (Protista, Ciliophora, facultative parasites of marine crustaceans)? *European Journal of Protistology* **35**, 379-389.

Wickham, D.E. (1986). Epizootic infestations by nemertean brood parasites on commercially important crustaceans. *Canadian Journal of Fisheries and Aquaculture Sciences* **43**, 2295-2302.

Wilhelm, G. and Mialhe, E. (1996). Dinoflagellate infection associated with the decline of *Necora puber* crab populations in France. *Diseases of Aquatic Organisms* **26**, 213-219.

Young, J.S. and Pearce, J.B. (1975). Shell disease from crabs and lobsters from New York Bight. *Marine Pollution Bulletin* **6**, 101-105.

Youngchim, S., Vanittanakom, N. and Hamilton, A.J. (1999). Analysis of the enzymatic activity of mycelial and yeast phases of *Penicillium marneffei*. *Medical Mycology* **37**, 445-450.

Zuo, X. and Woo, P.T.K. (1998). *In vitro* secretion of the metalloprotease by the pathogenic haemoflagellate *Cryptobia salmositica* Katz, 1951 and stimulation of the protease production by collagen. *Journal of Fish Diseases* **21**, 249-255.

Zuo, X. and Woo, P.T.K. (2000). *In vitro* haemolysis of piscine erythrocytes by purified metalloprotease from the pathogenic haemoflagellate *Cryptobia salmositica* Katz. *Journal of Fish Diseases* **23**, 227-230.

Appendix 1

Vol. 52: 175–177, 2002 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published November 22

NOTE

Detection of the parasitic dinoflagellate *Hematodinium* in the Norway lobster *Nephrops norvegicus* by ELISA

H. J. Small[1,*], S. Wilson[2], D. M. Neil[1], P. Hagan[2], G. H. Coombs[2]

[1]Division of Environmental and Evolutionary Biology, Graham Kerr Building, and [2]Division of Infection and Immunity, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK

ABSTRACT: Norway lobsters *Nephrops norvegicus* from the coastal waters of Scotland are seasonally infected by a parasitic dinoflagellate of the genus *Hematodinium*. An enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of the parasite in the haemolymph of *N. norvegicus*. The ELISA is simple to perform with a detection limit of 5×10^4 parasites ml^{-1} haemolymph. The ELISA is currently being used to study the prevalence and seasonality of *Hematodinium* infection in *N. norvegicus* and other crustacean hosts.

KEY WORDS: *Hematodinium* · *Nephrops norvegicus* · Infection · ELISA

Resale or republication not permitted without written consent of the publisher

The Norway lobster *Nephrops norvegicus* supports a major commercial fishery in the Northeast Atlantic. Lobster populations of this region harbour an infection by a parasitic dinoflagellate of the genus *Hematodinium* (Field et al. 1992). Infection of *N. norvegicus* by *Hematodinium* species was initially diagnosed by the dull orange colouration of the carapace and appendages; such lobsters were also observed to be moribund with milky white haemolymph. However, this diagnostic method lacks sensitivity as only advanced infections can be reliably identified (Field et al. 1992, Stentiford et al. 2001a). A more sensitive method was later developed in which the pleopod is examined under low power light microscopy for the aggregation of parasites in the vasculature (Field et al. 1992, Field & Appleton 1995). The severity of infection is staged using a 5-point scale, from uninfected to the most advanced infection. The pleopod method is a reliable field method for identifying advanced infections, but it is unable to detect low level haemolymph infection, and also requires a degree of training and standardisation.

Immunodiagnostic techniques can detect several pathogens of marine organisms (Bryant et al. 1999, Poulos et al. 1999, Hsu et al. 2000). An indirect fluorescent antibody technique (IFAT) developed using a polyclonal rabbit antiserum raised against an *in vitro* culture of several vegetative forms of *Hematodinium* from *Nephrops norvegicus* showed that some *N. norvegicus* harbour infections outside of the main infective season (Field & Appleton 1996). More recently, a Western blot method has been developed using the polyclonal anti-*Hematodinium* antiserum and applied to study the occurrence and progression of infection (Stentiford et al. 2001b). Both immunoassays offer greater sensitivity and specificity than the previous diagnostic methods, but are time-consuming and complex procedures, requiring trained personnel. Hence an enzyme-linked immunosorbent assay (ELISA) has been developed which can screen large numbers of samples in a short time and has greater sensitivity than the Western blot procedure.

Materials and methods. ***Collection and maintenance of lobsters:*** Norway lobsters *Nephrops norvegicus* were caught by otter bottom trawl (70 mm mesh size) at locations south of Little Cumbrae in the Clyde Sea Area (55.41° N, 4.56° W). The lobsters were transported in a cool, damp environment after capture, then maintained in a closed seawater system at 10°C and 33‰ salinity prior to experimental study.

Haemolymph preparation: For routine assessment, haemolymph samples were withdrawn from the base of the fifth pereiopod using a 1 ml disposable syringe and 25-gauge needle, allowed to clot, frozen to –20°C and thawed once. All subsequent treatments were performed at 22°C. The haemolymph samples were vortexed and a 15 µl aliquot of each haemolymph sample

*E-mail: 9909258s@student.gla.ac.uk

© Inter-Research 2002 · www.int-res.com

was then diluted in 285 µl of distilled water. From this, 100 µl was transferred into each of 2 wells of a 96-well microtiter plate (Immulon 4 HBX); for each haemolymph sample the ELISA was carried out in duplicate. After initial incubation for 30 min, plates were washed 4 times with phosphate-buffered saline (PBS) (pH 7.2) with 0.05% v/v Tween 20. Plates were then incubated for 30 min with 100 µl well^{-1} rabbit anti-*Hematodinium* antiserum (1/2000 dilution) (see Field & Appleton 1996 for antiserum production). Plates were again washed 4 times, followed by a third incubation for 30 min with 100 µl well^{-1} goat anti-rabbit horseradish peroxidase conjugated antibody (1/500 dilution) (Diagnostics Scotland). Plates were washed 4 times and 100 µl TMB substrate (3, 3', 5, 5'-tetramethyl benzidine) (Dynex Technologies) applied for colour development by incubation for 20 min in darkness. The optical density (OD) of the wells in the microtiter plate was then measured at 690 nm with an ELISA reader (Titertec Multiscan).

Sensitivity of ELISA: For determining the sensitivity of the ELISA test, a haemolymph sample was taken from a lobster that gave a positive result in the routine ELISA assessment, but displayed no external signs of infection. An aliquot of this sample was diluted 1:1 in marine anticoagulant (450 mM sodium chloride, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA pH 4.6, 1020 mOsm kg^{-1}) and transferred into an Improved Neubauer counting chamber. Parasite cell numbers were counted and the numbers per ml estimated using standard procedures. The sample was frozen to –20°C, then thawed and 6 aliquots were serially 2-fold diluted and assayed by the ELISA procedure above. A sample of uninfected haemolymph was assayed by the same method and a comparison of the OD values from infected and uninfected haemolymph was performed by the Mann-Whitney *U*-test. Significance was considered to be at $p < 0.005$.

Comparison of techniques for detection: Routine assessment of infection was made on a sub-sample from 2 monthly trawls using both the pleopod staging method of Field & Appleton (1995), and Western blotting of haemolymph samples described by Stentiford et al. (2001b). These assessments were then compared with the results from the ELISA conducted on the same haemolymph samples. Haemolymph samples from lobsters testing positive for *Hematodinium* infection by the ELISA method alone were assayed by the IFAT technique of Field & Appleton (1996) to confirm infection status. In this instance, the bovine serum albumin (BSA) used during the washing steps was replaced by an equivalent amount of porcine gelatin, because the anti-*Hematodinium* antibody reacts with BSA on Western blots (Stentiford et al. 2001b).

Results. ***Evaluation and sensitivity of ELISA:*** The ELISA could detect the presence of *Hematodinium* in haemolymph samples. There was some antibody binding in uninfected samples but this was considerably lower than for both low-level and advanced infection samples (Fig. 1).

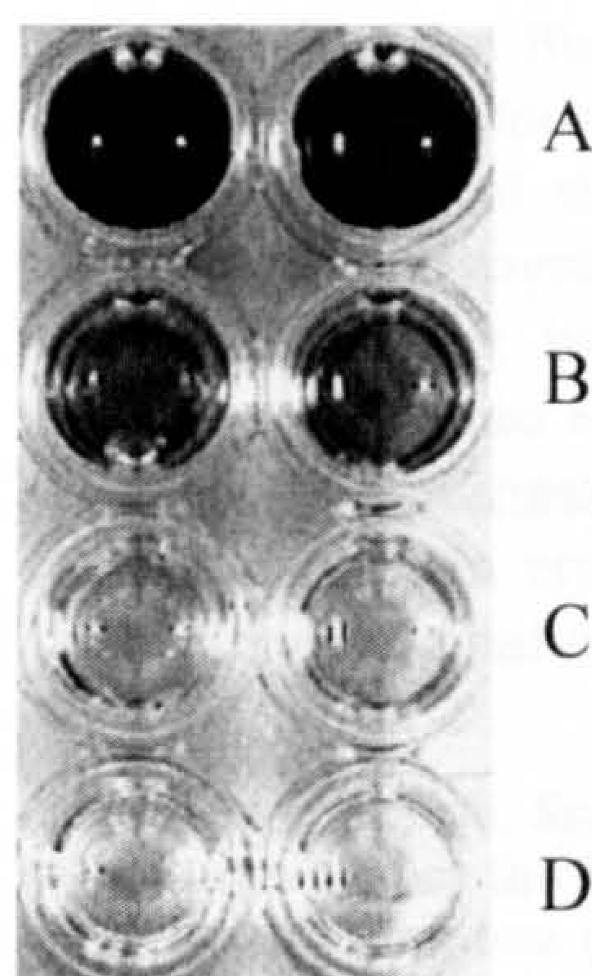


Fig. 1. Section of ELISA plate showing (A) advanced infection, (B) low-level infection, (C) uninfected, and (D) negative control ELISA reactions

Serial dilutions of a haemolymph sample with a known number of parasites present were used to determine the sensitivity of the assay (Fig. 2). The lowest density sample that was significantly different from the uninfected haemolymph sample was taken to represent the limit of detection of the assay (5×10^4 parasites ml^{-1} haemolymph).

Comparison of diagnostic methods: The diagnosis of *Hematodinium* species infection in 2 monthly subsets of 30 lobsters taken at times before the seasonal peak of infection, assessed by the pleopod, Western blot and ELISA methods, is shown in Table 1. By the pleopod method, all lobsters were assessed to be uninfected but by the antibody based methods a number of individuals were found to be infected. All haemolymph infections identified by Western blotting were also found to be positive by ELISA, but conversely not all infections identified by ELISA were detected by Western blotting. Comparison of the ELISA and IFAT results on these samples however, gave the same positive infection diagnosis (data not shown).

Discussion. The results obtained show that the ELISA is a sensitive and specific diagnostic test for the presence of *Hematodinium* parasites in the haemolymph of the Norway lobster. In common with the

Table 1. Comparison of *Hematodinium* infection diagnosis in a sub-sample of 30 lobsters by pleopod assessment, haemolymph Western blot, and haemolymph ELISA

Date (d/mo/yr)	Pleopod (prevalence)	Western blot (prevalence)	ELISA (prevalence)
10/10/00	0/30	4/30	8/30
06/11/00	0/30	5/30	6/30

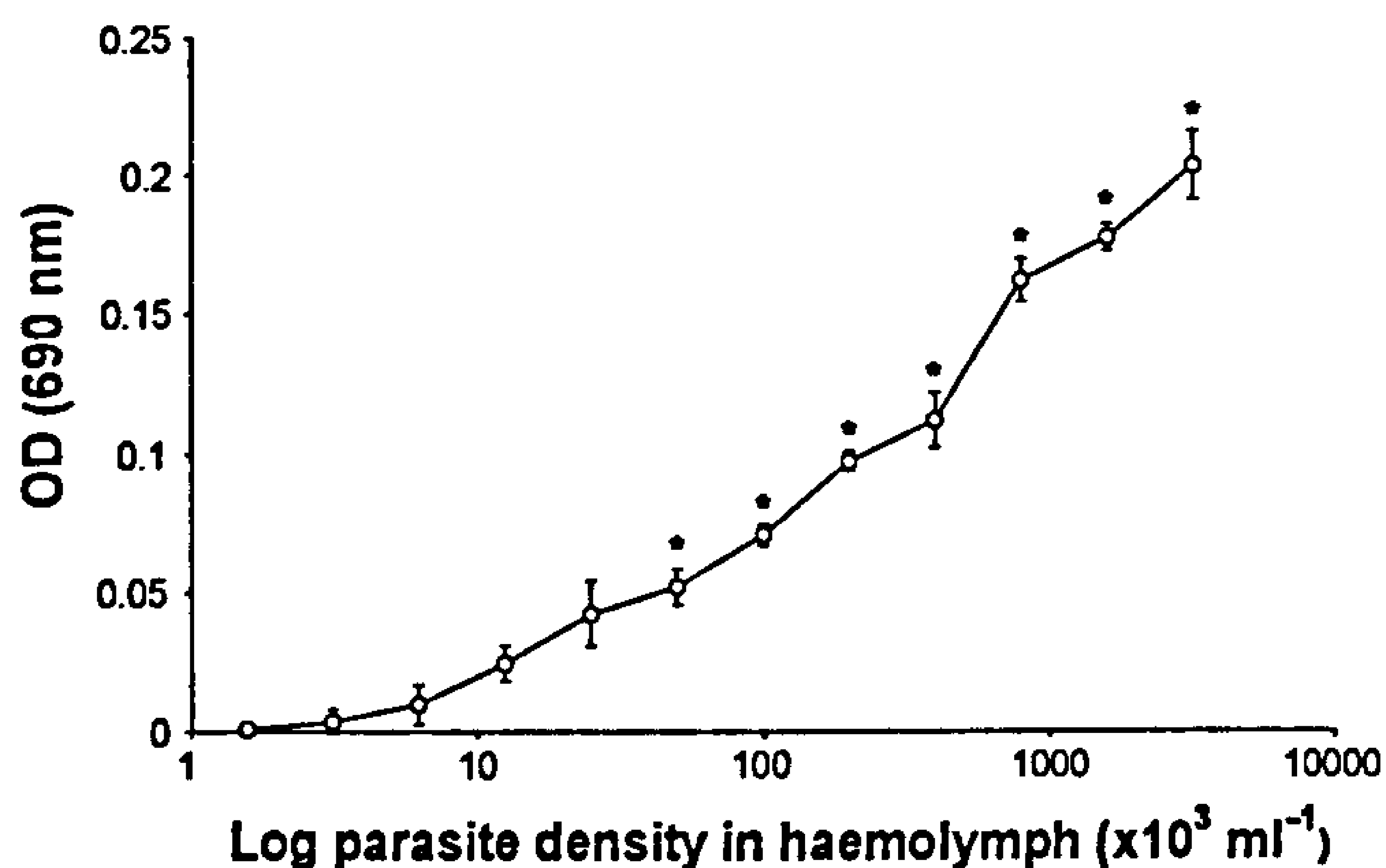


Fig. 2. Sensitivity of ELISA for *Hematodinium*. Infected haemolymph from an infected Norway lobster was serially 2-fold diluted and assayed by the ELISA. Uninfected haemolymph assayed by the ELISA had a mean optical density (OD) value of 0.037 with a standard deviation (SD) of 0.008. Data points represent means ± SD (n = 6). *p < 0.005 between uninfected and infected haemolymph

Western blot method of Stentiford et al. (2001b), it can detect both low-level and advanced infections. Moreover, it offers significant advantages over the Western blot procedure in terms of its sensitivity, simplicity and the number of samples that can be assayed. Importantly for routine use, the difference between positive and negative samples was visible by eye.

The sensitivity of the ELISA has been determined to be 5×10^4 parasites ml^{-1} haemolymph, making it more sensitive than the Western blot procedure by a factor of 4. This is consistent with the observation that several low-level infections identified by the ELISA were not detected by the Western blot.

The ability of the ELISA to test multiple samples within a short period, without sophisticated analytical equipment is a significant development. Previous immunological methods such as the IFAT and Western blot are complex and time-consuming. The ELISA requires only a small volume of haemolymph to conduct the test; host lobsters need not be damaged, and if necessary the sampled individuals can be kept alive for further observation under laboratory conditions.

The ELISA will be particularly useful in the identification of *Hematodinium* sp. infections in previously unexamined *Nephrops norvegicus* stocks. In addition, the ELISA could be used to identify *Hematodinium* infections in other crustacean species, since Stentiford et al. (2002) have shown that the primary anti-*Hematodinium* antibody is also reactive to a *Hematodinium*-like parasite of the European edible crab *Cancer pagurus*.

The finding by Bushek at al. (2002) that the polyclonal anti-*Hematodinium* antiserum used in this study is reactive against shared antibody binding epitopes on cells of the oyster pathogen *Perkinsus marinus* raises interesting issues concerning the affinity of *Hematodinium* and *Perkinsus*. However, this does not affect the usefulness of the antiserum in detecting *Hematodinium* in crustaceans as *P. marinus* is exclusively a mollusc pathogen.

Acknowledgements. Support for this research was provided by a contract from the Ministry of Agriculture Fisheries and Food, United Kingdom (Contract No. CSA 4172, project code MFO221) and a Natural Environment Research Council Studentship (NER/S/A/2000/03368) to H.J.S. The authors thank the crew of the RV 'Aora' (University Marine Biological Station, Millport) for assistance in collection of lobster.

LITERATURE CITED

Bryant MS, Lee RP, Lester RJG, Whittington RJ (1999) Anti-immunoglobulin antisera used in an ELISA to detect antibodies in barramundi *Lates calcarifer* to *Cryptocaryon irritans*. Dis Aquat Org 36:21–28

Bushek D, Dungan CF, Lewitus AJ (2002) Serological affinities of the oyster pathogen *Perkinsus marinus* (Apicomplexa) with some dinoflagellates (Dinophyceae). Eukaryot Microbiol 49:11–16

Field RH, Appleton PL (1995) A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops norvegicus*: observations on pathology and progression of infection. Dis Aquat Org 22:115–128

Field RH, Appleton PL (1996) An indirect fluorescent antibody technique for the diagnosis of *Hematodinium* sp. infection of the Norway lobster *Nephrops norvegicus*. Dis Aquat Org 24:199–204

Field RH, Chapman CJ, Taylor AC, Neil DM, Vickerman K (1992) Infection of the Norway lobster *Nephrops norvegicus* by a *Hematodinium*-like species of dinoflagellate on the west coast of Scotland. Dis Aquat Org 13:1–15

Hsu YL, Wang KH, Yang YH, Tung MC, Hu CH, Lo CF, Wang CH, Hsu T (2000) Diagnosis of *Penaeus monodon*-type baculovirus by PCR and by ELISA of occlusion bodies. Dis Aquat Org 40:93–99

Poulos BT, Kibler R, Bradley-Dunlop D, Mohney LL, Lightner DV (1999). Production and use of antibodies for the detection of Taura syndrome virus in penaeid shrimp. Dis Aquat Org 37:99–106

Stentiford GD, Neil DM, Atkinson RJA (2001a) The relationship of *Hematodinium* infection prevalence in a Scottish *Nephrops norvegicus* population to seasonality, moulting and sex. ICES J Mar Sci 58:814–823

Stentiford GD, Neil DM, Coombs GH (2001b) Development and application of an immunoassay diagnostic technique for studying *Hematodinium* infections in *Nephrops norvegicus* populations. Dis Aquat Org 46:223–229

Stentiford GD, Green M, Bateman K, Small HJ, Neil DM, Feist SW (2002) Pink Crab Disease (PCD) of the edible crab *Cancer pagurus* is caused by an infection by a *Hematodinium*-like parasitic dinoflagellate. J Invertebr Pathol 79:179–191

Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: February 5, 2002; Accepted: July 3, 2002
Proofs received from author(s): September 20, 2002

ACADEMIC
PRESS

Journal of Invertebrate Pathology 79 (2002) 179–191

Journal of
INVERTEBRATE
PATHOLOGY

www.academicpress.com

Infection by a *Hematodinium*-like parasitic dinoflagellate causes Pink Crab Disease (PCD) in the edible crab *Cancer pagurus*

G.D. Stentiford,[a,*] M. Green,[a] K. Bateman,[a] H.J. Small,[b] D.M. Neil,[b] and S.W. Feist[a]

[a] *Centre for Environment, Fisheries and Aquaculture Science (CEFAS) Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, UK*

[b] *Division of Environmental and Evolutionary Biology, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK*

Received 30 October 2001; accepted 2 May 2002

Abstract

The edible crab (*Cancer pagurus*) supports a large and valuable fishery in UK waters. Much of the catch is transported live to continental Europe in specially designed live-well ('vivier') vehicles. During the winter of 2000/2001, many trap-caught crabs from Guernsey, Channel Islands, UK, were reportedly moribund and pink in colour. These crabs generally died before and during vivier transportation. We provide histological, immunological, and molecular evidence that this condition is associated with infection by a *Hematodinium*-like dinoflagellate parasite similar to that previously reported in *C. pagurus* and to an infection causing seasonal mass mortalities of the Norway lobster (*Nephrops norvegicus*). Pathologically, every altered host bore the infection, which was characterised by very large numbers of plasmodial and vegetative stages in the haemolymph and depletion of reserve cells in the hepatopancreas. Due to the hyperpigmentation of the carapace and appendages, we have called this infection 'Pink Crab Disease' (PCD). Similar *Hematodinium* infections cause 'Bitter Crab Disease' in tanner and snow crabs, which has had a negative effect on their marketability. At present, little is known about the seasonality, transmission, and market impact of this infection in *C. pagurus*. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: *Cancer pagurus*; Crab fishery; Dinoflagellate; *Hematodinium*; Histopathology; *Nephrops norvegicus*; Mortality; Parasite; PCD; Pink Crab Disease

1. Introduction

The edible crab (*Cancer pagurus*) is one of the most valuable shellfish species captured in European waters, with a large fishery existing in the waters surrounding the United Kingdom (landings of over 27,000 t, worth £32 m in 1999—UK Sea Fisheries Statistics, 1999). Of the crabs landed in the United Kingdom, some are processed and sold locally, while a significant quantity, around 14,000 t in 1999 (UK Sea Fisheries Statistics, 1999), are transported live to continental Europe in specially designed live-wells known as 'viviers.' To ensure sale, exported crabs must be alive and in good condition on arrival at market.

Crustaceans are often exposed to an array of stressors during and after capture, which include crowding, mechanical damage to the cuticle, and exposure to light, air, and heat (see Chang et al., 1999; Jussila et al., 1997; Morris and Airriess, 1998; Paterson and Spanoghe, 1997; Stentiford and Neil, 2000). Another important stressor is infection by pathogens (for review, see Thompson, 1983). Considerable post-capture mortalities in holding tank conditions have been reported from decapods following epizootic infections by viruses (Arcier et al., 1999), bacteria (Cheng and Chen, 1998; Stewart, 1980), and ciliates (Armstrong et al., 1981; Bang et al., 1972; Cawthorn, 1997). In other cases, large-scale mortalities have occurred due to unknown agents or to idiopathic phenomena (see Anderson et al., 1990; Lindqvist and Mikkola, 1978; Stentiford and Neil, 2000).

The parasitic dinoflagellates of marine crustaceans are known to inhabit the eggs, stomach, soft tissue, and haemal sinuses of their hosts (Shields, 1994). Infections by parasitic dinoflagellates of the genus *Hematodinium*

* Corresponding author.
E-mail address: g.d.stentiford@cefas.co.uk (G.D. Stentiford).

0022-2011/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0022-2011(02)00028-9

have been reported in a number of commercially important crustacean hosts (Field et al., 1992; Hudson and Lester, 1994; Hudson and Shields, 1994; Maclean and Ruddell, 1978; Messick, 1994; Meyers et al., 1987; Newman and Johnson, 1975; Taylor and Khan, 1995; Wilhelm and Boulo, 1998; Wilhelm and Mialhe, 1996). Latrouite et al. (1988) have also reported an infection by a *Hematodinium*-like parasite in populations of *C. pagurus* taken from the English Channel, the Irish Sea, the Bay of Biscay, and the west coast of Scotland. In these cases, the haemolymph and muscle of affected crabs assumed a pink colouration, with the meat having an irregular texture and a bitter taste when cooked. Similar features of infection have been ascribed to *Hematodinium* infections in tanner crabs (*Chionoecetes bairdi* and *C. opilio*), where the condition, termed 'Bitter Crab Disease' renders the meat unmarketable (Meyers et al., 1987; Taylor and Khan, 1995).

During the autumn and winter 2000, creel-caught *C. pagurus* from the west coast of Cornwall and from the island of Guernsey displayed an altered colouration (pink hyperpigmentation) with a general morbidity. These crabs would usually die following handling, pounding, and subsequent vivier transportation. Signs of this condition were also noted in animals taken directly from creels. We provide histological, ultrastructural, and molecular evidences that PCD is associated with an infection by a *Hematodinium*-like dinoflagellate which is probably the same as that described by Latrouite et al. (1988) and provide recommendations for future monitoring and pathological studies.

2. Methods

Cancer pagurus were captured from the south coast of Guernsey (UK Channel Islands; 49°24′N, 2°32′–2°43′W) using conventional crab pots. Crabs were transported to the shore, following which tissues and organs were removed from crabs showing signs of PCD (morbid, pink hyperpigmented carapace) and from apparently healthy crabs. These were prepared for light and electron microscopy, immunological parasite detection, and molecular characterisation using standard methods.

2.1. Histopathology

Crabs were anaesthetised by chilling to 4 °C. For histopathology, the hepatopancreas, claw muscle, gill, gonad, hindgut, and heart of infected and uninfected crabs were removed and placed immediately into Davidson's seawater fixative (see Hopwood, 1996). Fixation proceeded for 24 h before samples were transferred to 70% industrial methylated spirit (IMS).

For electron microscopy, small pieces of tissue were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) with 1.75% sodium chloride for 2 h at room temperature (21 °C). Fixed tissue samples were rinsed in 0.1 M sodium cacodylate buffer with 1.75% sodium chloride (pH 7.4) and post-fixed in 1% osmium tetroxide, reduced with 1.75% potassium ferrocyanide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. Specimens were washed in three changes of 0.1 M sodium cacodylate buffer and stained en bloc in 0.5% aqueous uranyl acetate for 1 h. Following dehydration through an acetone series, specimens were embedded in epoxy resin 812 (Agar Scientific-pre-mix kit 812). Semi-thin sections (1–2 μm) were stained with toluidine blue for viewing with a light microscope, suitable areas were identified and ultrathin sections (70–90 nm) of these areas were cut and mounted on uncoated copper grids. Sections were stained with uranyl acetate and Reynolds lead citrate (Reynolds, 1963) and were examined using a JEOL 1210 transmission electron microscope.

2.2. Immunological characterisation

A 70 mg sample of Davidson's-seawater-fixed *C. pagurus* hepatopancreas was macerated in 300 μl sample buffer (62.5 mM Tris–HCl pH 6.8, 12.5% glycerol, 1.25% β-mercaptoethanol) and heated at 95 °C for 4 min. Lanes on a standard acrylamide gel (12.5%) were loaded with either 20 μl of whole sample or 20 μl of supernatant from a centrifuged sample (17,000*g*, 2 min). These were analysed for the presence of *Hematodinium* antigens using a polyclonal antibody raised against the *Nephrops norvegicus* isolate of *Hematodinium* (see Field and Appleton, 1996) applied by the Western blotting method of Stentiford et al. (2001c) with *Hematodinium*-infected *N. norvegicus* haemolymph run as a positive control (see Stentiford et al., 2001c). *Hematodinium*-positive samples generally appeared as multiple-bands or smears due to the reaction of numerous parasite proteins to the polyclonal antibody (as described by Stentiford et al., 2001c).

2.3. Molecular characterisation

Aliquots of 100 mg samples of fixed *C. pagurus* hepatopancreas from asymptomatic crabs and those showing the signs of PCD, were homogenised separately in 500 μl extraction buffer (50 mM Tris, 5 mM EDTA, 100 mM NaCl, and pH 8), 200 μl of 10% SDS, and 20 μl Proteinase-K (10 μg/ml) and incubated at 56 °C for 24 h. DNA was purified by phenol/chloroform extraction, ethanol precipitated, and resuspended in sterile deionised water. PCR reactions were performed in 20 μl total reaction volume by adding 2 μl of 10X reaction buffer (final concentration 10 mM Tris–HCl, pH 9, 50 mM KCl, 0.1% Triton X-100), 1.2 μl $MgCl_2$ (final concentration 1.5 mM), 1 μl dNTP mix (final concentration

100 μM), 1 μl each of forward and reverse primers (final concentration 0.5 pmol μl^{-1}), volume of target DNA to approximately 100 ng, 1 unit of *Taq* polymerase, and sterile deionised water to a final volume of 50 μl. Reactions were overlaid with 10 μl mineral oil.

Thermal cycling conditions were as follows: denaturation at 94 °C for 1 min; primer annealing at 52 °C for 1 min; chain extension at 70 °C for 3 min; repeated for 35 cycles with a final cycle incorporating a 7 min extension. Primer sequences were as described by Hudson and Adlard (1994). Amplification products were run on a 1.5% agarose gel, stained with ethidium bromide and viewed under a UV light source.

3. Results

3.1. Gross clinical signs of PCD

Crabs showing clinical signs of PCD were moribund and were reported to die quickly following capture and during transit. Heavily infected crabs typically displayed hyperpigmentation (pink) of the carapace and discolouration (yellowing) of the arthrodial membranes and the genital pores. This yellowing was later found to be due to the creamy consistency and colouration of the haemolymph caused by infection with large numbers of single, bi-, and multi-nucleate plasmodia. Internally, organs and tissues were friable, with creamy multicellular parasite deposits covering their outer surfaces.

3.2. Histopathology

Histologically, during severe PCD, the haemal sinuses of the hepatopancreas was heavily dilated and filled with large numbers of plasmodial cells of the parasite which had condensed chromatin profiles (Fig. 1). The hepatopancreatic tubule cells of infected crabs were relatively devoid of lipid reserves and in a number of specimens, hepatopancreatic tubule cells appeared degenerate with plasmodial forms of the parasite observed within the lumen of the tubules (Fig. 2). Due to the severely dilated haemal sinuses, haemolymph vessels, and their associated fixed phagocytes were rarely observed.

Pathological changes to the muscles of the claw and the body cavity were extensive. In the claws of infected crabs, muscle tissue was almost completely replaced by large numbers of plasmodia, with only small islands of identifiable muscle fibres remaining (Fig. 3). Intense

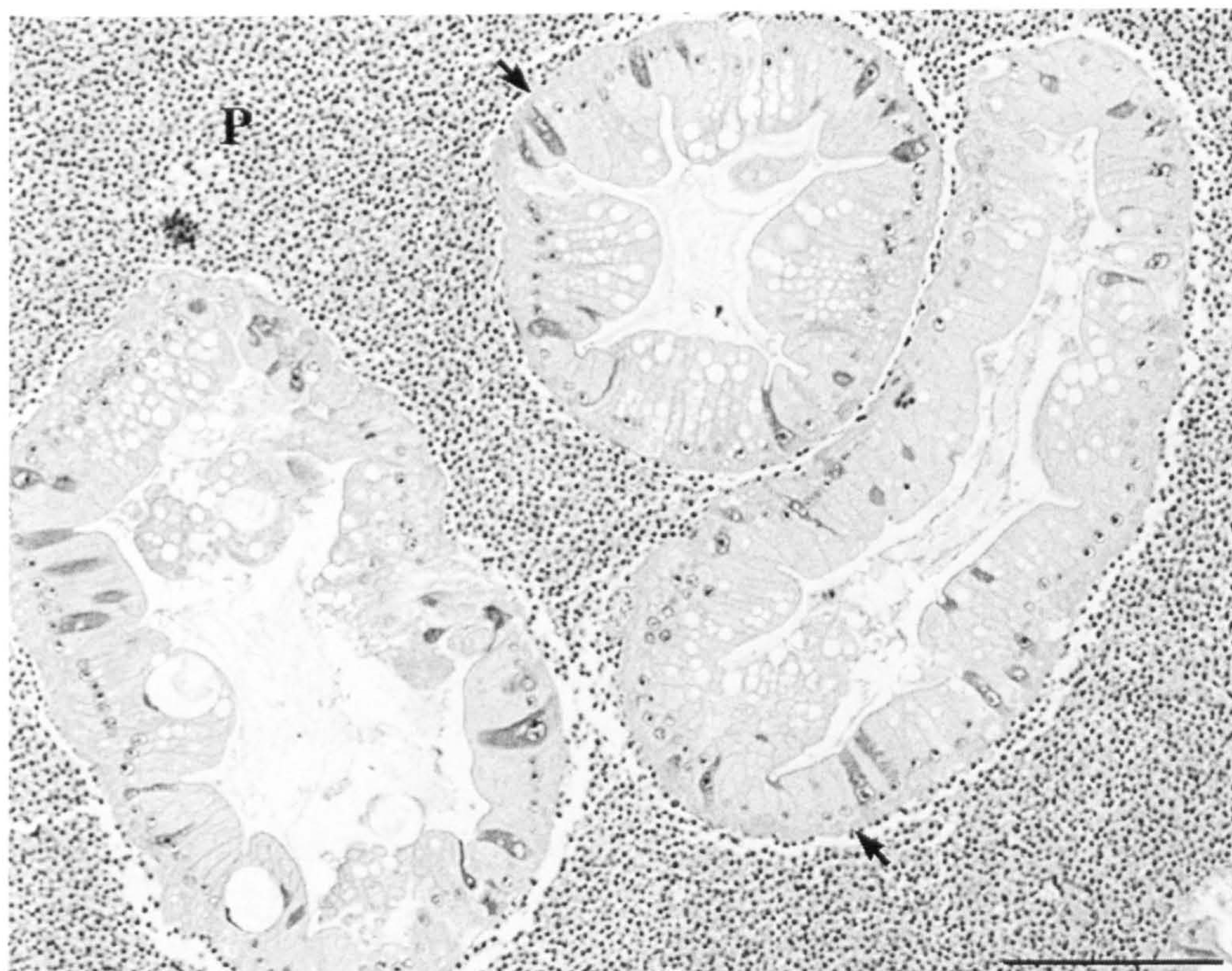


Fig. 1. Hepatopancreas of crab with PCD. Note the dilated haemal sinuses filled with masses of parasitic plasmodial cells (P). Vessels and associated fixed phagocytes were rarely observed and reserve cells were not observed in the inter-tubular connective matrix. Tubule cells were often seen to be devoid of lipid reserves (arrows). Haematoxylin and Eosin, 5 μm section. Bar = 200 μm.

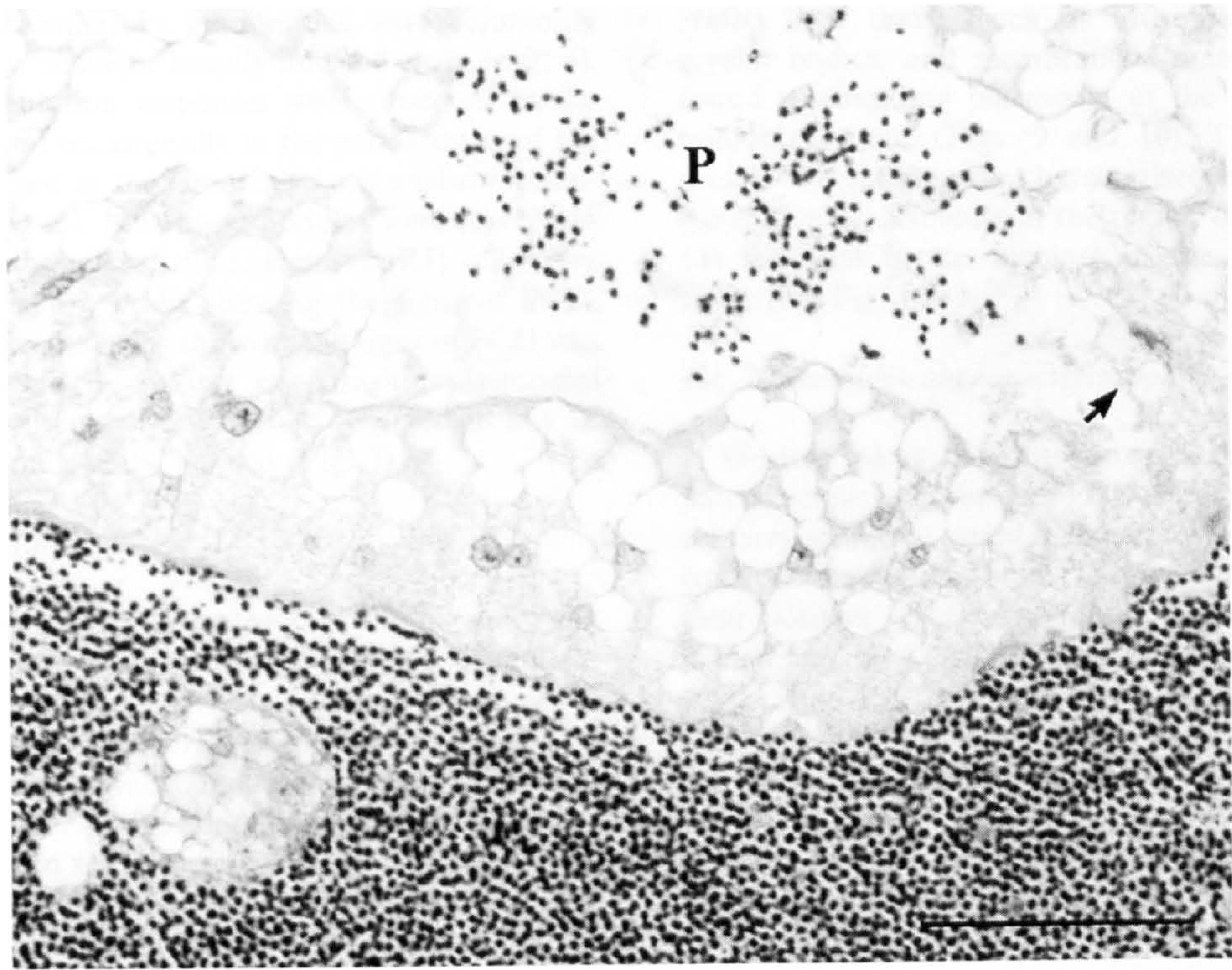


Fig. 2. Hepatopancreas of crab with PCD. Note the degraded nature of tubule cells (arrow) and the invasion of the tubule lumen by parasitic plasmodial cells (P). Haematoxylin and Eosin, 5 μm section. Bar = 100 μm.

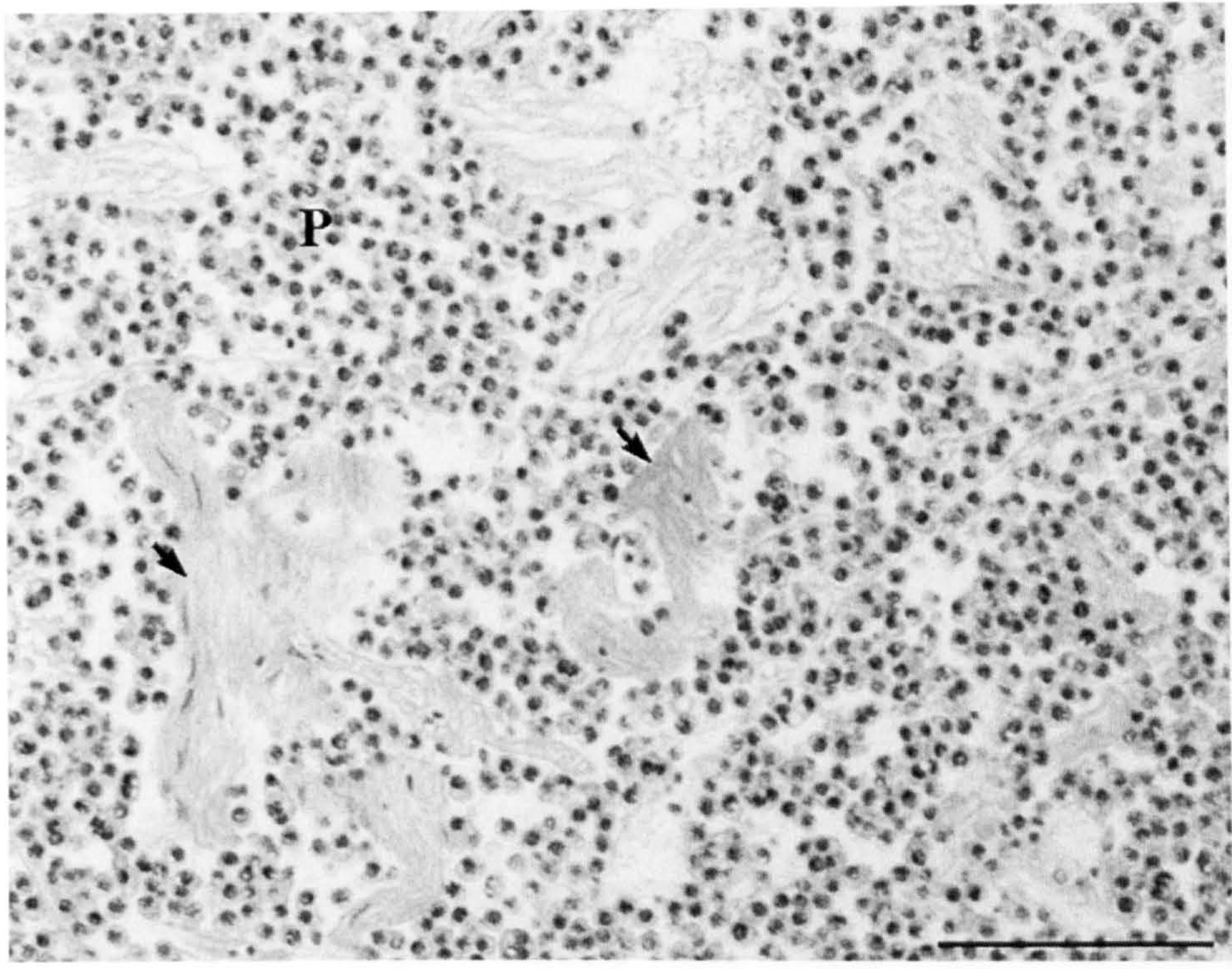


Fig. 3. Claw muscle of crab with PCD. Note the 'islands' of unattached muscle blocks (arrows) surrounded by masses of parasitic plasmodial cells (P). Haematoxylin and Eosin, 5 μm section. Bar = 100 μm.

multi-focal inflammatory granulomas were commonly seen in the claw tissue of heavily infected crabs (Fig. 4). Similar encapsulation responses were observed in the myocardium and occasionally in the pericardium of the heart (Fig. 5) and in the connective material surrounding the gut (Fig. 6). Melanised nodules were rarely observed within the gill lamellae. Reserve (RI) cells were not observed in any crabs showing the signs of PCD. The ovary of female crabs showing the signs of PCD was heavily infiltrated by masses of parasitic plasmodial cells. Vitellogenic oocytes were not observed in any of the infected female crabs studied (Fig. 7).

3.3. Ultrastructure

Electron microscopy revealed that the haemolymph and tissues of crabs with PCD harboured a *Hematodinium*-like dinoflagellate parasite similar to that previously described in *N. norvegicus* (Field et al., 1992). Plasmodia typically had condensed chromatin profiles (up to 5 nuclei per plasmodia), abundant lipid droplets, membrane-bound trichocysts, and mitochondria, and a surrounding alveolar membrane. The centriolar apparatus was observed in a number of parasites (Fig. 8). Plasmodia were frequently found in close association with the outer surface of the hepatopancreatic tubules and with the muscle sarcolemma. Remnants of degenerated host tissue (such as atrophied mitochondria, myelin bodies, and membranous material) were often found surrounding plasmodia at the periphery of the remaining tissue (Figs. 9 and 10). The pathology of muscle breakdown was characterised by a severe disorganisation of filaments in the region of the Z-line (Fig. 11) followed by an increase in the sub-sarcolemmal space (see Fig. 10).

3.4. Immunological characterisation

Western blots of proteins extracted from the hepatopancreas of crabs with PCD were performed using a primary antibody raised against *Hematodinium* isolated from *N. norvegicus*. Tissue from infected crabs showed a clear positive reaction to this polyclonal anti-*Hematodinium* antibody, characteristically as a multi-band smear (Fig. 12).

3.5. Molecular characterisation

PCR amplification of the first internal transcribed spacer (ITS1) region of ribosomal DNA and flanking 3′ end of the small subunit (SSU) was achieved using primer sequences previously used for the diagnosis of *Hematodinium* infections in other crustacean species (Hudson and Adlard, 1994). A single 680 bp amplifica-

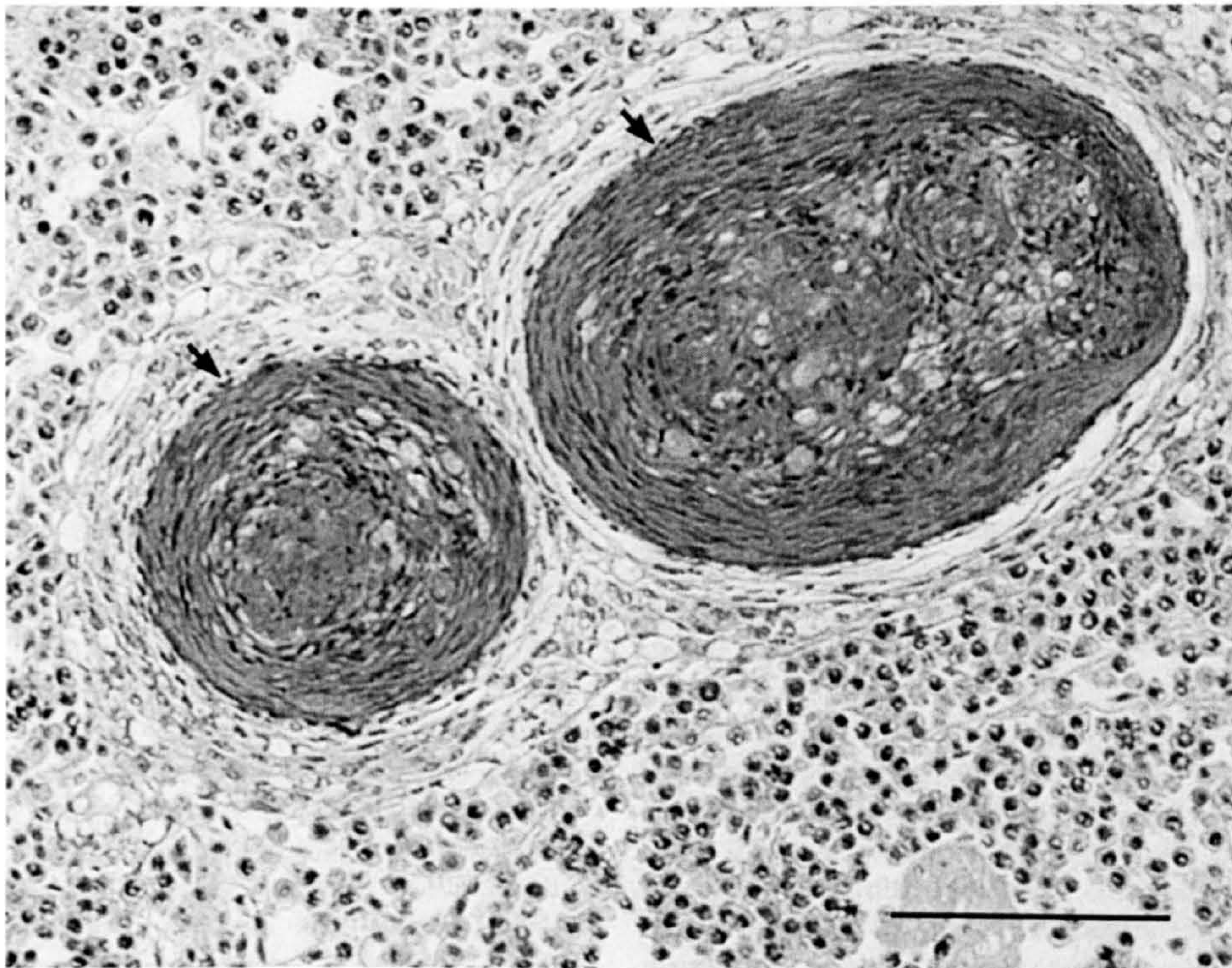


Fig. 4. Two large focal granulomas (arrows) in the claw muscle of a crab with PCD. Haematoxylin and Eosin, 5 μm section. Bar = 100 μm.

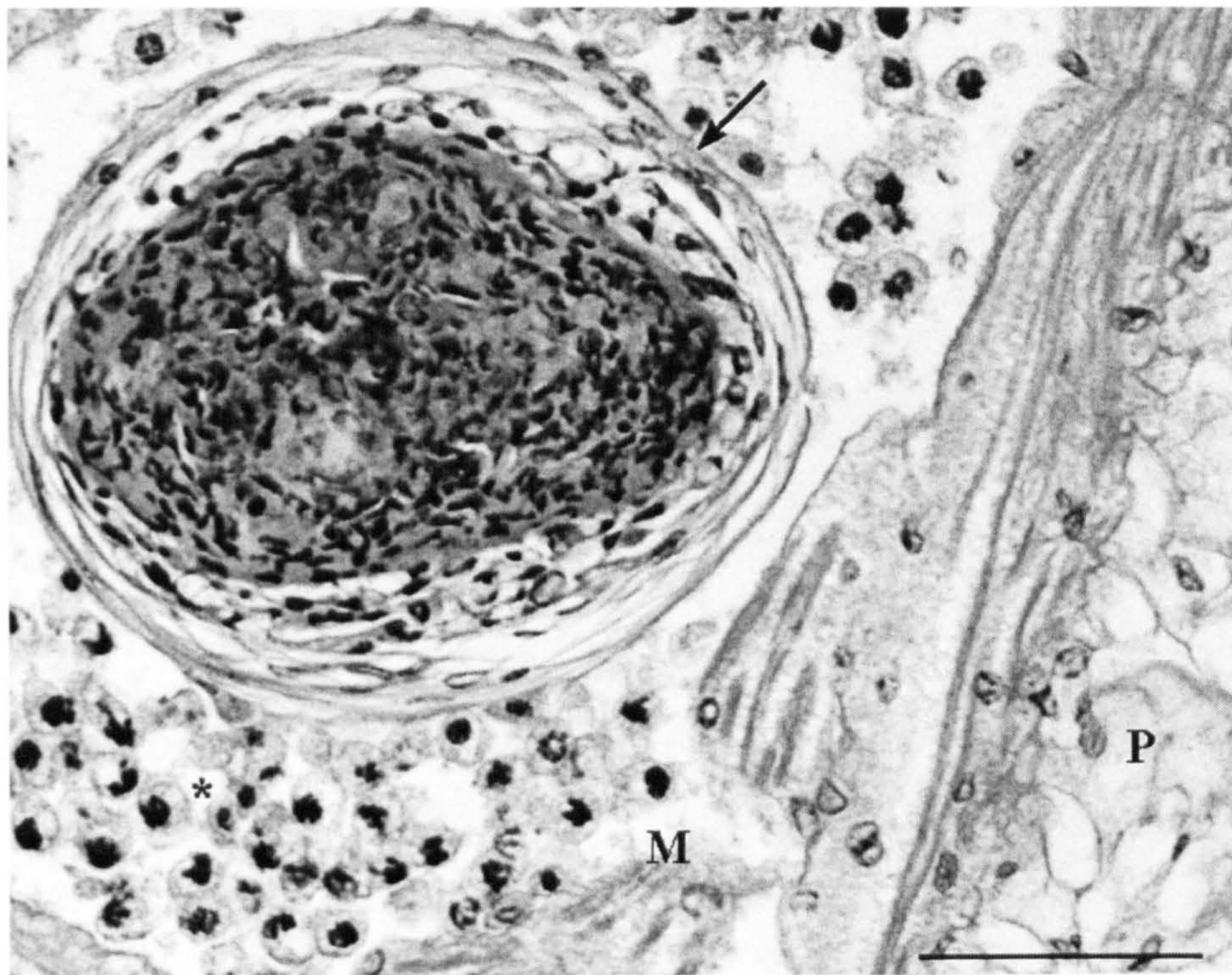


Fig. 5. Heart of crab with PCD. Note the pericardium (P) and the myocardium (M) containing a large melanised granuloma (arrow). In some crabs, multiple granulomas were observed throughout the myocardium and occasionally within the pericardium. Haematoxylin and Eosin, 5 μm section. Bar = 50 μm.

tion product was produced in crabs showing the symptoms of PCD, while no reaction product was seen in crabs asymptomatic for PCD (Fig. 13).

4. Discussion

4.1. Aetiology

The histological, ultrastructural, and molecular data presented in this study have shown that PCD is caused by a parasitic dinoflagellate of the genus *Hematodinium* and formally confirms the presence of this parasite in the English Channel fishery for *C. pagurus*. Furthermore, application of *Hematodinium*-specific primers led to the appearance of a 680 bp PCR amplification product from the ribosomal DNA of this parasite, suggesting a strong similarity to the *Hematodinium* strains isolated from other crustacean species (see Hudson and Adlard, 1994). It is highly likely that this is also the same organism as previously reported to colonise the haemolymph of *C. pagurus* captured from various European locations (Latrouite et al., 1988) and similar to that reported from a number of other commercially important crustacean hosts (Field et al., 1992; Hudson and Lester, 1994; Hudson and Shields, 1994; Maclean and Ruddell, 1978; Messick, 1994; Meyers et al., 1987; Newman and Johnson, 1975; Taylor and Khan, 1995; Wilhelm and Boulo, 1998). Whether this parasite is the same as the *Hematodinium*-like species thought to be responsible for commercially significant declines in populations of velvet swimming crab (*Necora puber*) from the English Channel remains to be shown (see Wilhelm and Mialhe, 1996).

Uni-cellular, bi-cellular, and multi-cellular (up to 5 nuclei) stages of the parasite were observed in the haemolymph and within the tissue interstices of crabs with PCD. In their description of the type species, *Hematodinium perezi*, in portunid crabs captured from French waters, Chatton and Poisson (1931) describe motile stages within the haemolymph (a similar finding to that of Newman and Johnson, 1975; Messick, 1994 and Shields and Squyars, 2000, in *C. sapidus*). In addition, Appleton and Vickerman (1997) have described motility in the *Hematodinium* sp. isolated and cultured from *N. norvegicus*, suggesting that such motile forms may represent the early trophont stages in all *Hematodinium* species. Observations of fresh haemolymph preparations of low-level *Hematodinium* infections are required if these findings are to be confirmed. In addition, further molecular and ultrastructural comparisons of *Hematodinium*-like sp. to the type species (*H. perezi*) are required to apply accurate taxonomic status to these isolates.

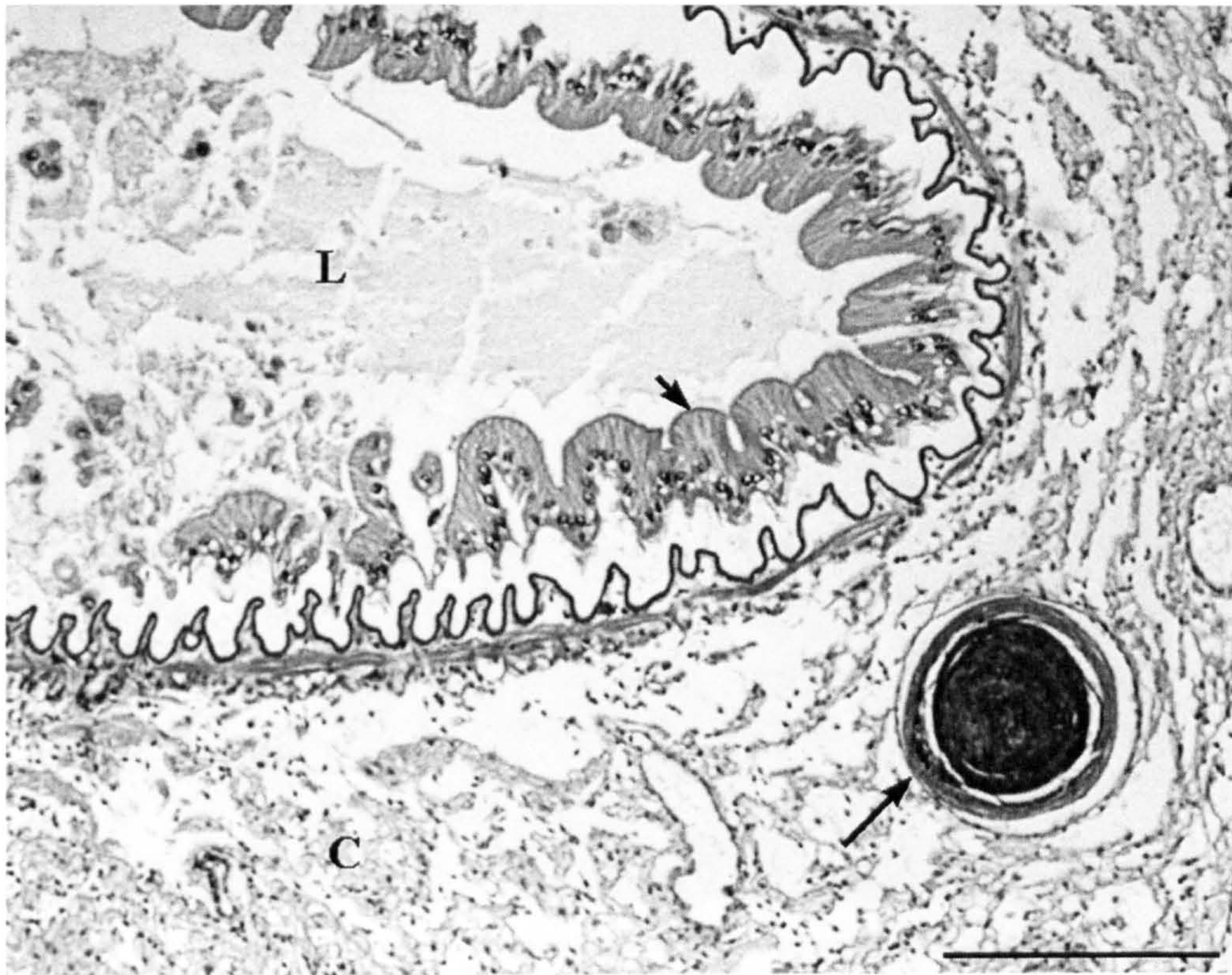


Fig. 6. Transverse section through the hindgut of crab with PCD. The gut lumen (L) contains food material and multiple large melanised granuloma were commonly seen in the connective tissue (C) (long arrow). Separation of gut epithelium (short arrow) from basement membrane is likely to be an artefact of tissue preparation. Haematoxylin and Eosin, 5 μm section. Bar = 100 μm.

Anecdotal reports suggest that PCD may show a seasonal epizootiology, with peak infection occurring during the winter and the spring, and with a latent infection or absence during the summer and early autumn (G.D. Stentiford, personal observation). Observations on the prevalence of *Hematodinium* infection in other crab species also suggest highly seasonal disease outbreaks, with peak infection occurring over a relatively narrow time period, followed by a longer period of undetectable or low level prevalences (see Messick and Shields, 2000; Shields, 1994). Studies on Scottish *N. norvegicus* populations have revealed similar features of *Hematodinium* infection epizootiology (Field et al., 1992, 1998; Stentiford et al., 2001b).

4.2. Pathology

Crabs infected with PCD revealed significant alterations from the normal structure of muscle and hepatopancreatic tissue. The alteration in the lipid content of hepatopancreas tubule cells during PCD is consistent with a progressive parasite-induced physiological starvation. The absence of reserve (RI) cells in the connective tissue throughout crabs with PCD confirms this hypothesis. Similar effects of *Hematodinium* infection have previously been reported in *N. norvegicus* (Stentiford et al., 1999, 2000, 2001a) and in *Callinectes sapidus* (Whittington et al., 1997). Histology revealed that the tubule cells of the hepatopancreas were frequently degenerate, possibly explaining the presence of parasitic plasmodia within the lumen of tubules themselves. This feature of *Hematodinium* infection has also been reported in *N. norvegicus* (Field and Appleton, 1995). The significance of the presence of parasites within the tubule lumens is not presently known, though this may reflect a possible route of transmission (via the gut) to other hosts. In addition to their presence within the lumens of the tubules, parasites were also observed in close association with the myoepithelial layer surrounding these tubules. Whether these cells are attached to the tubule surface or whether their presence is an artefact of tissue preparation is difficult to elucidate. However, similar features of *Hematodinium* sp. infection have previously been reported in *N. norvegicus* (Field and Appleton, 1995). In this species, it has been suggested that the hepatopancreas and other tissues may represent the seat of latent *Hematodinium* infection (Field and Appleton, 1995, 1996; Stentiford et al., 2001c). Further studies of apparently uninfected *C. pagurus*, possibly out of the main infection period for this disease may allow such latent stages to be located.

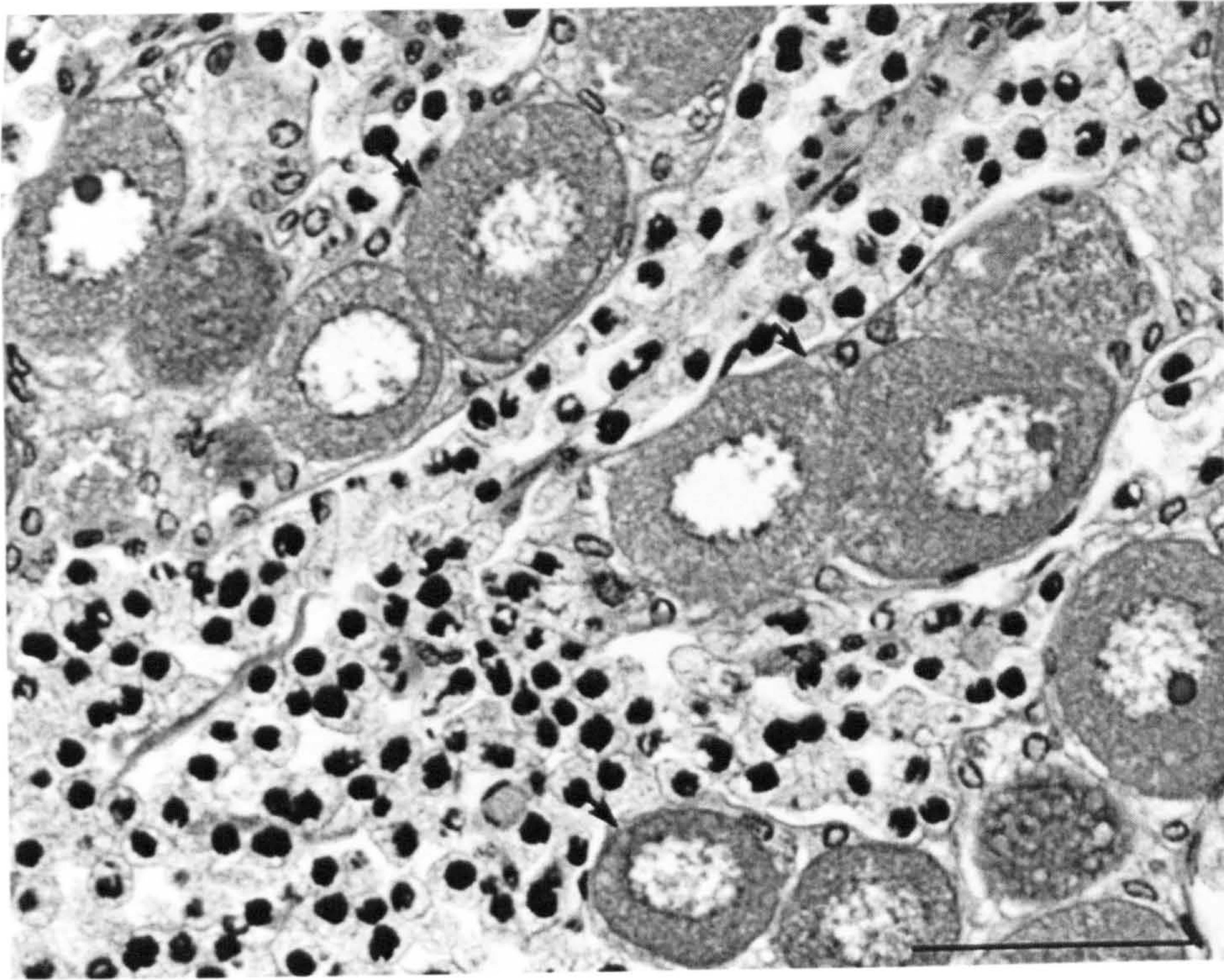


Fig. 7. Ovary of female crab with PCD. Note the presence of pre-vitellogenic oocytes (short arrows) and the presence of masses of parasitic plasmodial cells between the oocytes. Haematoxylin and Eosin, 5 μm section. Bar = 50 μm.

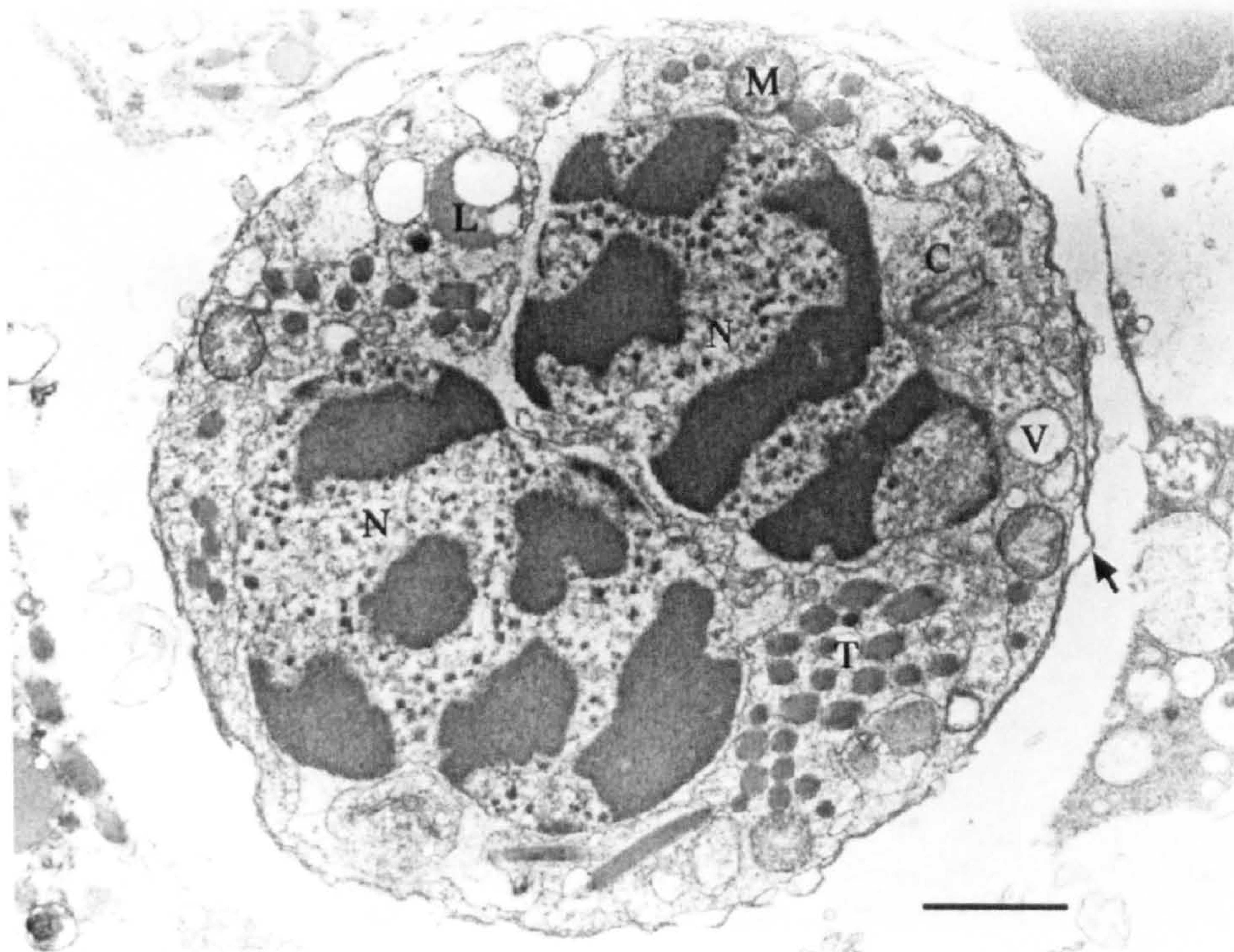


Fig. 8. Bi-nucleate parasitic plasmodium in the haemolymph of a crab with PCD. Note the presence of lipid droplets (L), trichocysts (T), mitochondria (M), vacuoles (V), centriole apparatus (C), and a surrounding alveolar membrane (arrow). Parasites typically contained between one and four nuclei (N). TEM, scale bar = 1 μm.

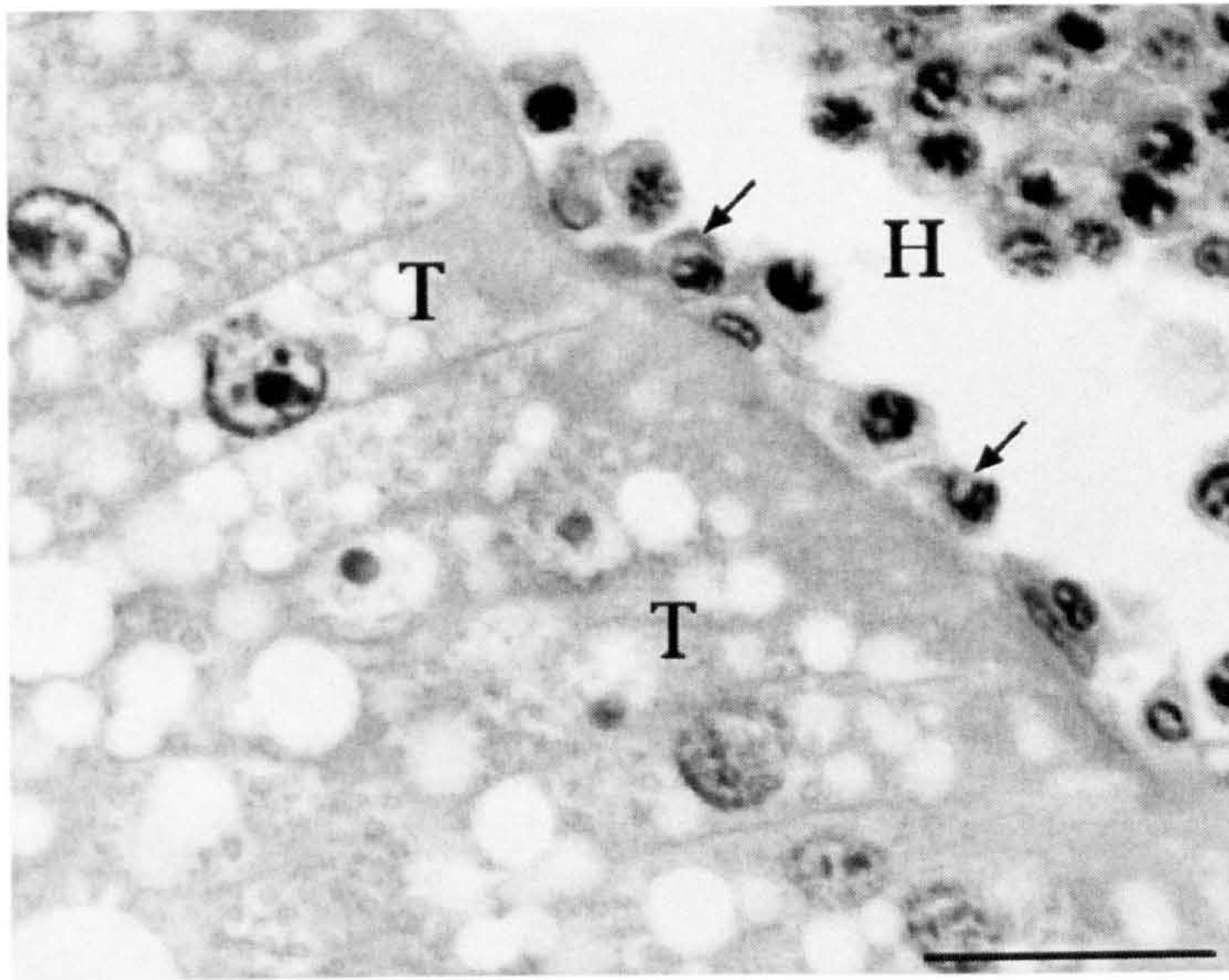


Fig. 9. Parasitic plasmodial cells at the periphery of a hepatopancreatic tubule. Note the close association of parasites (arrows) and tubule cells (T). Parasitic plasmodial cells filled the haemal spaces (H). Haematoxylin and Eosin, 5 μm section. Bar = 50 μm.

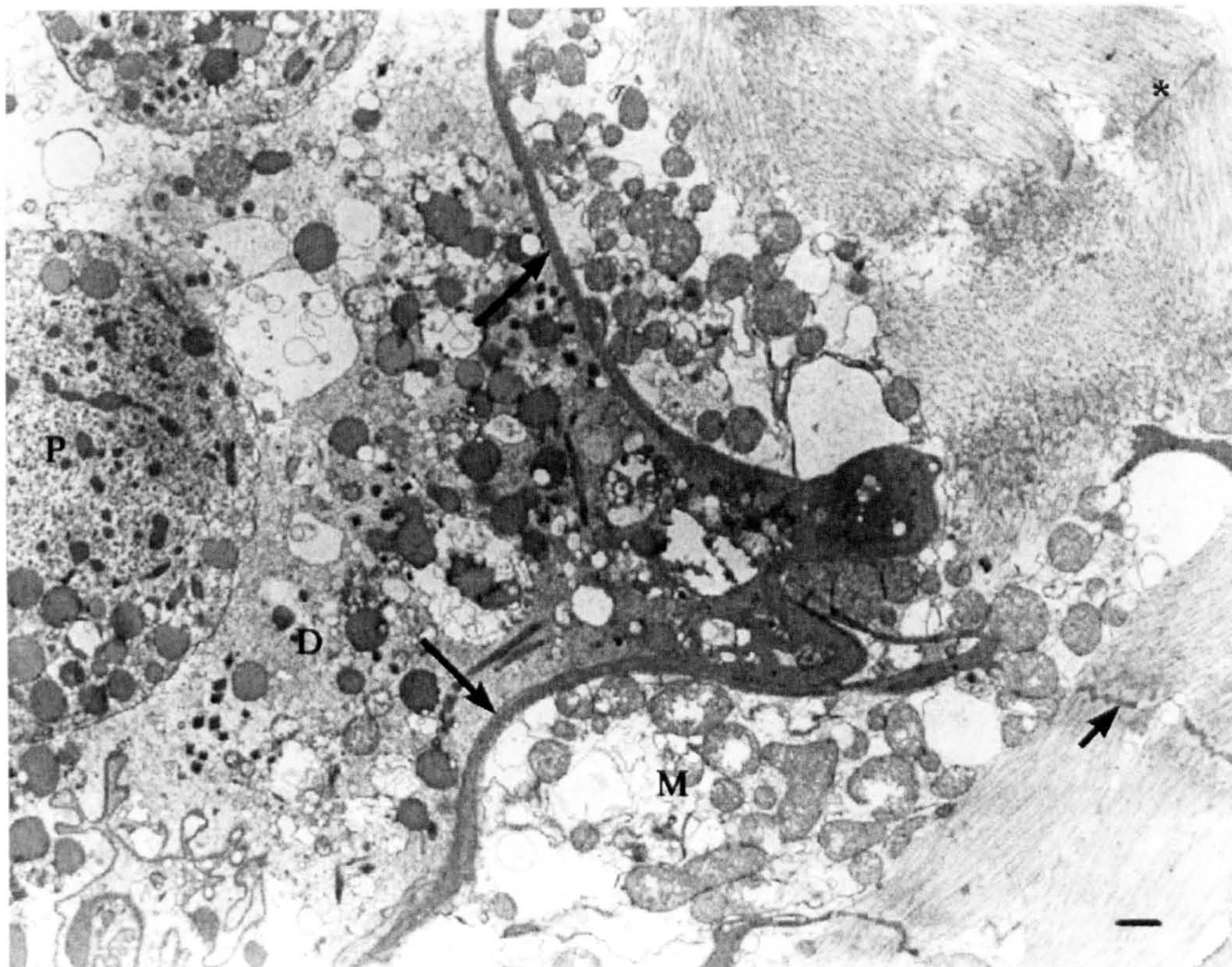


Fig. 10. Claw muscle of crab with PCD. Parasitic plasmodial cells (P) were often seen in close association with the sarcolemmal membrane (arrows) which was often well separated from the contractile muscle blocks. Note the presence of mitochondria at the muscle periphery (M) and the presence of Z-lines in various states of degeneration (arrow and asterisk). Cellular debris of host and parasite origin was commonly seen on the surface of the sarcolemma (D). TEM, scale bar = 1 μm.

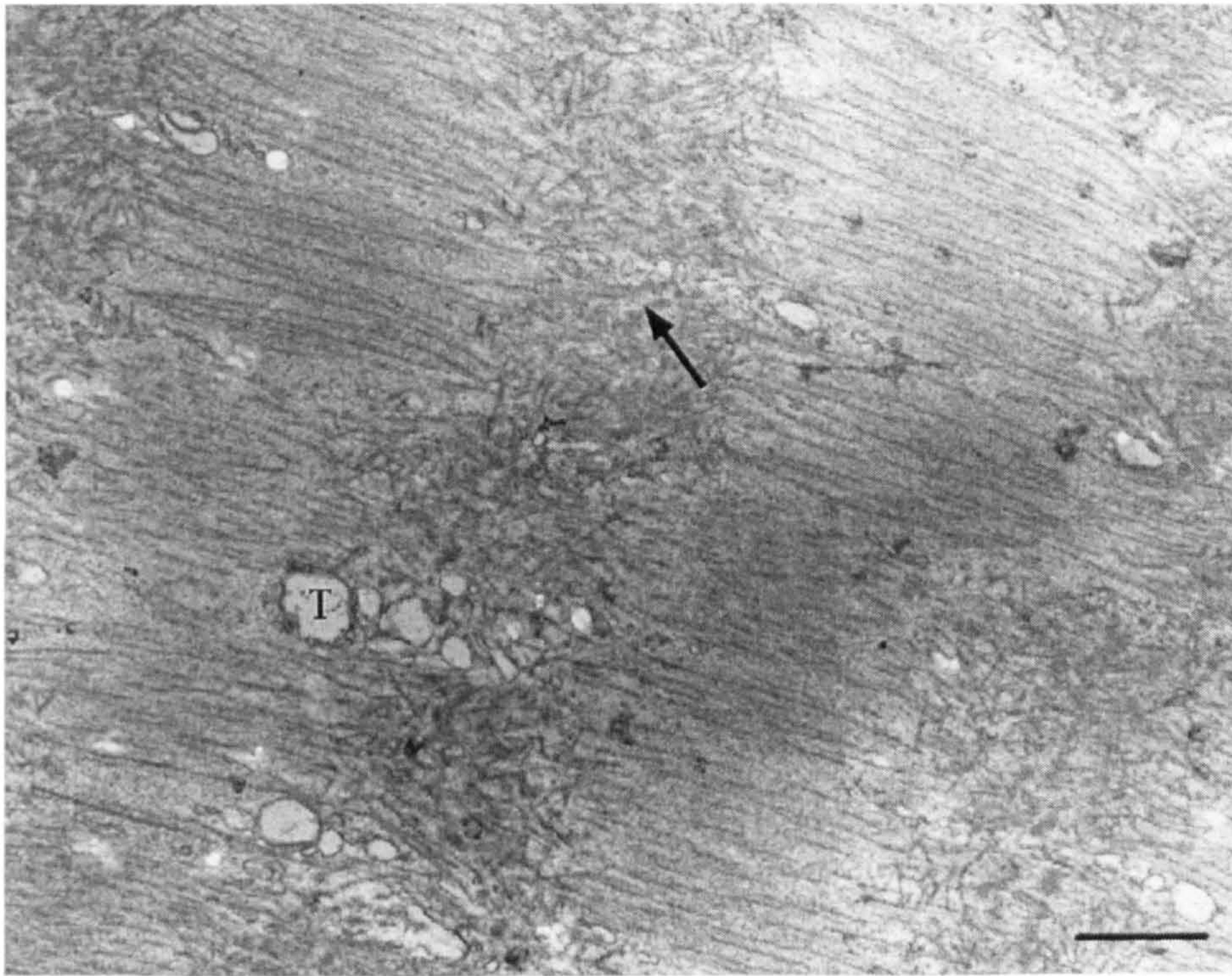


Fig. 11. Claw muscle of crab with PCD. Note the disorganisation of fibrils in the region of the Z-lines (arrow) and remnants of the tubular system (T). TEM, scale bar = 1 μm.

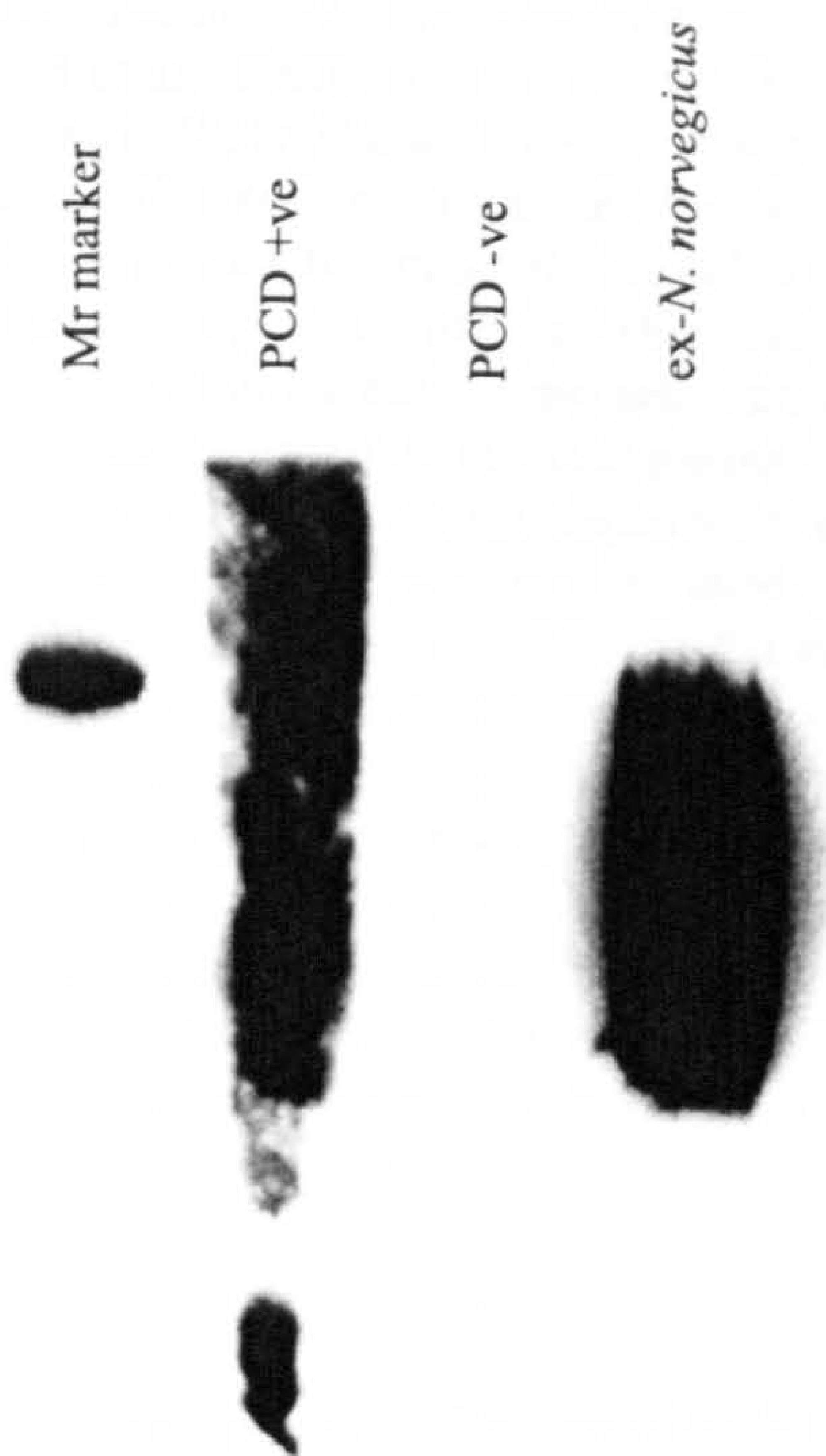


Fig. 12. Western blot of hepatopancreas from crab with PCD (*C. pagurus*) using anti-*Hematodinium* (ex-*Nephrops norvegicus*) polyclonal primary antibody. Multiple band or smear reactions were seen in PCD +ve crabs. No reaction was seen in PCD −ve crabs. In vitro cultured *Hematodinium* (ex-*N. norvegicus*) was used as a positive control.

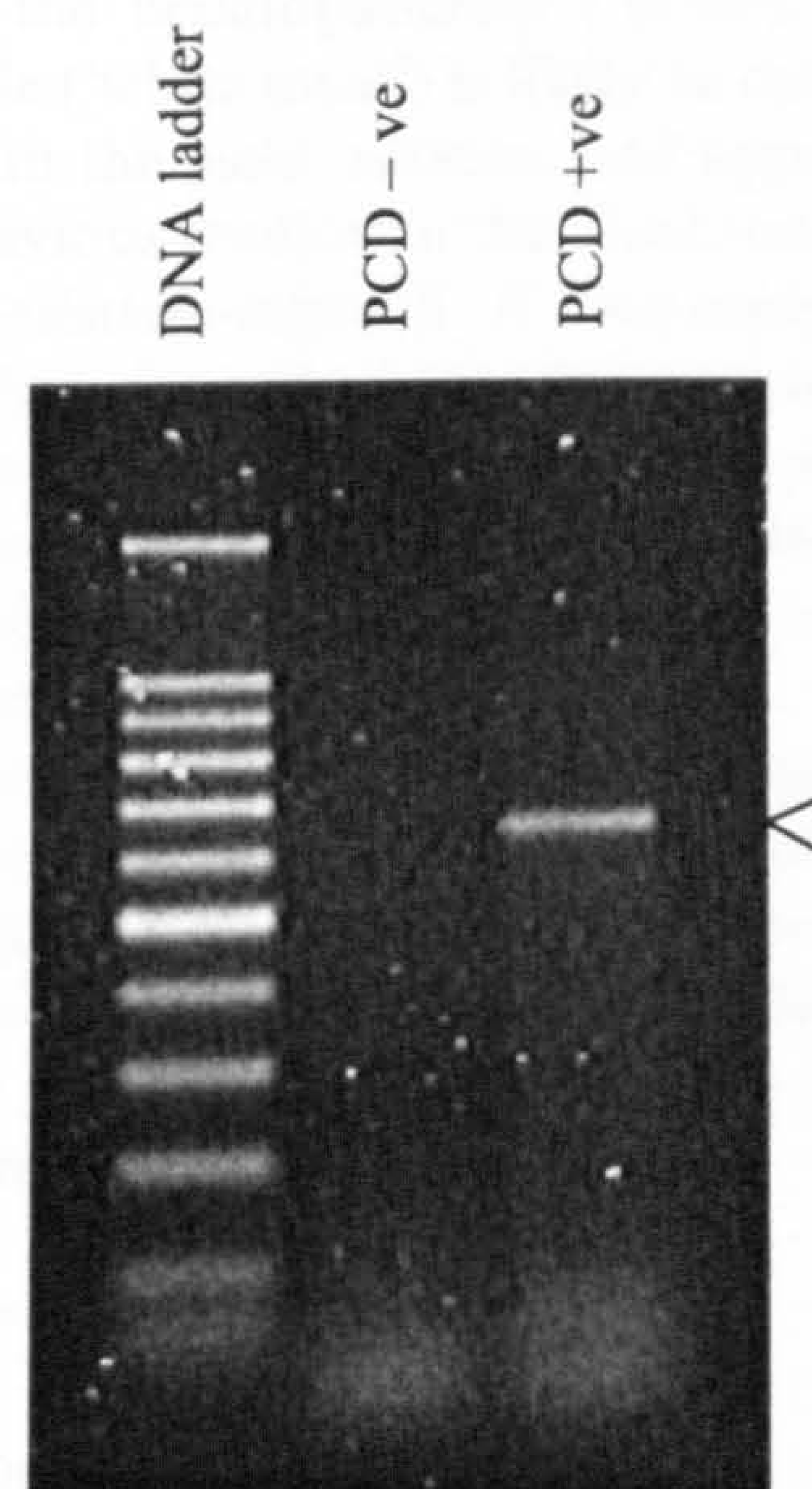


Fig. 13. Agarose gel showing 680 bp amplification product (arrowhead) from hepatopancreas of crab exhibiting the symptoms of PCD (PCD +ve). No amplification product was seen in uninfected crabs (PCD −ve). DNA marker also shown.

Muscle tissue, particularly that found within the claws, was almost completely destroyed in crabs with PCD, with 'islands' of apparently unattached muscle

tissue surrounded by masses of parasitic plasmodial cells. Granuloma-like foci of hyalinocytes were observed within the remaining blocks of claw muscle and also within the heart and surrounding the gut. Such foci have been described as aggregations of flattened hyaline cells encapsulating foreign material and which lead to the deposition of melanin either on the object or within the haemocyte matrix. In the case of parasite infection, the parasite is destroyed as the inner layers of these foci become necrotic (Smith and Söderhall, 1986). Such encapsulating lesions have previously been recorded in the gills and heart of *Hematodinium*-infected *N. norvegicus* (Field and Appleton, 1995; Field et al., 1992), their presence thought to indicate a previous microbial or parasitic infection. Whether these lesions relate to the original infection site of *Hematodinium* sp. has not been shown to date. Interestingly, these melanised encapsulation responses were rarely observed in the gill lamellae of crabs with PCD. As such, if these lesions do mark the infection route of *Hematodinium* sp., it is possible that this route differs between *C. pagurus* (via the gut) and *N. norvegicus* (via the gills). Further studies are required to elucidate the route of entry of this parasite to its respective hosts.

Ultrastructurally, the remaining intact muscle fibres showed an exaggerated separation of the sarcolemma from the contractile myofibrils at the fibre periphery. However, in contrast to the infection in *N. norvegicus*, where the Z-lines of the sarcomeres remain intact (Stentiford et al., 2000), the sarcomeres from the muscle of crabs with PCD showed severe disorganisation of filaments in the region of the Z-line. Loss of Z-line material is reported to occur in a number of pathological and physiological conditions (Kumudavalli Reddy et al., 1975) and apparently represents an early step in normal premoult atrophy in crustaceans (Mykles and Skinner, 1990a). The calcium-dependent proteases involved in premoult atrophy are localised in the sarcoplasm (Mykles and Skinner, 1990b) and it is conceivable that severe disruption of the muscle during PCD in *C. pagurus* may assist in the activation of these proteases. Alternatively, proteases of parasitic origin may be responsible for the differential breakdown of muscle within crab and lobster hosts. The reason for the difference in the breakdown characteristics of muscle in PCD and in *Hematodinium* infection of *N. norvegicus* warrants further investigation.

4.3. Potential commercial impact

PCD has the potential for considerable commercial impact at several levels. *Hematodinium* is ultimately fatal to its *N. norvegicus* host (Stentiford et al., 2001b), with seasons of high infection prevalence at a particular site being linked to reductions in landings per unit effort in the following season (Field et al., 1998). Additionally, *Hematodinium* infections of *C. bairdii* (Meyers et al., 1987), *C. opilio* (Taylor and Khan, 1995), *C. sapidus* (Messick and Shields, 2000; Shields and Squyars, 2000), and *N. puber* (Wilhelm and Mialhe, 1996) have been associated with large commercial losses. As little is known about the prevalence and seasonality of PCD in the field, no inference can be made as to its likely role as a mortality factor in the fishery. However, due to the severe pathology associated with PCD in *C. pagurus*, similar effects as those observed within the fisheries for *C. bairdii*, *C. opilio*, and *C. sapidus* may manifest themselves in populations of *C. pagurus* which harbour PCD. The current study has also shown that *Hematodinium* sp. infections are likely to disrupt the reproductive ability of infected crabs. Whilst the presence of infection per se may be expected to cause significant population effects (through increased natural mortality), at present, little is known about how sub-lethal levels of infection may impact upon the reproductive output of host species. Further research into the reproductive status of infected *C. pagurus*, coupled with monitoring of offshore and inshore sites and a retrospective analysis of landings data would facilitate study of the potential for such effects on commercial stocks.

In addition to their reduced survivability during holding and transportation, the quality and yield of meat from crabs infected with PCD is also of potential commercial significance. The severe pathology associated with the hepatopancreas ('brown meat') and the claw muscle ('white meat') is likely to cause considerable alteration in the yield, texture, and appearance of these tissues. Previous studies on the biochemical composition of *Hematodinium*-infected *N. norvegicus* tissues also suggests that disruptions in the normal carbohydrate and amino acid profiles of these tissues may be implicated in the 'bitter' taste of the meat that accompanies this and other *Hematodinium* infections (Meyers et al., 1987; Stentiford et al., 2001a,b). The cooking of *Hematodinium*-infected and uninfected tanner and snow crabs under batch conditions has been suggested to cause tainting of the whole batch (see Meyers et al., 1987). The batch preparation of uninfected *C. pagurus* with those infected with PCD may be expected to cause similar effects.

4.4. Future studies

The mode of transmission of *Hematodinium* infections in the field is the subject of some conjecture. However, a number of studies have suggested that there is a significant risk of spread through in-transit culling and disassembly of the catch at sea (see Hudson and Shields, 1994; Love et al., 1993; Taylor and Khan, 1995). Anecdotal evidence suggests that the potential for the spread of PCD via these practices, and others that involve the use of crabs as bait for the capture of other

species, is significant. This is noteworthy when considering the suggested route of entry (via the gut) for the parasite causing PCD. Knowledge gained via studies on the transmissibility of the *Hematodinium* species causing PCD may be applied to improve current commercial capture and holding practices, which may to some extent be facilitating transmission of this parasite in the field.

Acknowledgments

The authors thank Mr. Richard Lord and Mr. Clive Brown for the provision of samples of *Cancer pagurus* from Guernsey and to Professor Keith Vickerman for assistance in the interpretation of ultrastructural material. This work was supported by the Department of Environment, Food, and Rural Affairs (DEFRA) under Contract Number F1137.

References

Anderson, I.G., Nash, G., Shariff, M., 1990. Mass larval mortalities in giant freshwater prawn, *Macrobrachium rosenbergii* de Man, cultured in Malaysian modified static 'green water' systems. J. Fish Dis. 13, 127–134.

Appleton, P.L., Vickerman, K., 1997. In vitro cultivation and development cycle in culture of a parasitic dinoflagellate (*Hematodinium* sp). associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters. Parasitology 116, 115–130.

Arcier, J.-M., Herman, F., Lightner, D.V., Redman, R.M., Mari, J., Bonami, J.R., 1999. A viral disease associated with mortalities of hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii*. Dis. Aquat. Org. 38, 177–181.

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

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

Cawthorn, R.J., 1997. Overview of "bumper car" disease—impact on the north American lobster fishery. Int. J. Parasitol. 27, 167–172.

Chatton, E., Poisson, R., 1931. Sur l'existence, dans le sang des crabes, de Péridiniens parasites: *Hematodinium perezi* n.g., n.sp. (Syndinidae). C. R. Seances Soc. Biol., Paris 105, 553–557.

Chang, E.S., Chang, S.A., Keller, R., Reddy, P.S., Snyder, M.J., Spees, J.L., 1999. Quantification of stress in lobsters: crustacean hyperglycemic hormone, stress proteins and gene expression. Am. Zool. 39, 487–495.

Cheng, W., Chen, J.C., 1998. Isolation and characterization of an *Enterococcus*-like bacterium causing muscle necrosis and mortality in *Macrobrachium rosenbergii* in Taiwan. Dis. Aquat. Org. 34, 93–101.

Field, R.H., Appleton, P.L., 1995. A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops norvegicus*: observations on pathology and progression of infection. Dis. Aquat. Org. 22, 115–128.

Field, R.H., Appleton, P.L., 1996. An indirect fluorescent antibody technique for the diagnosis of *Hematodinium* sp. infection of the Norway lobster, *Nephrops norvegicus*. Dis. Aquat. Org. 24, 199–204.

Field, R.H., Chapman, C.J., Taylor, A.C., Neil, D.M., Vickerman, K., 1992. Infection of the Norway lobster *Nephrops norvegicus* by a *Hematodinium*-like species of dinoflagellate on the west coast of Scotland. Dis. Aquat. Org. 13, 1–15.

Field, R.H., Hills, J.M., Atkinson, R.J.A., Magill, S., Shanks, A.M., 1998. Distribution and seasonal prevalence of *Hematodinium* sp. infection of the Norway lobster *Nephrops norvegicus* around the west coast of Scotland. ICES J. Mar. Sci. 55, 846–858.

Hopwood, D., 1996. Fixation and fixatives. In: Bamcroft, J.D., Stevens, A. (Eds.), Theory and Practice of Histopathological Techniques, fourth ed. Churchill Livingstone, Hong Kong, pp. 23–46.

Hudson, D.A., Adlard, R.D., 1994. PCR-techniques applied to *Hematodinium* spp. and *Hematodinium*-like dinoflagellates in decapod crustaceans. Dis. Aquat. Org. 20, 203–206.

Hudson, D.A., Lester, R.J.G., 1994. A parasitological survey of the mud crab *Scylla serrata* (Forskal) from southern Moreton bay, Queensland, Australia. Aquaculture 120, 183–199.

Hudson, D.A., Shields, J.D., 1994. *Hematodinium australis* n.sp., a parasitic dinoflagellate of the sand crab *Portunus pelagicus* from Moreton bay, Australia. Dis. Aquat. Org. 19, 109–119.

Jussila, J., Jago, J., Tsuetnenko, E., Dunstan, B., Evans, L.H., 1997. Total and differential haemocyte counts in western rock lobsters (*Panulirus cygnus* George) under post-harvest stress. Mar. Freshwater Res. 48, 863–867.

Reddy, M., Etlinger, J.D., Rabinowitz, M., Fischman, D.A., 1975. Removal of Z-lines and α-actinin from isolated myofibrils by a calcium-activated neutral protease. J. Biol. Chem. 250, 4278–4284.

Latrouite, D., Morizur, Y., Noël, P., Chagot, D., Wilhelm, G., 1988. Mortalite du tourteau *Cancer pagurus* provoquee par le dinoflagelle parasite: *Hematodinium* sp. Conseil International pour l'Exploration de la Mer. CM. 1988/K: 32.

Lindqvist, O.V., Mikkola, H., 1978. On the etiology of the muscle wasting disease in *Procambarus clarkii* in Kenya. Freshwater Cray. 4, 363–372.

Love, D.C., Rice, S.D., Moles, D.A., Eaton, W.D., 1993. Seasonal prevalence and intensity of Bitter Crab dinoflagellate infection and host mortality in Alaskan Tanner crabs *Chionoecetes bairdi* from Auke bay, Alaska, USA. Dis. Aquat. Org. 15, 1–7.

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

Messick, G.A., 1994. *Hematodinium perezi* infections in adult and juvenile blue crabs *Callinectes sapidus* from coastal bays of Maryland and Virginia, USA. Dis. Aquat. Org. 19, 77–82.

Messick, G.A., Shields, J.D., 2000. Epizootiology of the parasitic dinoflagellate *Hematodinium* sp. in the American blue crab *Callinectes sapidus*. Dis. Aquat. Org. 43, 139–152.

Meyers, T.R., Koeneman, T.M., Botelho, C., Short, S., 1987. Bitter crab disease: a fatal dinoflagellate infection and marketing problem for Alaskan tanner crabs, *Chionoecetes bairdi*. Dis. Aquat. Org. 3, 195–216.

Morris, S., Airriess, C.N., 1998. Integration of physiological responses of crustaceans to environmental challenge. S. Afr. J. Zool. 33, 87–106.

Mykles, D.L., Skinner, D.M., 1990a. Calcium-dependent proteinases in crustaceans. In: Intracellular Calcium-dependent Proteolysis. CRC Press, Boca Raton, FL, pp. 139–154.

Mykles, D.L., Skinner, D.M., 1990b. Atrophy of crustacean somatic muscle and the proteinases that do the job. A review. J. Crust. Biol. 10, 577–594.

Newman, M.W., Johnson, C.A., 1975. A disease of blue crabs *Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp. J. Parasitol. 63, 554–557.

Paterson, B.D., Spanoghe, P.T., 1997. Stress indicators in marine decapod crustaceans, with particular reference to the grading of western rock lobsters (*Panulirus cygnus*) during commercial handling. Mar. Freshwater Res. 48, 829–834.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17, 208–212.

Shields, J.D., 1994. The parasitic dinoflagellates of marine crustaceans. Ann. Rev. Fish Dis. 4, 241–271.

Shields, J.D., Squyars, C.M., 2000. Mortality and hematology of blue crabs, *Callinectes sapidus*, experimentally infected with the parasitic dinoflagellate *Ematodinium perezi*. Fish. Bull. 98, 139–152.

Smith, V.J., Söderhall, K., 1986. Cellular immune mechanisms in the Crustacea. Sympos. Zool. Soc. London 56, 59–79.

Stentiford, G.D., Neil, D.M., 2000. A rapid onset, post-capture muscle necrosis in the Norway lobster, *Nephrops norvegicus* (L.) from the west coast of Scotland, United Kingdom. J. Fish Dis. 23, 251–264.

Stentiford, G.D., Chang, E.S., Chang, S.A., Neil, D.M., 2001a. Carbohydrate dynamics and the crustacean hyperglycaemic hormone (CHH): effects of parasitic infection in lobsters. Gen. Comp. Endocrinol. 121, 13–22.

Stentiford, G.D., Neil, D.M., Atkinson, R.J.A., 2001b. The relationship of *Hematodinium* infection prevalence in a Scottish *Nephrops norvegicus* population to seasonality, moulting and sex. ICES J. Mar. Sci. 58, 814–823.

Stentiford, G.D., Neil, D.M., Coombs, G.H., 1999. 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*). Dis. Aquat. Org. 38, 151–157.

Stentiford, G.D., Neil, D.M., Coombs, G.H., 2000. 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*. Dis. Aquat. Org. 42, 133–141.

Stentiford, G.D., Neil, D.M., Coombs, G.H., 2001c. Development and application of an immunoassay diagnostic technique for studying *Hematodinium* infections in *Nephrops norvegicus* populations. Dis. Aquat. Org., in press.

Stewart, J.E., 1980. Diseases. In: Cobb, J.S., Phillips, B.F. (Eds.), The Biology and Management of Lobsters. Academic Press, New York, pp. 301–342.

Taylor, D.M., Khan, R.A., 1995. Observations on the occurrence of *Hematodinium* sp. (Dinoflagellata: Syndinidae); the causative agent of Bitter crab disease in Newfoundland snow crab (*Chionoecetes opilio*). J. Invertebr. Pathol. 65, 283–288.

Thompson, S.N., 1983. Biochemical and physiological effects of metazoan endoparasites on their host species. Comp. Biochem. Physiol. 74B, 183–211.

UK Sea Fisheries Statistics, 1999. United Kingdom sea fisheries statistics 1999. Ministry of Agriculture, Fisheries and Food (MAFF). UK Government Statistical Service.

Whittington, D.M., Bernstein, K.L., Shields, J.D., 1997. Changes in aspects of the blood chemistry of blue crabs infected with *Hematodinium perezi*. J. Shell Res. 16, 357–358.

Wilhelm, G., Boulo, V., 1988. Infection de l'etrille *Liocarcinus puber* (L.) par un dinoflagelle parasite de type *Hematodinium*. ICES Committee Meeting CM.1988/K:32.

Wilhelm, G., Mialhe, E., 1996. Dinoflagellate infection associated with the decline of *Necora puber* crab populations in France. Dis. Aquat. Org. 26, 213–219.

GLASGOW
UNIVERSITY
LIBRARY