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INSTITIÚID TEICNEOLAÍOCHTA NA GAILLIMHE-MAIGH EO

Impacts of the parasitic dinoflagellate *Hematodinium* sp. on Irish crustacean fisheries.

By

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Degree of Doctor of Philosophy

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Submitted to Galway-Mayo Institute of Technology

May 2010

NDP NATIONAL DEVELOPMENT PLAN

BIM Bord Iascaigh Mhara Irish Sea Fisheries Board

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ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to my supervisor, Dr. Martin Robinson for his time and support throughout this process even from afar in the Shetlands. His guidance and direction have ensured that I have remained (mostly!) motivated until the end. Thank you also to my co-supervisor Dr. Pauline King for her assistance particularly at the final stressful stages.

I gratefully acknowledge Bord Iascaigh Mhara who funded this project under the National Development Plan 2000 – 2006 through the programme for Innovation and Sustainability in the Fisheries Sector.

I would like to give special thanks to Shelltechians Ed, Dave and Rebecca for their friendship and for all the laughs and support in the office over the years. To Róisín, Bláithín, Sonya, Dónal, Jenni and John I thank you all for creating such a warm, friendly environment in Shelltec. Thank you to Brendan for all the road trips over the years and thank you Mary for help with the early project. To Eugene, I would like to say thanks for all the advice (hunting, fishing and otherwise) and for being a great boss and friend during my time in GMIT. To Bernie, I'm glad you made it to GMIT when you did!

Without the fishermen and managers who provided crab samples or who facilitated me onboard their vessels over the years; Danny Farren, Mickey Farren, Thomas Miskella, Joe O'Regan, Gerry Sweeney and Charlie O'Donnell from the Malin Head Fisheries Co-op Ltd., this work would not have been possible.

I would like to express my appreciation to Professor Jim Wilson and Dr. Martyn Linnie from the Zoology Department in Trinity College Dublin for providing me with a much needed work space away from home during my write-up time in Dublin.

Thank you to everyone who kept me reminded me of life on the 'outside'; my friends both from home and from college and also my diving buddies. Thank you Helen & Diarmuid, for all the lovely Sunday dinners and for giving me a home away from home.

To Dónal, how you put up with me still amazes me but I express my love and gratitude that you still do and I thank you for your love, patience and support (and bribes!) these past few years. Grá mo chroí thú.

Agus tá buíochas ó chroí le glacadh agam do mo mhuintir Pádraic agus Geraldine. Murach an tacaíocht agus grá atá taispeáinte acu dom le linn na blianta fada seo ag foghlaim, ní bheadh an lá seo sroichte go brách.

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INTRODUCTION

The United Nations Food and Agriculture Organisation (FAO) define the term fishery as an activity leading to the harvesting of fish, a term which includes finfish, molluscs, crustaceans and any aquatic animal which is harvested. It may involve capture of wild fish or farming of fish through aquaculture. Globally, fish provides more than 1.5 billion people with almost 20 % of their average per capita intake of animal protein (FAO 2008). In 2006, approximately 77 % of world fish production was used for direct human consumption with live and fresh forms the most valuable and sought after while the remaining 23 % was primarily used for non food applications such as the manufacture of fish oil and fish meal (FAO 2008). While world aquaculture has continued to grow rapidly during the past half century, marine capture fisheries remain the largest contributors to world fish production. From a total of 144 million tonnes of fish produced in 2006, 57 % was produced by marine capture fisheries. Marine and inland water aquaculture represented around 36 %, while inland water capture fisheries accounted for the remaining 7 %. Employment in the primary production of fish either in the form of wild capture or aquaculture in 2006 was estimated at 43.5 million people worldwide (FAO 2008).

The decline in recent years, of traditionally dominant fish stocks, such as the Atlantic cod *Gadus morhua* for example, is thought to have contributed to a change in overall catch composition in some of the major fishing divisions such as the Northwest and Northeast Atlantic. Catch declines of demersal fishes have been accompanied by an increase in catches of molluscs, crustaceans and other demersal species formerly perceived as being low in value such as blue whiting *Micromesistius poutassou* (FAO 2005). Despite these declines, analysis of production by commodity (species groups) reveals that marine fishes

still contribute most to global catches. In 2006, 66 million tonnes of marine fishes were captured and over US $43 billion were generated in global exports fetching a price of $1.09 per kilogram (kg) (FAOstat). Global catches of marine crustaceans totalled 5.7 million tonnes in same year with crab and lobster catches at the highest level ever recorded. International exports of crustaceans in 2006 were valued at almost US $20 billion fetching a considerably higher price per kilogram than marine fishes at US $4.48 / kg. In fact, even when compared to diadromous fishes such as salmon and eels, crustaceans are the most valuable fishery commodity in terms of volume and value (FAOstat).

The Irish inshore fisheries sector is represented by demersal, pelagic, shellfish, salmon, and sea-angling fisheries. Shellfisheries in particular represent a vital component of the national inshore fishery. Over 80 % of the national fishing fleet rely on the year round availability of shellfish such as crustaceans and molluscs (Anon, 1999). Operating within 12 nautical miles of shore and comprising of over 1800 vessels, shellfisheries directly employ approximately 3,700 fishermen and support some 15, 000 dependants. The majority of these vessels are small boats typically less than 12 metres in length which operate within 6 nautical miles of the coast (Anon 1999). Management of shellfisheries is achieved through the implementation of a framework, a cooperative approach involving both state and industry which concentrates on 15 species and over 25 stocks of crustaceans and molluscs, excluding the Norwegian lobster *Nephrops norvegicus* which is a quota species and is assessed internationally by the International Council for the Exploration of the Sea (ICES) (Anon 2005).

The most important crustacean fisheries in Ireland, in terms of volume and value include those for brown crab and lobster. Approximately 18 thousand tonnes of crustaceans were landed in Irish ports in 2008 with a first sale value of nearly € 50 million. These included the brown crabs *Cancer pagurus*, velvet swimming crabs *Necora puber*, spider crabs *Maja brachydactela*, green crabs *Carcinus maenas*, European lobsters *Homarus gammarus*, crawfish *Palinurus eliphas*, shrimps *Palaemon serratus* and *Crangon crangon*, and Norwegian lobsters *Nephrops norvegicus* (pers com. S. Coughlan SFPA). Many of these are considered to be fully or over-exploited (Anon, 1999), and with the exception of *N. norvegicus*, almost all have no input or output control/quota in place. Seasonal peaks in unit value tend to occur at the Christmas and Easter festival periods, although oversupply from Ireland and/or other countries to continental markets, where the majority of the consumer base is located, can depress these significantly. Effort directed toward each species and stock annually and the resulting fishing mortality, is dictated by a wide variety of parameters including market factors for the target and other species (a high price for one may deflect effort from another), weather and/or changes in legislation (an example being the 'days at sea' restriction placed on the brown crab offshore fleet). As a number of the more important species are often captured together in 'mixed' fisheries where one is targeted directly but there is a significant, marketable quantity of another as by-catch, fishing pressure can be exerted on a number of stocks at one time by one gear unit.

Fishing pressure however is not the sole process responsible for fluctuations in populations of commercially exploited crustaceans. Their complex life histories mean both physical and biotic processes jointly regulate populations throughout their various life history stages. Stochastic forces such as oceanographic or meteorological events are believed to

regulate larval life histories while density-dependent processes like competition, predation or emigration act primarily on settled juveniles and adults (Roughgarden et al. 1988, Pile et al. 1996). Additional studies have highlighted the importance of infectious diseases as population regulators in many species of invertebrates (Anderson & May 1981, Mouritsen & Jensen 1997, Beldomenico & Begon 2010). Anderson & May (1979) in fact theorise that in natural communities, parasitic infections represent an equivalent or at least complimentary constraint to population growth as resource limitation or predators. On a global scale parasitic epizootics are widespread among invertebrates. Perhaps the most catastrophic epizootic of wild crustacean populations is Krebspest or crayfish plague in European crayfish *Astacus astacus* caused by a phycomycete fungus *Aphanomyces astaci*. It was first reported in Lombardy, Italy in the 1860s and later spread throughout Europe eradicating *A. astacus* populations across the continent in the following decades (Unestam & Weiss 1970, Johnson 1983). All species of European, Australasian and Asian crayfish are equally susceptible to the disease, Death from infection occurs within several days to weeks often preceded by paralysis of the abdomen (Uneston & Weiss 1970)

Emersion, handling, overcrowding, temperature and/or salinity changes all contribute to physiological stress responses during the capture, marketing and distribution of crustacean species and when stress levels exceed an animal's ability to regulate, the effect can be lethal (Paterson & Spanoghe 1997, Schmitt & Uglow 1997, Chang et al. 1999, Danford & Uglow 2001, Danford et al. 2002). In cultured crustaceans in particular, infections by pathogens have led to severe losses in recent years. Some of the most devastating diseases from captive rearing operations include gaffkaemia of homarid lobsters caused by a Gram-positive tetrad-forming bacteria *Aeroccocus viridans* var. *homari* (Johnson 1983), and viral

diseases of penaeid shrimp such as White Spot disease (WSD) (Chou et al. 1995, Mayo 2002, Munn 2006, Sánchez- Martínez et al. 2007), Yellowhead disease (YHD) (Chantanachookin et al. 1993, Nunan et al. 1998) and Taura syndrome (TS) (Lightner et al. 1995, Bonami et al. 1997). WSD prevalence is highly variable from less than 1 % in infected wild populations to up to 100 % in captive populations (OIE 2006) and can cause 100 % mortality within a few days (Munn 2006). Prior to 1995, the viruses responsible for WSD and YHD were limited in their geographical distribution to the Eastern Hemisphere but have since been reported in the USA (Nunan et al. 1998, Stentiford et al. 2009). In the USA Nunan et al. (1998) demonstrated that these exotic shrimp pathogens are not only detectable but also infectious in frozen shrimp imports from Asia. High stocking densities, poor water quality, frequent handling and inadequate nutrition can alter agent/host interrelations as they exist in nature, such that they become favourable to pathogens whilst simultaneously reducing a host's capacity for defence (Kinne 1990). This has led to a considerable amount of research effort being concentrated on such diseases, vastly increasing the available knowledge of crustacean disease pathology. Under the recent EC Council Directive 2006/88/EC adopted during 2008, YHD and TS are listed as exotic diseases due to their apparent absence from the European Union (EU) while WSD is currently listed as a 'non exotic' pathogen based on its reported occurrence in penaeid shrimp farms in the south of Europe (Stentiford et al. 2009). Under this directive, European Union member states cooperate with a central European Community Reference Laboratory (CRL) for crustacean diseases in a bid to improve bio-security across the European Union. Currently in Ireland, the Marine Institute is the designated national reference laboratory for crustacean diseases.

Dinoflagellates are considered to be one of the most important protistan pathogens of wild marine crustaceans (Meyers 1990). Dinoflagellates are a group of predominantly unicellular, eukaryotic flagellates which are significant primary producers in aquatic ecosystems, second only to diatoms. They are placed within the superphylum Alveolata along with apicomplexans and ciliates (Gajadhar et al. 1991). Despite their phylogenetic relationship, the nuclear organisation of dinoflagellates, apicomplexans and cilates is very different. Structure of the dinoflagellate nucleus and its division during asexual reproduction are highly unusual. They exhibit a unique form of mitosis (dinomitosis) in which the nuclear envelope remains intact during cell division and a 'spindle' of microtubules develops in channels that permeate the nucleus and coordinates the segregation of chromosomes (Rizzo 1987, Vickerman & Coombs 1999, Bhaud et al. 2000). Despite displaying typical eukaryotic cell organisation, the chromosomes of most dinoflagellates are permanently condensed throughout both interphase and mitosis and they also lack histones and nucleosomes. Another striking feature of dinoflagellate nuclei is the enormous amount of cellular DNA they contain (Rizzo 1987). The function of all this DNA remains unknown but it is unlikely that it codes for proteins in its entirety and may provide a structural role (Rizzo 2003). Genome replication and/or transcription are thought to involve only the peripheral DNA strands as the permanently condensed nature of the main chromosomal body is unlikely to be accessible to RNA polymerase (Rizzo 2003, Hackett et al. 2004). Yet another distinguishing feature of dinoflagellates is the presence of two dissimilar flagella, one of which is laterally directed and another which beats posteriorly. The former lies in a transverse channel on the cell's surface known as the cingulum. Its proximal edge is contracted by a striated strand while the distal edge has a ruffled or spiral shape due to the longer axoneme. Propulsion of the transverse flagellum accounts for both forward motion and turning. The longitudinal flagellum lies in the sulcus

and provides a steering function (Levandowsky & Kaneta 1987). Beating of the two flagella gives rise to a distinct spinning motion during swimming, from which the name dinoflagellate derives (Gr. *dinos* = 'whirling') (Munn 2004).

Dinoflagellates have been claimed by both botanists as algae and by zoologists as protozoans because many have cell walls, they can swim and some are photosynthetic while others are not. Approximately half of extant dinoflagellates are obligate heterotrophs, i.e. they lack plastids or pigments necessary for photosynthesis (Gaines & Elbrächter 1987). In some parasitic species photosynthesis can in fact supply up to half of the energy required for growth (Coats 1999). Parasitic dinoflagellates have gained attention in recent years as disease agents in marine fisheries. Approximately 150 of the 2000 extant species of dinoflagellates are parasitic (Coats 1999). Hosts are varied and include algae, annelids, ascidians, crustaceans, fish, molluscs, protozoans, rotifers and salps (Cachon & Cachon 1987, Shields 1994, Coats 1994). Parasitic dinoflagellates often lack the distinguishing features of their free-living counterparts such as pustules, sulcus, flagella and the cytopharyngeal funnel; however life history stages resembling free-living stages do occur in the form of dinospores (Chatton & Poisson 1931, Cachon & Cachon 1987). These spore stages along with their unique nuclear characteristics are what enabled early researchers to recognise the dinoflagellate nature of many parasites (Cachon & Cachon 1987). There are four orders of dinoflagellates which contain parasitic forms: the Phytodiniales, Gymnodiniales, Blastodiniales and Syndiniales; however only members of the latter two contain known parasites of crustaceans (Shields 1994). The majority of the Blastodiniales are ectoparasites with specific organelles modified for host attachment (Cachon & Cachon 1987), while members of the Syndiniales are strictly endoparasitic (Coats 1999).

Bitter Crab Disease (BCD) and Pink Crab Disease (PCD) are conditions associated with infections of the Syndiniid parasitic dinoflagellate *Hematodinium* sp. that have been shown to have a negative impact on crustacean fisheries globally. In the past twenty years, epizootics caused by *Hematodinium*-like organisms have been documented in commercially exploited stocks of the velvet swimming crab *Necora puber* in France (Wilhelm & Boulo 1988, Wilhelm & Mialhe 1996), Southeast Alaskan Tanner crabs *Chionoecetes bairdi* (Meyers et al. 1987, 1990, Eaton et al. 1991, Love et al. 1993), snow crabs *C. opilio* in Alaska and Newfoundland (Meyers et al. 1996, Pestal et al. 2003, Shields et al. 2005), blue crabs *Callinectes sapidus* on the eastern seaboard of the United States (Messick 1994, Messick & Shields 2000), brown crabs *Cancer pagurus* in France, Britain and Ireland (Latrouite et al. 1988, Stentiford et al. 2002, Ní Chualáin et al. 2009) and Norway lobsters *Nephrops norvegicus* in Scotland (Field et al. 1992, 1998, Field & Appleton 1995, Stentiford et al. 2001a). Most recently, *Hematodinium* sp. has been implicated as the causative agent of milky disease in cultured mud crabs *Scylla serrata* (Li et al. 2008), Chinese swimming crabs *Portunus trituberculatus* (Xu et al. 2007) and in ridgetail white prawns *Exopalaemon carinicauda* (Xu et al. 2010) in Southeastern China.

At present there are only two described species for *Hematodinium*. The type species *H. perezi* was found in only a few of several thousand shore and harbour crabs *Carcinus maenas* and *Liocarcinus depurator* from France (Chatton & Poisson 1931) and a second species *H. australis* was described from the sand crab *Portunus pelagicus*, from Australia (Hudson & Shields 1994). Taxonomy of the genus *Hematodinium* has been difficult because few morphological features exist that discriminate between species. Pathogenicity also varies between hosts and electron microscope studies of the type species have not been undertaken. It is only in recent years that attempts have been made to differentiate

between species of *Hematodinium* through molecular analysis of conserved and variable regions of the ribosomal RNA gene (Hudson & Adlard 1994, 1996, Gruebl et al. 2002, Small et al. 2006, 2007a, 2007b). Recently Small et al. (2007b) proposed that the same species of *Hematodinium* infects *Nephrops norvegicus, Cancer pagurus, Pagurus bernhardus* and *Chionoecetes opilio* based on sequence similarity of the first internal transcribed spacer region (ITS1) of the ribosomal RNA complex. Similarly, Xu et al. (2010) suggest that *Scylla serrata*, *Portunus trituberculatus* and *Exopalaemon carinicauda* are all infected with the same *Hematodinium* sp. which is closely related to the *Hematodinium* sp. identified from the blue crab *Callinectes sapidus.* However, it appears that even the same species of *Hematodinium* produce varying pathologies in different host species. For example the degenerative effects of *Hematodinium* sp. on the claw muscle appears to vary between infected *N. norvegicus* and *C. pagurus,* (Stentiford et al. 2000, Stentiford et al. 2002) but whether this can be attributed to higher parasite virulence or host susceptibility remains unknown (Small et al. 2007b).

Growth of the parasite occurs within the haemolymph of the host where cells of *Hematodinium* sp. rapidly divide via schizogony to produce vast numbers of non-motile uninucleate trophonts and multinucleate plasmodia. Trophic stages do not contain chloroplasts, nutrition is obtained solely through osmotrophy. Presence of lipid and polysaccharide inclusions suggest active feeding at the expense of the host (Cachon & Cachon 1987, Stentiford & Shields 2005). Stentiford et al. (2000) described a depletion of glycogen reserves by approximately 85% in the deep abdominal flexor muscles of Norway lobsters *Nephrops norvegicus* infected with *Hematodinium* sp., suggesting that the parasites either act as a carbohydrate sink by consuming haemolymph glucose or they inhibit glycogen synthesis in the host. Shields et al. (2003) reported similar reductions in

the glycogen content of the hepatopancreas of heavily infected blue crabs *Callinectes sapidus* with female and male crabs showing 50% and 70% reductions respectively. Glycogen depletion in patent infections of *Hematodinium* sp. is thought to be a contributing factor in the altered colouration of affected hosts as glycogen is required for the synthesis of chitin (Stentiford & Shields 2005).

Heavily infected animals are lethargic and die more readily from handling stress than their uninfected counterparts (Meyers et al. 1987). The overwhelming number of proliferating parasite cells within the haemolymph places a huge metabolic load on the infected hosts which subsequently become lethargic and eventually die (Stentiford et al. 2000, 2001b, Shields et al. 2003, Stentiford & Shields 2005). Respiratory dysfunction associated with *Hematodinium* sp. infections has been reported in both crab and lobster species (Field et al. 1992, Field & Appleton 1995, Taylor et al. 1996, Sheppard et al. 2003). Oxygen consumption has been shown to increase in infected lobsters indicating the high oxygen demand of the proliferating parasite cells. Despite this increased demand, the oxygen carrying capacity of the haemolymph of infected animals is reduced by approximately 50%. Gill filaments of *Nephrops norvegicus* have been shown to become clogged by large numbers of parasite cells in the haemolymph (Field & Appleton 1995).

Dinospore stages of *Hematodinium* spp. are thought to be the infective agents responsible for the dispersal of the species; they precede a vegetative, growth stage involving trophonts and plasmodia which are followed by a sporogenic reproductive phase that generates large numbers of dinospores (Coats 1999). The motile *Hematodinium* sp. dinospore from Tanner crabs *Chinoecetes bairdi* is biflagellate and comes in two forms; a large slow moving

macrospore and a smaller fast moving microspore (Meyers et al. 1987, Eaton et al. 1991). Generally only one spore type is produced within the same crab, although both spore types have been observed in individual Tanner crabs *C. bairdi* (Love et al. 1993). Once thought to represent anisogametes (Meyers et al. 1987), this has since been discounted as both spore types have been shown to be infectious when injected into naïve *C. bairdi.* Equally, both spore types from infected *Nephrops norvegicus* are capable of initiating a developmental cycle *in vitro* (Appleton & Vickerman 1998). As the macrospores, microspores and trophonts of *Hematodinium* sp. from Tanner crabs contain approximately the same concentration of DNA per nucleus it suggests that the ploidy of all three forms is similar (Love et al. 1991, Shields 1994). The functional differences between the spore types have not been determined and as yet their fate after sporulation remains unknown.

The exact mode of transmission of *Hematodinium* sp. to new susceptible hosts is still unknown. Studies have shown that injection of infected haemolymph can act as a successful innoculant for uninfected *Chionoecetes bairdi* (Meyers et al. 1987), *Chinoecetes opilio* (Shields et al. 2005), *Callinectes sapidus* (Shields & Squyars 2000) and for the sand crab *Portunus pelagicus* (Hudson & Shields 1994). Direct injection demonstrates host susceptibility to a pathogen and may also indicate a natural transmission pathway in the case of wounding or limb loss. Cannibalism has also been suggested as a possible mode of transmission given that many crustacean species actively feed on conspecifics. Moulting has been suggested as a major predisposing factor for *Hematodinium* sp. infections in many host species, with higher infection prevalence observed in soft-shelled post-moult animals (Meyers et al. 1987, 1990, Eaton et al. 1991, Field et al. 1992, 1998, Messick 1994, Dawe 2002, Shields et al. 2005, 2007). Messick (1994) and Eaton et al. (1991) suggest that compromised host defence mechanisms during ecdysis render new shell crabs

more susceptible to invading organisms while Dawe (2002) suggests that motile stages from the sediment infiltrate the soft cuticle.

The most distinguishing features of BCD & PCD, particularly in late stage infections, are the altered colouration of the host's carapace and lethargy. Parasitised *Chionoecetes opilio* and *C. bairdi* develop a cooked appearance (Meyers et al. 1987, 1990, 1996, Eaton et al. 1991, Love et al. 1993, Taylor & Khan 1995, Pestal 2003), the carapace of *Nephrops norvegicus* takes on a dull orange colouration and opacity (Field et al. 1992, Tarnlund 2000, Stentiford et al. 2001a, Briggs & McAliskey 2002) while *Cancer pagurus* develops a pink hyperpigmentation of the carapace (Latrouite et al. 1988, Stentiford et al. 2002, Ní Chualáin et al. 2009). No obvious external signs of the disease have been observed in the American blue crab *Callinectes sapidus* apart from the occasional pink carapace (Messick 1994) or in the velvet swimming crab *Necora puber* (Wilhelm & Mialhe 1996). It is the progressive degradation of internal tissues that gives rise to the term BCD. Muscle, the main commercial product extracted from crustaceans, develops a watery, chalky texture and bitter flavour in snow and Tanner crabs. Affected muscle in other commercial crustacean species are also rendered unmarketable leading to severe losses within affected fisheries worldwide (Meyers et al. 1987, Eaton et al. 1991, Field et al. 1992, Taylor and Khan 1995, Stentiford et al. 2002, Shields et al. 2005, 2007, Ní Chualáin et al. 2009).

Prior to the discovery of significant quantities of pink, lethargic/dead crabs in commercial catches of brown crabs *Cancer pagurus* in the Southwest of Ireland in 2004 (Tully et al. 2006), there were no previous records of pink crab disease in Irish crustacean fisheries. Despite numerous interviews with crab fishermen from all over Ireland, only one from the

southwest recalled the 'pink crab' phenomenon in the preceding 2 - 3 years (J. Regan pers. comm.). However, by 2005, the occurrence of pink, dead or dying crabs was widespread in Ireland's brown crab fisheries, with a positive diagnosis also occurring in velvet crab *Necora puber* from the south coast. The distinguishing pink colouration and the presence of amoeboid trophonts and plasmodia within the haemolymph of affected crabs revealed the dinoflagellate *Hematodinium* sp. as the causative agent of pink crab disease (Ní Chualáin et al. 2009). This raised concern for all of the commercially important crustacean stocks around the coast of Ireland in terms of population health, economic losses and subsequent impacts on local fishing communities where few other sources of income often exist. Initial management advice could only draw from the limited information pertaining to *Hematodinium* sp. management in Alaskan Tanner crabs *Chionoecetes bairdi* (Meyers et al. 1987) which advised the landfill of patently infected specimens in an attempt to reduce the prevalence of *Hematodinium* sp. Considering the wide range of biological, physiological, environmental and population differences between the two species, the need for a more geographically relevant response was identified for *Cancer pagurus* in Ireland.

The overall objective of this project was to investigate the recent occurrence and proliferation of the parasitic dinoflagellate *Hematodinium* sp. in crustacean fisheries throughout Ireland and to assess its potential biological and economic impacts in order to provide the most appropriate epizootiological data to inform management of affected fisheries. There were four main sub-objectives in this project.

The first task was to identify which commercially important crustacean species are infected with *Hematodinium* sp. and to examine temporal and spatial differences in prevalence and infection intensity. Brown crabs *Cancer pagurus*, European lobsters *Homarus gammarus*,

velvet crabs *Necora puber* and spider crabs *Maja brachydactela* were examined for the presence of *Hematodinium* sp. The results are discussed in Chapter 1.

The second task involved investigating the effects of biotic and abiotic factors on *Hematodinium* sp. infection prevalence in the brown crab *Cancer pagurus*. Stratified samples were obtained from four distinct substrate types where seabed characteristics had been identified by validated acoustic habitat mapping. The ultimate goal was to determine if and at what scale sampling data were likely to be suitable in attempting to estimate the impact of the disease. The results are discussed in Chapter 2

The third task was to determine the most suitable diagnostic method for continued monitoring of *Hematodinium* sp. in Irish brown crab fisheries. The following three diagnostic methods were assessed: stained haemolymph smears, histological sections of gill, heart, midgut, hepatopancreas, muscle and gonad, and a polymerase chain reaction (PCR) assay using genus specific primers for *Hematodinium* sp. Results are compared and discussed in Chapter 3.

The fourth task in the present study was to to explore possible infection routes of *Hematodinium* sp. in *Cancer pagurus* and to monitor disease progression in infected crabs. Cannibalism, cohabitation with infected crabs and fluctuating temperatures, conditions typical of crab storage and transportation, were examined to see if they contributed to *Hematodinium* sp. transmission in healthy *Cancer pagurus* or if such conditions were

capable of exacerbating the disease in crabs already infected with *Hematodinium* sp. The results are discussed in Chapter 4.

CHAPTER 1

DISTRIBUTION AND SEASONALITY OF *HEMATODINIUM* SP. IN IRISH CRUSTACEAN FISHERIES

ABSTRACT

Infection of *Cancer pagurus* by a parasitic dinoflagellate of the genus *Hematodinium* is described for the first time in Ireland. An industry-based monitoring programme was established to determine seasonality of infection intensity and prevalence in the country's three largest brown crab *Cancer pagurus* fisheries in the southwest, north and southeast. Velvet crabs *Necora puber*, European lobsters *Homarus gammarus* and spider crabs *Maja brachydactela* were also screened for *Hematodinium* sp. Infections were scarce in *N. puber* and absent in both *H. gammarus* and *M. brachydactela* samples. In relation to *C. pagurus*, the parasite was present in all areas for the majority of sampling periods, with highest prevalences recorded in pre-recruit animals of both sexes. Microscopic examination of haemolymph revealed trophont, plasmodial and dinospore stages of the parasite. Overall prevalence in males (16 %) was higher than females (9 %). Prevalence of *Hematodinium* sp. infection ranged from 0 - 51 % but a distinct seasonal trend was not apparent. Infection intensity was seasonal with significantly higher peaks occurring in late autumn/early winter months than in other quarters, which corresponded with industry reporting moribund and dead pink-shelled crabs in commercial catches. It is postulated that seawater temperature or a temperature linked process is a key factor in the triggering the final stages of infection as significant autumn peaks were followed by a reduction in infection intensity as temperature decreased in the late winter/early spring months with no increase in intensity again until the following autumn. It is proposed that infection intensity rather than

prevalence provides a more appropriate indication of the period when there is greatest potential for biological and economic impacts and discuss the parameter's application as a fisheries management tool.

INTRODUCTION

Parasitic dinoflagellates of the genus *Hematodinium* have been shown to have negative impacts on a number of commercial crustacean fisheries globally (for review Stentiford & Shields 2005). Pink Crab Disease (PCD) is a condition associated with infections of *Hematodinium* sp. in the brown crab *Cancer pagurus* (Latrouite et al. 1988, Stentiford et al. 2002). Glycogen depletion in severe infections is thought to cause the pinkish lightening in the appearance of the shell (Stentiford & Shields 2005) and is accompanied by progressive degradation of internal tissues eventually leading to host death (Latrouite et al. 1988, Stentiford et al. 2002). In other host species such as Tanner crabs *Chionoecetes bairdi* and snow crabs *Chionoecetes opilio* meat develops a chalky, unpalatable texture giving rise to the term Bitter Crab Disease (Meyers et al. 1987, 1990, Taylor & Khan 1995). Data from France (Latrouite et al. 1988) and Ireland (M. Robinson, unpublished data) also suggest that the visual signs of tissue degradation observed in pink-shelled, infected brown crabs are associated with a negative alteration in texture and taste. There is currently concern as to the impacts *Hematodinium* sp. may have on stock health in Ireland and on consumer confidence as it is unknown at what stage pathological alterations in tissues occur that are destined for human consumption. Shell discolouration, tissue degradation and altered taste can all result in consumer dissatisfaction and reduced product value.

The majority of pot fishing vessels in Ireland are partially or fully reliant on income generated from landing brown crabs *Cancer pagurus*. Economic viability of the Irish offshore vivier fleet is entirely dependent on its ability to exploit the species year round. The majority of landings are exported live to continental European markets. Approximately 9000 t of brown crabs were landed in Ireland in 2006 with a first sale value estimated at €10 million (source SFPA). *Hematodinium* sp. infections in Irish brown crab fisheries were first confirmed in 2004. Reports of the occurrence of pink, dead crabs in fishing pots in the south were similar to those attributed to *Hematodinium* sp. infections by Latrouite et al. (1988) and Stentiford et al. (2002) previously. An industry focused monitoring programme was established in Ireland to gauge infection prevalence and seasonality with the aim of developing management measures to limit the biological and economic impact of disease outbreaks. This report presents data from this programme and discusses findings in relation to disease monitoring and management.

METHODS

Seasonal samples were obtained from three geographic locations that together yield > 95 % of annual national landings for *Cancer pagurus*. Crabs were captured in baited pots during commercial fishing activity in the north, southwest and southeast of the country from November 2004* to December 2007. Sex, carapace width (CW) and macroscopic signs of PCD (moribund, pink hyperpigmented carapace, milky coloured haemolymph) were noted. Commercial fishers were requested to bring in crabs from a sample of initial catch prior to grading so that marketable and unmarketable crabs were sampled. The latter group included sub-legal minimum landing size (< 130 mm CW), soft-shelled and damaged crabs. Adherence to this request was verified periodically by onboard observers. Fishermen

were asked to report the occurrence of pink crabs in catches at times when sampling was not taking place. *Necora puber, Homarus gammarus* and *Maja brachydactela* samples were obtained from fishermen and cooperatives around Ireland.

Haemolymph was extracted aseptically from the arthrodial membrane at the junction of the basis and ischium of one of the walking legs from each species while onboard ship or onshore immediately after landing to port. Samples were immediately fixed in chilled 4 % neutral-buffered formalsaline. Haemolymph smears were prepared by air drying the fixed haemolymph on a slide and staining with Giemsa (Humason 1979) so that infection intensity and parasite morphology could be determined. Slides were examined using an Olympus BX41 compound microscope and were defined as positive for infection when at least one clearly identifiable *Hematodinium* sp. cell of any stage (i.e. trophont, plasmodia, dinospores) was observed. Identification of the different forms was based principally on the descriptions made by Appleton & Vickerman (1998) with cross-reference to Latrouite et al. (1988) and Stentiford et al. (2002). Once a slide was defined as positive, a total of 300 cells comprising of parasite cells and haemocytes, was counted from 5 fields of view. The percentage of *Hematodinium* sp. cells in those 300 cells defined the infection intensity of that slide. For each sampling occasion prevalence was expressed as a percentage by dividing the number of crabs infected with *Hematodinium* sp. by the number of crabs examined (Bush et al. 1997). Infection intensities were calculated for all forms of the parasite observed. Mean intensity for a sampling period was the sum of the intensities of infected crabs divided by the number of infected crabs (Sheppard et al. 2003). Mean percentages of plasmodia in infected smears were compared with mean monthly seawater temperatures provided by the Irish Marine Institute from data buoys near each of the three

sampling areas. Monthly mean infection intensities recorded from sampling points in each quarter were plotted against the same temperature data.

*Samples prior to November 2005 were not collected by the author but have been used and analysed by the author with the permission of M.Robinson. Data have not been used within a formal publication other than with the author and have not appeared in any other thesis.

RESULTS

From November 2004 until December 2007, haemolymph from 4422 *Cancer pagurus* (Table 1.1), 1021 *Necora puber*, 650 *Homarus gammarus* and 306 *Maja brachydactela* (Table 1.2) was examined for the presence of *Hematodinium* sp. Infections were widespread in *C. pagurus,* rare in *N. puber* (2/1021) and undetected in *H. gammarus* and *M. brachydactela*. The following results pertain to the brown crab host *C. pagurus.* Samples comprised 1424, 1714 and 1284 crabs from the southwest, north and southeast of Ireland respectively ranging in size between 75 mm – 219 mm CW, (Figure 1.1). Size distributions of crabs were not significantly different (ANOVA, $p > 0.5$) between locations and a F:M sex ratio of 3:1 was observed, this being representative of commercial catches in Ireland. *Hematodinium* sp. infections were confirmed microscopically in brown crabs from all three locations, (Figure 1.1), but < 1 % of individuals in the study showed external signs of PCD. When samples from all areas were pooled, highest prevalence was apparent in small size classes for both sexes and decreased gradually with increasing size, (Figure 1.2). Overall 9 % and 16 % of females and males respectively were infected when samples were pooled. Trophonts were spherical and had a high nuclear cytoplasmic ratio; chromatin

varied between uncondensed and distinctly condensed. Plasmodia contained two or more nuclei with chromatin often distinctly condensed and appeared in round clump forms. Most positive infections were characterised by trophonts with the host appearing healthy when examined externally. When clinical signs of infection were observed plasmodia were extremely numerous within the haemolymph. Pyriform dinospores were observed in one individual in each of December of 2006 and 2007. Industry reports of dead or dying pink-shelled crabs in fishing pots were received between November and December from the southwest in 2004 to 2007, the north in 2005 to 2007 and the southeast in 2006.

Table 1.1. *Hematodinium* sp. prevalence (% infected) and mean intensity (%) in *Cancer pagurus* from 3 regions in Ireland.

Region	Date	Total Female	% Infected	Total Male	% Infected	Total n	% Infected	Mean Intensity
Southwest	Nov-04	46	13	5	20	51	14	39
	Jun-05	45	11	5	40	50	14	8
	Aug-05	47	0	3	0	50	0	-
	Oct-05	56	39	13	77	69	46	63
	Nov-05	72	51	62	50	134	51	40
	May-06	149	7	123	16	272	11	9
	Jul-06	69	6	31	10	100	7	4
	Oct-06	74	8	26	4	100	7	17
	Nov-06	82	4	18	6	100	4	46
	Mar-07	27	0	73	7	100	5	6
	May-07	70	17	25	52	95	29	7
	Jun-07	70	1	31	6	101	3	6
	Aug-07	77	3	22	9	99	4	14
	Oct-07	93	8	5	0	98	7	74
North	Nov-04	48	0	0	-	48	0	-
	Apr-05	59	12	0	-	59	12	11
	May-05	50	24	0	-	50	24	7
	Aug-05	50	0	0	-	50	0	-
	Nov-05	85	32	55	7	140	23	40
	Feb-06	82	4	46	0	128	2	9
	Mar-06	50	10	0	-	50	10	7
	May-06	54	13	10	10	64	13	11
	Jun-06	74	3	26	12	100	5	3
	Jul-06	38	0	9	11	47	2	25
	Aug-06	81	5	19	11	100	6	7
	Oct-06	81	6	19	26	100	10	23
	Dec-06	62	3	38	13	100	7	80
	Feb-07	80	4	20	0	100	3	7
	May-07	75	11	25	0	100	8	4
	Aug-07	86	3	12	42	98	8	11
	Sep-07	107	14	72	19	179	16	10
	Oct-07	80	8	17	18	97	9	20
	Dec-07	46	17	54	13	100	15	52
Southeast	Nov-04	178	6	36	3	214	6	64
	Dec-04	50	4	0	-	50	4	45
	Mar-05	25	24	22	5	47	15	6
	Jun-05	57	5	30	7	87	6	2
	Aug-05	58	0	2	0	60	0	-
	Nov-05	50	12	0	-	50	12	8
	Apr-06	136	13	129	19	265	15	11
	Jul-06	59	2	12	25	71	6	7
	Sep-06	59	3	14	14	73	5	7
	Mar-07	84	6	73	15	157	10	11
	May-07	44	14	12	33	56	18	8
	Aug-07	68	9	7	14	75	9	4
	Oct-07	74	8	4	25	78	9	47

Table 1.2. Prevalence and infection intensity of *Hematodinium* sp. infections in *Necora puber*, *Homarus gammarus* and *Maja brachydactela* from different regions around Ireland.

Species	Region	Month	Total	% Infected	Infection Intensity (%)
N. puber	West	Dec-05	50	0	-
		Jan-06	99	0	-
		Apr-06	44	0	-
		May-06	49	0	-
		Jul-06	100	0	-
		Sep-06	50	0	-
		Nov-06	50	0	-
		Dec-06	50	0	-
		Jan-07	51	0	-
		Feb-07	50	0	-
	North	May-06	75	0	-
		Jun-06	50	2	1
		Aug-06	75	0	-
		Oct-06	50	0	-
		Dec-06	48	0	-
		Aug-07	50	0	-
	East	Jul-06	50	0	-
	Southeast	Jul-06	30	3	4
H. gammarus	North	May-06	50	0	-
		Jun-06	100	0	-
		Aug-06	50	0	-
		Oct-06	50	0	-
		Feb-07	50	0	-
	West	May-06	250	0	-
		Sep-06	100	0	-
M. brachydactela	Southwest	Jun-06	100	0	-
		May-07	56	0	-
		Jun-07	50	0	-
		Aug-07	50	0	-
		Oct-07	50	0	-

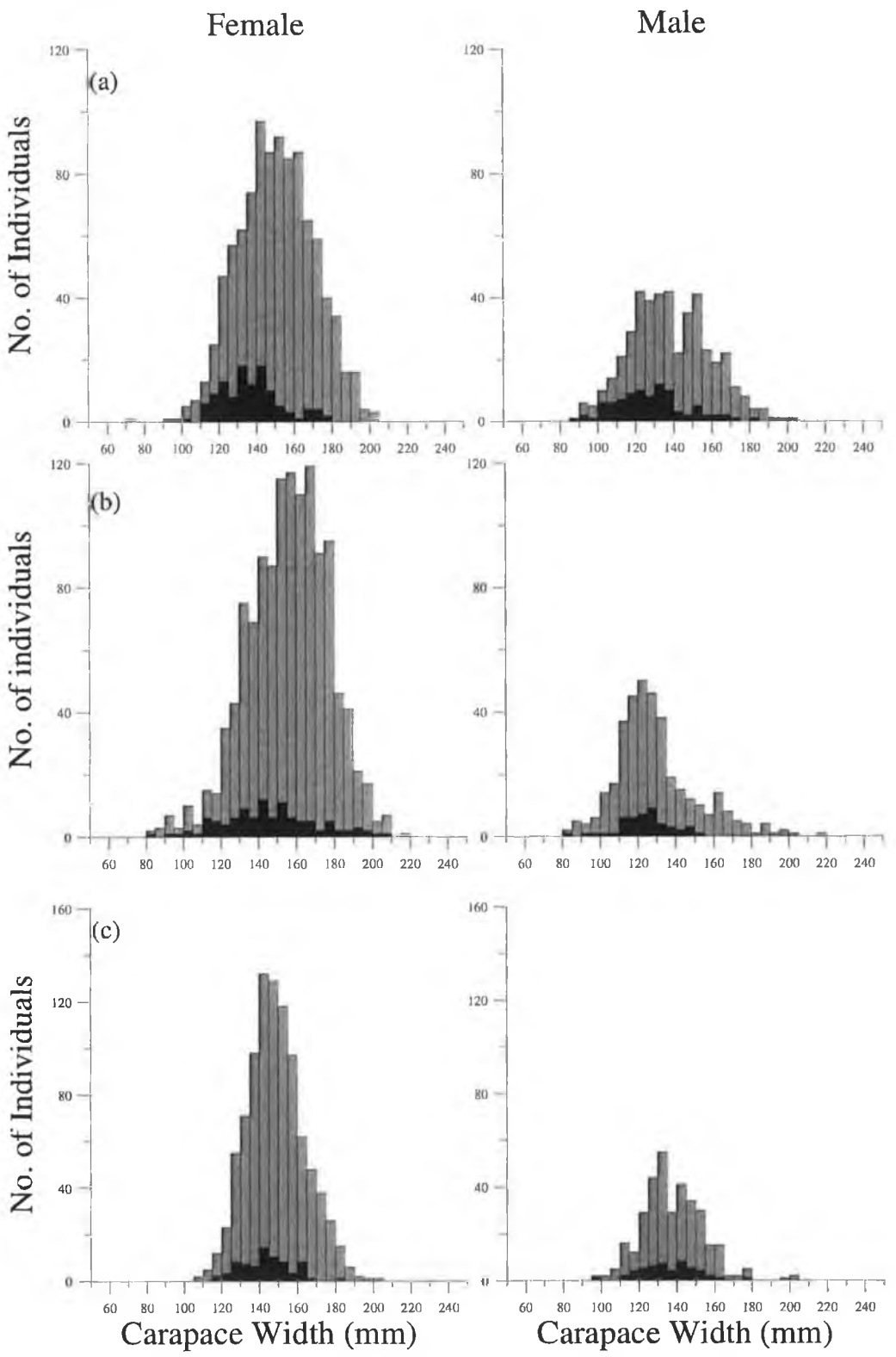


Figure 1.1. Sex-specific size-frequency distribution of *Hematodinium* sp. -infected (black bars) and non-infected (grey bars) from (a) southwest, (b) north and (c) southeast Ireland. Note scale change on y-axis of (c)

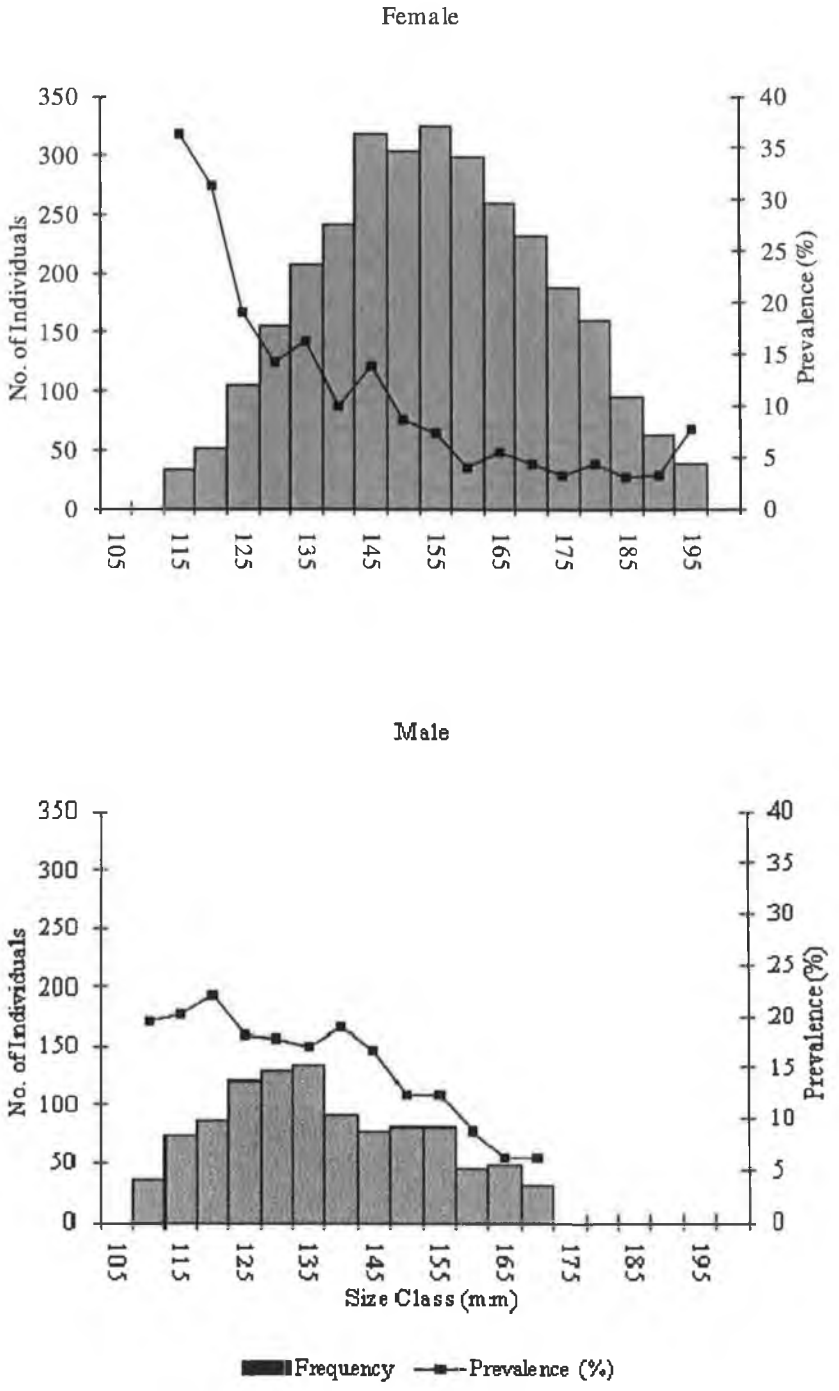


Figure 1.2. Sex-specific size-frequency distribution and prevalence of *Hematodinium* sp. infection for all locations pooled. Only size classes containing > 30 individuals are included

Prevalence ranged from 0 - 51 %, 0 - 24 % and 0 - 19 % for the southwest, north and southeast respectively, (Figure 1.3). The southwest experienced highest prevalence in 2005 reaching 51 % in the winter, over twice that of the north (23 %) and over four times that of the southeast (12 %) for the same period (Table 1.). In 2006, prevalence peaked at 15 % in the southeast, 13 % in the north and 11 % in the southwest in the late spring/early summer. Prevalence had increased to 29 % in the southwest in 2007, 16 % in the north and 17 % in the southeast in the summer/autumn months. *Hematodinium* sp. infections were not encountered in any area during August 2005 and no infections were encountered in November 2004 in the north.

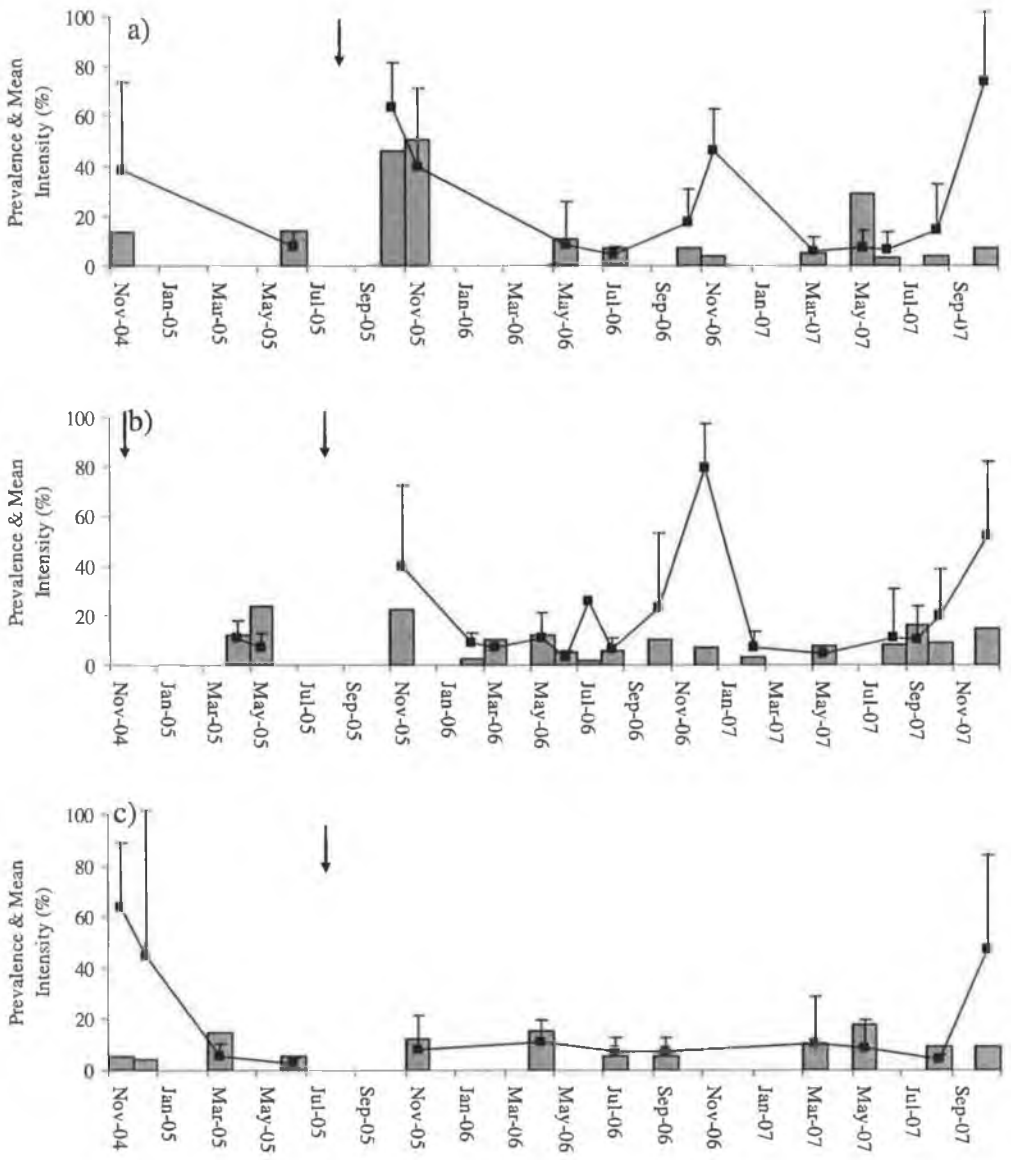


Figure 1.3. Seasonal variation in prevalence (bars) and mean intensity (lines) (+SD) of *Hematodinium* sp. infection from a) southwest, b) north and c) southeast Ireland. Male and female samples pooled. Only one side of the standard deviation is shown for clarity. Arrows indicate sampling when *Hematodinium* was not encountered.

Monthly mean seawater temperatures ranged between 8.8 – 16.8 °C and were identical for the three geographic sampling locations. Infection intensities of *Hematodinium* sp. recorded within quarter (Q) 4 were significantly higher than those in the other three sampling quarters ('Mood's median', $p < 0.001$), while Q2 was significantly lower than Q1 ($p < 0.05$), (Figure 1.4). Significantly high infection intensities observed during Q4 each year corresponded with moderate and decreasing seawater temperatures (Figure 1.5). The mean percentage of plasmodia within infected individuals peaked in the Q4 cooling autumn phase each year (Figure 1.5).

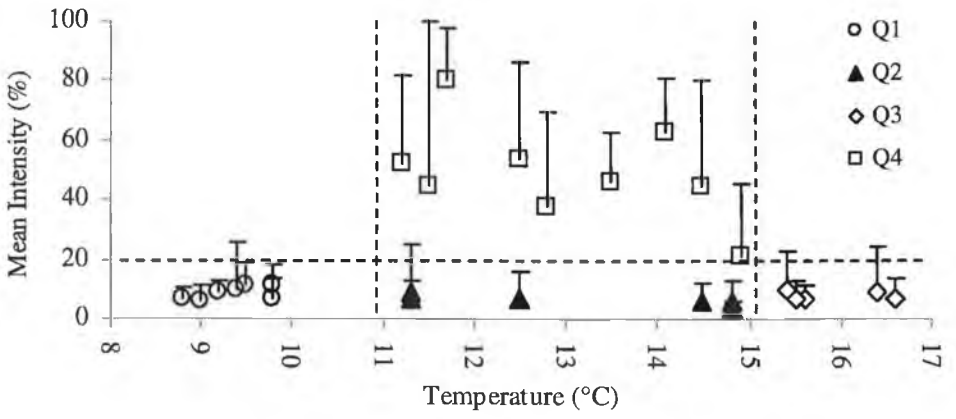


Figure 1.4. Mean intensity (+SD) of *Hematodinium* sp. infection in relation to seawater temperature. Only one side of the standard deviation is shown for clarity. Samples are grouped by quarters of the year (Q1: January to April; Q2: May to June; Q3: July to September; Q4: October to December)

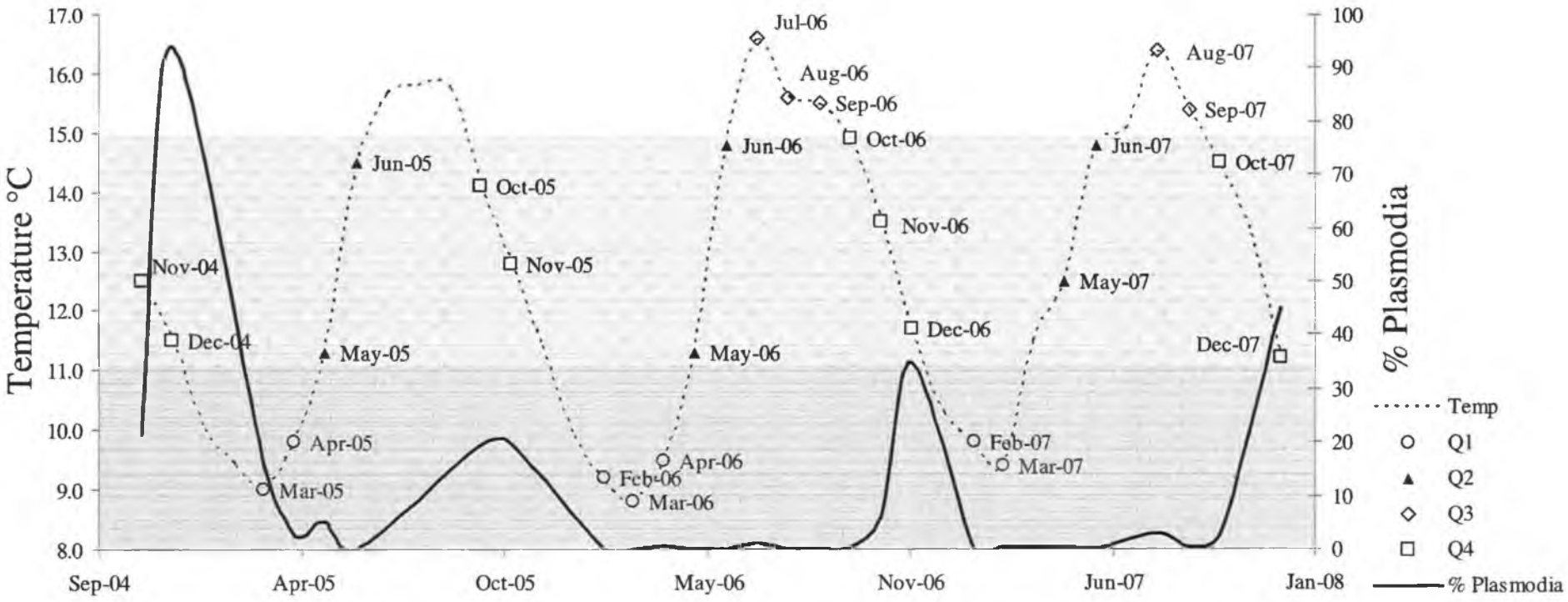


Figure 1.5. *Cancer pagurus*. Seawater temperature profile from November 2004 – December 2007 and mean percentage of plasmodial cells present in *Hematodinium* sp. - infected individuals. Sampling dates are indicated by month and year and grouped by quarter (Q1-Q4). Associated errors are not shown here for clarity as data is intended to act as an indicator of seasonal disease activity

Infection intensity was examined in relation to different parasite morphologies. Of the 479 infected crabs, 311 were found to be infected with only the uninucleate trophont stage while 166 contained varying amounts of plasmodia, with or without trophonts. Infection intensity was 96 and 85 % for 2 individuals containing dinospores but due to low sample numbers these were not compared to other stages. To ensure independence and homogeneity for statistical analysis, 166 infection intensity values were chosen at random from the 311 trophont-only infected animals and were tested against the 166 plasmodial infections. Analysis of variance showed that animals infected with plasmodia had a higher infection intensity than animals infected with only trophonts (ANOVA, $p < 0.001$). Infection intensity was significantly higher in females than males (ANOVA, $p < 0.05$).

DISCUSSION

This is the first report of *Hematodinium* sp. in Irish *Cancer pagurus* fisheries. Prior to 2004 the parasite had been either absent or undetected. As with a number of other important European commercial fisheries (Latrouite et al. 1988, Wilhelm & Boulo 1988, Field et al. 1992, Wilhelm & Mialhe 1996, Briggs & McAliskey 2002, Stentiford et al. 2002) there is concern it may significantly impact stock dynamics. Anecdotal reports from industry would suggest that PCD is a relatively new phenomenon in Irish waters rather than being present but undetected previously. Despite extensive enquires the condition had only been observed by one operator in the southwest for 2 - 3 years previously and had been unnoticed in other regions. As the occurrence of moribund and dead pink-shelled animals in fishing pots is obvious to industry (Latrouite et al 1988, Stentiford et al 2002), it is unlikely that stakeholders in other areas fishing in an identical manner were failing to notice PCD. It is unclear whether a natural northern expansion in the geographic distribution of the parasite or artificial translocation has resulted in it reaching or being

spread of the disease, in the current study macroscopic signs of infection only occurred during a discrete period and consistently underscored prevalence, indicating that only a proportion of infected individuals within a population can be managed during a restricted time. With further refinement, scientifically derived infection intensity data could be incorporated into stock assessment estimates and subsequent input (e.g. fishing effort) and output (e.g. quota) management advice. Disease-related contributions to natural mortality are not currently factored into assessment methods for this species.

Although the intention was to sample a minimum of 100 individuals on a quarterly basis, closed fishing seasons and sporadic availability of samples due to bad weather resulted in some discontinuity. The apparent variability in prevalence may have been an artefact of relatively small sample sizes, but as crabs were taken from standardised locations and processed in an identical manner this seems unlikely. Previous studies have shown the relevant stocks to be highly mobile however (Tully et al. 2006), showing strong directional migration patterns. Crab movements into and out of localised sampling areas within each of the fisheries could account for much of the variability in prevalence as the potential for exposure to *Hematodinium* spp. infection can vary geographically (Shields et al. 2005).

A parasitic marine dinoflagellate life cycle involves an infective agent (dinospore), a growth stage (trophonts and plasmodia) and a sporogenic reproductive phase that generates large numbers of dinospores (Coats 1999). It is suggested that the high infection intensity Q4 season corresponds to the main growth phase preceding the production of dinospores as vast numbers of trophonts and plasmodia were observed only during this period, as were macroscopic signs of infection and dinospores. Seawater temperature, or processes linked to it (e.g. moulting) was possibly a determining factor in triggering stages in the

reproductive lifecycle of the parasite. This theory is supported by industry reports of late stage, terminally infected or dead *Cancer pagurus* in pots only during November and December, with these mortalities explaining the lower prevalence and absence of heavily infected crabs observed in spring. While the peak in mean percentage of plasmodia appeared less pronounced in 2005, poor weather conditions prevented sample collection that December, which appeared to correspond to the peak in the other years. In common with the present study Latrouite et al. (1988) reported the presence of *Hematodinium* sp. in French brown crabs in all months examined with a marked increase in the number of pink crabs and mortalities during the winter months. Stentiford et al (2002) reported similar seasonality in peak infection.

Transmission of *Hematodinium* spp. in other host species has been long associated with moulting (Meyers et al. 1987, 1990, Eaton et al. 1991, Field et al. 1992, Dawe 2002, Shields et al. 2005, 2007). The main moulting period for brown crabs in Irish fisheries occurs from September to December (Tully et al. 2006), coinciding with high infection intensities observed in Q4 in this study. The hypothesis that crabs contract *Hematodinium* sp. infections during ecdysis may account for the relatively high prevalence of low level infections in the spring. It is postulated that the warmer months in Q2 and Q3 allow for the growth of the early vegetative stage and the cooling associated with Q4 initiates division of cells prior to the production of dinospores, eventually leading to death of the host. This would suggest a lifecycle of approximately 12 mo for the parasite in this host. Tagging and growth studies in Ireland have shown decreasing moult frequency with increasing size of brown crab (Tully et al. 2006). If infection is linked directly to or increased by ecdysis, then this may afford large animals or those moulting early in the season a temporary refuge from infection and account for the high prevalence observed in the small size classes in

this study. As standard fishing pots have a mesh panelling that allows escapement of individuals < 80 mm carapace width, individuals lower than this size were not fully represented rendering prevalence data 'apparent' rather than 'total'. Consequently, there are concerns as to disease impact on pre-fishery recruits in nursery areas that are known to overlap or are directly adjacent to the main fishing areas. Shields et al. (2005) highlighted the potential for significant losses from both the fished and pre-recruit components of stocks of snow crabs *Chionoecetes opilio* specifying that disease impact on recruitment is difficult to quantify without carefully designed, directed surveys.

Presence of the parasite in Ireland has raised concerns not only due to its potential biological impact on *Cancer pagurus* stocks, but also due to the risk of damaging consumer confidence if deteriorated products reach consumers. Although the majority of infected animals screened during this study showed no visible signs of disease or deterioration, the observed pattern of significant increases in infection intensity and the appearance of late-stage infections in Q4 coincided with that of maximum economic unit value for Irish *Cancer pagurus*. Demand for live crab products in continental Europe peak just prior to and during the Christmas festival period and market prices can as much as quadruple in response. As live export duration between capture and consumption can exceed 20 days there is potential that individuals with infections insufficiently progressed to be macroscopically identifiable at capture may already have internal tissue degradation or develop it prior to reaching the consumer. Additionally, infected *C. pagurus* and other crab species have been reported to suffer and die more readily during captivity than uninfected conspecifics (Meyers et al. 1987, Stentiford et al 2002). Stakeholders involved in live export must be made aware of the risks associated with medium-term storage during this period in the absence of data relating to disease progression.

CHAPTER 2

INVESTIGATION INTO *HEMATODINIUM* SP. PREVALENCE AND INFECTION INTENSITY IN *CANCER PAGURUS* FROM MALIN HEAD, IN RELATION TO HABITAT AND HOST FACTORS

ABSTRACT

High mortalities of brown crabs *Cancer pagurus* have been caused by the parasitic dinoflagellate *Hematodinium* sp. in the commercially important Malin Head fishery to the north of Ireland since 2005. Screening was conducted in 2007 to assess the susceptibility of different components of the brown crab population so that an estimate of the biological impacts of the parasite could be obtained. Haemolymph was collected from 1972 crabs from a range of substrate types and examined for the presence of the parasite. Crabs were grouped by sex and further stratified into three size categories representing juvenile, pre-recruit and marketable crabs. Prevalence of *Hematodinium* sp. was also compared between intermoult and recently moulted crabs to address any ecdysial influences on infection levels. Juvenile and pre-recruit cohorts appeared to be most susceptible to infection. There was no significant difference in the susceptibility of males and females and infection parameters were not influenced by substrate or moult status of crabs. The most significant implication for the host stock appears to be the potential for *Hematodinium* sp. to remove 42 % of sub-legal sized individuals before recruiting to the fishery.

INTRODUCTION

The brown crab *Cancer pagurus* is one of the most important constituent species of Ireland's trap-based fisheries. Without the revenue derived from the sale of the species, many small inshore vessels and all of the offshore crab fishing sector would cease to

remain economically viable. Approximately 8000 t of brown crabs were landed to Ireland in 2008 with a first sale value estimated at €9 million (source: Irish Sea Fisheries Protection Authority (SFPA)). The northwest (NW) coast supports the largest fishery accounting for 60 - 75 % of national landing annually (SFPA). While the offshore brown crab fishing sector is characterised by large, capitalised and modern vessels that can remain at sea for extended periods, the inshore areas are generally exploited by more numerous, smaller boats that return to shore every day. Inshore operations are generally more basic and less capitalised than the offshore ones and unpublished data from recent studies for Bord Iascaigh Mhara (M. Robinson, unpublished data) suggested that many vessels run close to or at an economic loss annually and as a result the fleet has contracted significantly in the last decade. As the vessels from both the inshore and offshore sectors within the NW region generally come from marginal and relatively isolated locations where few other sources of employment and income exist (Anon 1999), the continued viability of the industry is important in the context of social cohesion. Factors that impact significantly on stock availability, abundance and product quality can therefore have a rapid and significant impact on the socio-economics of Irish coastal communities. In 2005, fishermen in the NW Irish brown crab fishery began to notice weak or dead discoloured individuals in fishing traps (Tully et al. 2006). Investigation indicated the endoparasitic dinoflagellate *Hematodinium* sp. as the causative agent. A nationwide monitoring programme established at that time to assess the impact of the disease has since reported infection levels as high as 24 % in commercial landings from the northwest coast (Ní Chualáin et al. 2009). As *Hematodinium* sp. infections are highly pathogenic to their hosts (Stentiford & Shields 2005), such epizootics are of significant biological and economic concern. The latter factor is of particular concern to fishers as the highest infection intensity levels and visible patent disease symptoms occur during December when the

seasonally variable European live export market price is always relatively high. Quality control is very problematic during the 30 days that can elapse between capture and consumption in the live export market and mortalities can be high. In contrast, local factories process crabs within 24 hours of capture and have quality control systems before and after cooking to prevent low grade products reaching the consumer, but unlike the year round export trade, operate only on a restricted seasonal basis.

Historically, the first reports of *Hematodinium*-related *Cancer pagurus* mortalities came from Brittany in France during 1986. Infections were characterised by a pink colouration of the haemolymph, loss of consistency of the muscle and a negative alteration in taste (Latrouite et al. 1988). In a more recent study, Stentiford et al. (2002) assigned the term 'pink crab disease' to such infections in brown crabs due to a hyperpigmentation of the appendages and carapace in patent infections, symptoms which appear toward the terminal stages when individuals are moribund and often die during handling or transportation. Similar *Hematodinium* sp. related infections cause "bitter crab disease" in snow crabs *Chionoecetes opilio* and Tanner crabs *C. bairdi* in the northwest Atlantic and in southeast Alaska respectively. The bitter flavour imparted by the parasite renders crabs unmarketable and has resulted in significant economic losses in those affected fisheries (Meyers et al. 1987, Taylor & Khan 1995). *Hematodinium*-like organisms have also been documented as impacting on the stock abundance of other European commercial species such as the velvet crab *Necora puber* in France (Wilhelm & Boulo 1988, Wilhelm & Mialhe 1996) and Norwegian lobster *Nephrops norvegicus* in Scotland (Field et al. 1992, 1998, Field & Appleton 1995, Stentiford et al. 2001a). The high prevalence levels reported from the NW coast of Ireland brown crab fishery have also raised the additional concern that natural mortality may be sufficiently increased to render some stock assessment methods (e.g. egg

production per recruit (Tully et al. 2006)) significantly less reliable in the absence of an up-to-date indication of disease activity.

Observations from other commercially exploited fisheries affected by *Hematodinium* sp. have implicated several possible host factors in their susceptibility to infection including size (Field et al. 1992, 1998, Messick 1994, Stentiford et al. 2001a, Briggs & McAliskey 2002, Ní Chualáin et al. 2009), sex (Field et al. 1992, Shields et al. 2003, Stentiford et al. 2001a) and moult condition (Meyers et al. 1987, 1990, Eaton et al. 1991, Field et al. 1992, Dawe 2002, Shields et al. 2005). In many host species, ecdysis is considered the major predisposing factor in acquiring *Hematodinium* sp. infections as significantly higher prevalences have been observed in recently moulted hosts compared with intermoult conspecifics (Meyers et al. 1987, 1990, Eaton et al. 1991, Field et al. 1992, 1998, Messick 1994, Dawe 2002, Shields et al. 2005). However, the apparent lag phase between newly infected hosts and the manifestation of the presumptive infectious stage, dinospores, has led to the hypothesis by several authors (Hudson & Shields 1994, Shields 1994) that alternative hosts exist. Abiotic factors such as salinity (Messick & Shields 2000) and hydrographic features like confined bays or fjords with limited tidal flushing (Messick & Shields 2000, Shields et al. 2005) have also been implicated in the epizootiology of the disease in several crustacean species. It is thought that partially restricted coastal systems such as lagoons, fjords and embayments which have a high potential for entrained water masses, contribute to the spread of *Hematodinium* sp. by retaining the transmissive stages in those restricted areas (Shields 1994, Dawe 2002, Shields et al. 2005, Shields et al. 2007). However, outbreaks are also widely reported from more open ocean regions (Meyers et al. 1996, Field et al. 1998, Briggs & MacAliskey 2002, Stentiford et al. 2002, Ní Chualáin et al. 2009). Positive association between *Hematodinium* sp. prevalence with

mud/sand substrate types of snow crabs has also been reported (Shields et al. 2005) and this has been taken to indicate the possibility of alternate or reservoir hosts. However, a depth related association with prevalence could not be discounted in that study.

Compared to the geographic spread of other crab fisheries around Ireland, the NW represents the most extensive, covering an area of 45000 km^2 which extends northward to the west coast of Scotland (Tully et al. 2006). Fishing is shelf-based and generally confined to waters between 20 – 200 m. Habitat type varies locally across the fishery, particularly in inshore waters < 100 m deep and the well mixed, highly exposed Atlantic waters ensure uniform temperature and salinity. As the purpose of a previous study by Ní Chualáin et al. (2009) was to assess the impact of *Hematodinium* sp. on the commercially available portion of the population, it drew random samples from the landings and discard components of the local Malin Head fishery and was therefore restricted to size ranges determined by gear selection. Also female crabs were over-represented due to natural bias in sex ratios or catchability. In order to make a more refined estimate of the potential biological impacts that the parasite may have at a regional and national level, it was necessary to establish a more complete, compartmentalised assessment of host susceptibility factors across the population. The aim of this study was to provide the data necessary to achieve this within the most intensive and geographically contiguous inshore fishery within Ireland.

METHODS

Fishing strings (rope with weighted ends and traps spaced at approximately 10 – 15 m along their length) of 25 creel traps were deployed off the Malin Head coast, Co. Donegal for 24 hr in pre-defined areas where seabed characteristics had been previously identified

by validated acoustic habitat mapping during a Bord Iascaigh Mhara crab recruitment survey (Robinson et al. 2008). Crabs from kelp, pebble, sand and cobble substrates were examined for *Hematodinium* sp. infection during May 2007, these substrates being typical of those in the NW fishery. A second survey was undertaken in December 2007 on the eastern side of Malin Head during which crabs were sampled from sand, kelp and cobble substrates; pebble substrates were non existent on the eastern side of Malin Head and therefore were not sampled (Figure 2.1). Sampling periods were selected based on a previous study by Ní Chualáin et al. (2009) which identified high prevalence of light and advanced *Hematodinium* sp. infections in May and December respectively. On each sampling occasion an attempt was made to sample 50 intermoult 'juvenile' (< 100 mm carapace width (CW)), 50 pre-recruit (101 – 130 mm CW) and 50 legal-sized (> 130 mm CW) brown crabs of both sexes from each substrate type. Size classes were selected in an attempt to gauge potential losses from the marketable component of the population (legal size), the cohort due to recruit to the fishery at the next moult (pre-recruits) and those at least one moult away from fisheries recruitment (juvenile). Soft-shelled animals were not sampled. Depth range varied between 20 – 40 m, with all sites being deemed highly exposed to tidal flushing. Haemolymph was extracted from crabs onboard ship from the arthrodial membrane at the junction of the basis and ischium of one of the walking legs using a 1ml syringe and 19 gauge needle and subsequently injected into chilled vacutubes preloaded with 4 % buffered formalsaline. Haemolymph samples were stored chilled for processing in the laboratory.

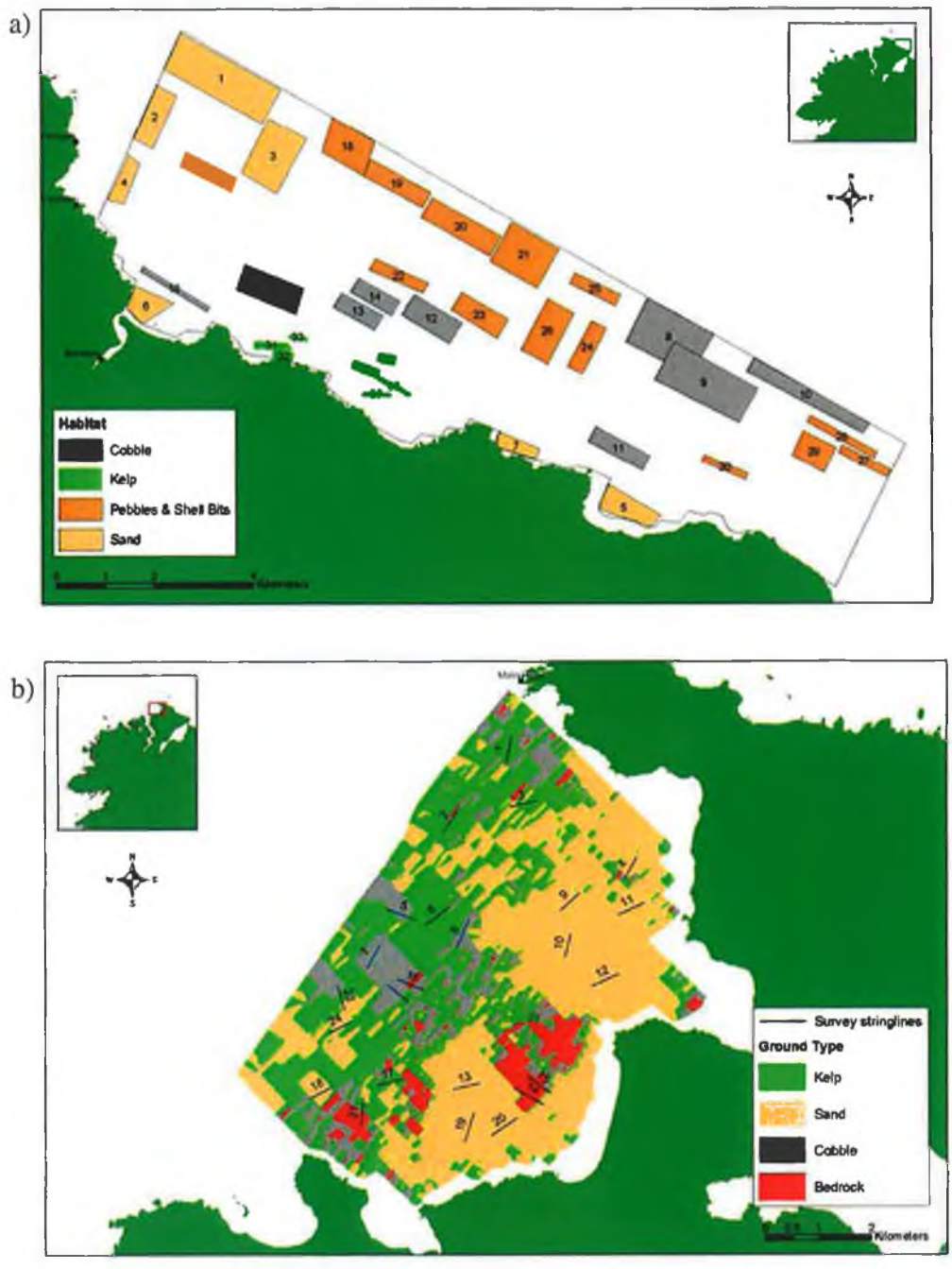


Figure 2.1 Survey area of Malin Head, Donegal where brown crabs *Cancer pagurus* were sampled from a range of substrates in a) May and b) December 2007. Polygons represent each of the different substrates which were classified using acoustic data and validated using video footage (Robinson et al. 2008). Taken from Robinson et al. 2008 (Project no. 01.SM.T1.05)

Blood samples were taken from two additional sets of individuals in September and November 2007 to investigate the relationship between *Hematodinium* sp. infection and ecdysis. Although soft-shelled individuals can be found from spring through to winter, the majority of moulting takes place between September and November and therefore sampling was conducted during these months. Haemolymph samples were drawn from soft-shelled and intermoult individuals within sex and size stratified groupings as above.

The mesh covering of standard commercial creel fishing traps allows escapement of individuals less than 80 – 90 mm CW. In an attempt to gauge infection prevalence in early benthic phase *Cancer pagurus*, individuals were collected on the shore at low water during a spring tide from a rocky beach within the main Malin Head fishing area. A sample of commercially captured brown crabs was examined for the presence of *Hematodinium* sp. on the same day.

Fixed haemolymph samples from all of the above sampling procedures were smeared onto slides and allowed to air-dry for 24h. Slides were then stained with Giemsa staining solution (Humason 1979), dehydrated in a series of ethyl alcohol solutions, cleared in Histoclear® and mounted with Clarion® mounting medium. Slides were examined using an Olympus BX41 compound microscope without reference to field sampling data (indicating substrate, size, moult status and/or sex) and were defined as positive for infection when at least one clearly identifiable *Hematodinium* sp. cell of any stage (i.e. trophont, plasmodia, dinospores) was observed. Once a slide was defined as positive, a total of 300 cells comprising of parasite cells and haemocytes, was counted from 5 fields of view. The percentage of *Hematodinium* sp. cells in those 300 cells defined the infection intensity of that slide. Mean intensity for each sampling period was the sum of intensities

of infected crabs divided by the number of infected crabs (Sheppard et al. 2003). Infection prevalence was expressed as a percentage by dividing the number of crabs infected with *Hematodinium* sp. by the number of crabs examined (Bush et al. 1997).

A generalised linear model based on a negative binomial distribution was carried out to investigate the main effects and higher level interactions of month, substrate, size and sex on *Hematodinium* sp. counts from each crab. The reason a generalised linear model was used and not the more familiar general linear model is that the latter model assumes the data follow a normal distribution with homogenous variance. Generalised linear models have the same basic structure as general linear models but allow for a wider variety of underlying probability distributions (Power & Moser 1999). In the present study, when variances across factors were examined they were found to be heterogenous (Cochran's test). Variances were found to either increase or exceed the mean with increasing mean values similar to either a Poisson or an over-dispersed negative binomial distribution. Log-transformation of the data was unsuccessful in normalising the data. Quantile-quantile (Q-Q) plots were used to assess the suitability of the three distributions to the data by plotting the observed quantiles of the residuals against the theoretical quantiles of a normal, Poisson and negative binomial distribution. The corresponding residual plots are shown in Figure 2.2. Generalised linear models based on normal, Poisson and negative binomial distributions were further compared using Akaike's Information Criterion (AIC) which is a likelihood-based statistic which includes a penalty for each parameter in a model. The model with the lowest AIC value indicates the best fit (Akaike 1983). Analysis of variance (ANOVA) was carried out to determine if mean size of *Cancer pagurus* varied significantly over the various substrate types. For this test Cochran's test was used to

ensure homogeneity of variances. For all analyses, differences were considered significant when $p < 0.05$.

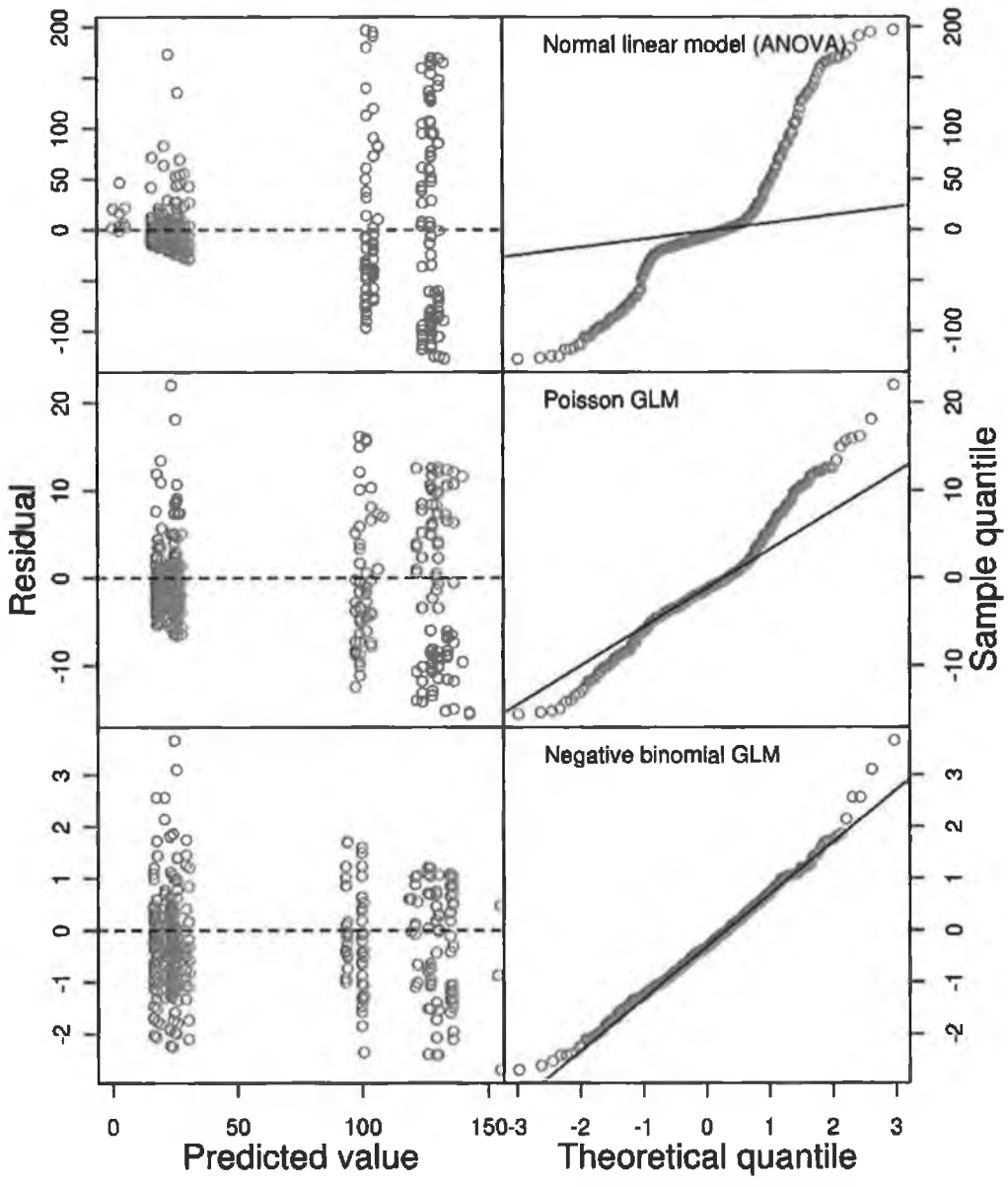


Figure 2.2. QQ plots and residual plots of the *Hematodinium* sp. counts and theoretical normal, Poisson and negative binomial distributions.

RESULTS

A total of 975 *Cancer pagurus* was examined for the presence of *Hematodinium* sp. over four substrate types; sand, pebble, kelp and cobble after sampling in May 2007 (Table 2.1); the maximum number of individuals per sex and size class within habitat was not always achieved at the standardised fishing effort. While the kelp habitat yielded the fewest crabs, the mean size of crabs did not differ significantly between the four habitat types (ANOVA $p > 0.05$). Overall, fewer crabs (7 %) were infected with *Hematodinium* sp. from kelp substrates when compared with sand (22 %), pebble (27 %) and cobble (20 %) substrates (Figure 2.3). Prevalence was almost identical between the sexes (20 and 21 % of males and females respectively). *Hematodinium* sp. prevalence was highest in crabs in the smallest size category and decreased in larger animals; this was particularly evident for male *C. pagurus* (Figure 2.4a). When sex and substrate type were pooled the < 100 mm, 101 – 130 mm and > 130 mm CW size categories had prevalence values of 27, 21 and 16 % respectively.

Table 2.1. Prevalence (%) and Mean Intensity (%) data by sex, size and habitat of *Hematodinium* sp. infection in brown crabs collected in Donegal in May 2007.

Female	**Sand**	**Pebble**	**Kelp**	**Cobble**
Size (mm)	≤100	≤100	≤100	≤100
Total n	35	39	4	18
Infected n	7	10	1	4
Prevalence	20	26	25	22
Mean Intensity	5	7	7	19
Size (mm)	101-130	101-130	101-130	101-130
Total n	48	50	14	50
Infected n	11	10	0	14
Prevalence	23	20	0	28
Mean Intensity	8	4		8
Size (mm)	>130	>130	>130	>130
Total n	47	50	32	47
Infected n	11	16	2	7
Prevalence	23	32	6	15
Mean Intensity	7	4	6	7
Male	**Sand**	**Pebble**	**Kelp**	**Cobble**
Size (mm)	≤100	≤100	≤100	≤100
Total n	49	49	9	49
Infected n	14	18	1	12
Prevalence	29	37	11	25
Mean Intensity	10	5	1	9
Size (mm)	101-130	101-130	101-130	101-130
Total n	50	49	51	65
Infected n	11	15	4	15
Prevalence	22	31	8	23
Mean Intensity	8	5	7	8
Size (mm)	>130	>130	>130	>130
Total n	50	50	20	50
Infected n	6	9	1	4
Prevalence	12	18	5	8
Mean Intensity	17	5	4	11

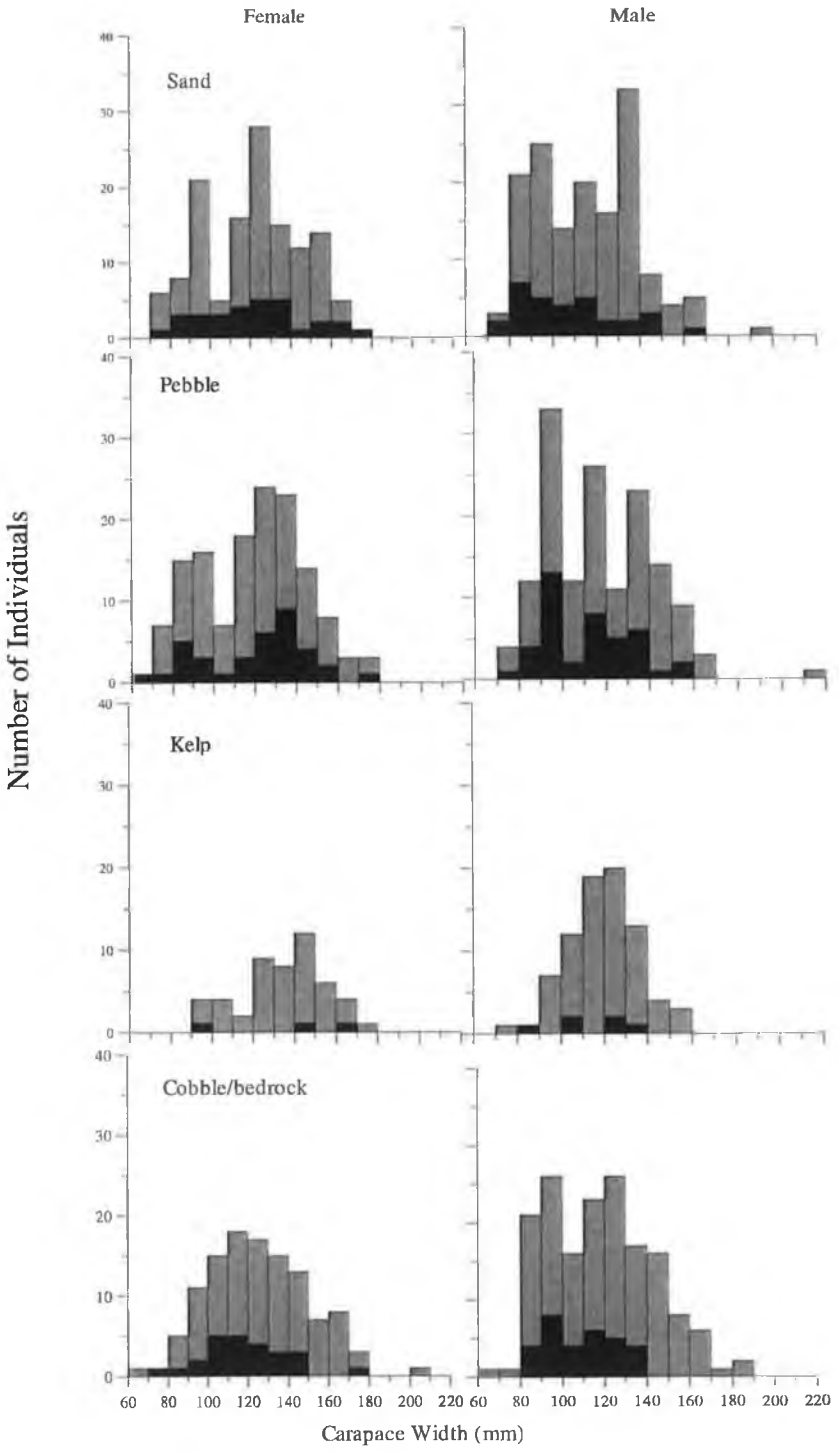


Figure 2.3. Size-frequency distribution of healthy (grey) and *Hematodinium* sp. infected (black) female and male brown crab sampled in Malin Head, Donegal from four habitat types, May 2007.

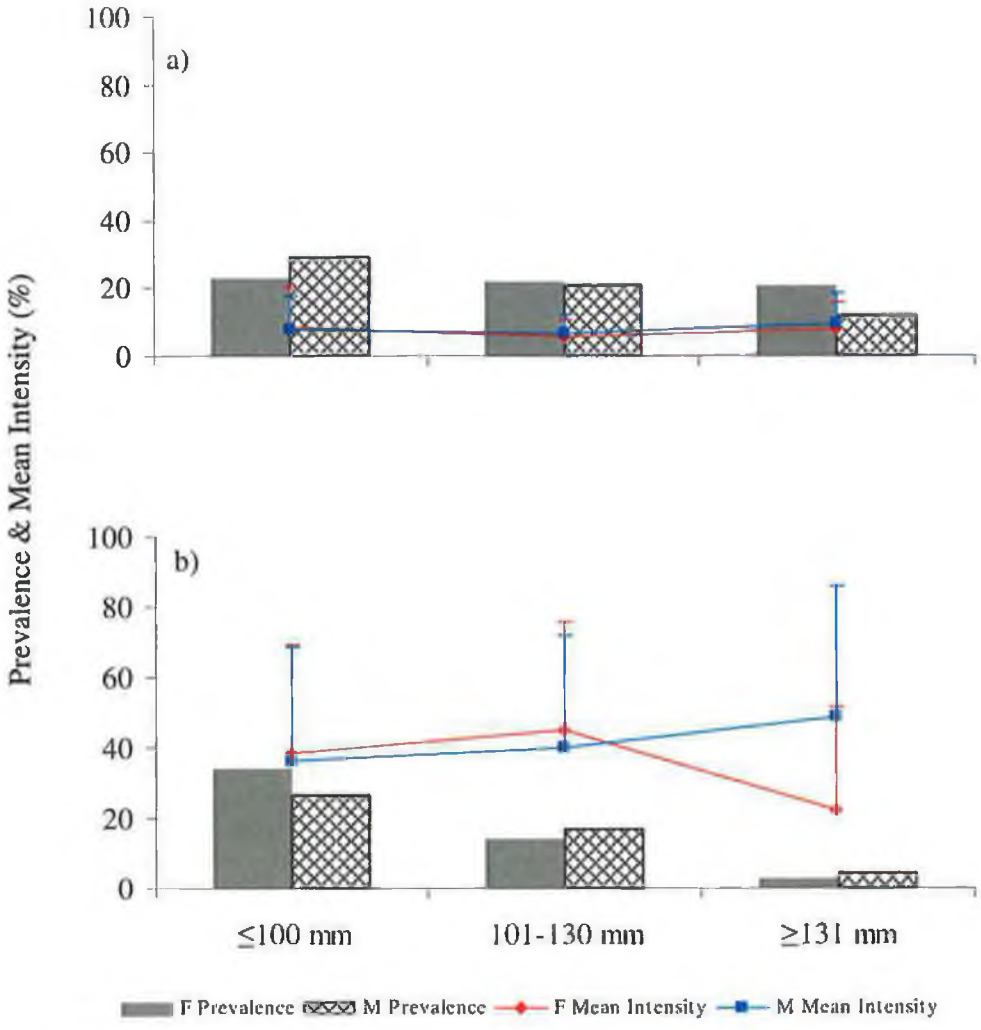


Figure 2.4. Prevalence and mean intensity (+SD) of *Hematodinium* sp. in female (F) and male (M) brown crabs from Donegal captured in a) May and b) December 2007.

In December 2007, 853 *Cancer pagurus* from sand, kelp and cobble substrates were screened for *Hematodinium* sp. infections (Table 2.2). Similarly to data from May, mean size of crabs (Figure 2.5) did not differ significantly between substrate types, (ANOVA, $p > 0.05$). Prevalence of *Hematodinium* sp. decreased in December to 16 % for both female and male crabs. A decrease in prevalence from juvenile to commercial-sized crabs was apparent in December, being more pronounced than observed in May (Figure 2.4b). Pooled data for sex and substrate type for the < 100 mm, 101 – 130 mm and > 130 mm CW size categories yielded prevalence values of 30, 15 and 3 % respectively. When season, habitat and sex were pooled prevalence of *Hematodinium* sp., within the size categories < 100 mm, 101 – 130 mm and > 130 mm CW yielded values of 29, 19 and 10 % respectively.

Table 2.2. Prevalence (%) and Mean Intensity (%) data by sex, size and habitat of *Hematodinium* sp. infection in brown crabs collected in Donegal in December 2007.

Female	**Sand**	**Kelp**	**Cobble**
Size (mm)	≤100	≤100	≤100
Total n	36	48	48
Infected n	9	22	14
Prevalence	25	46	29
Mean Intensity	35	35	47
Size (mm)	101-130	101-130	101-130
Total n	47	57	48
Infected n	6	8	7
Prevalence	13	14	15
Mean Intensity	51	38	48
Size (mm)	>130	>130	>130
Total n	49	53	47
Infected n	1	1	2
Prevalence	2	2	4
Mean Intensity	22	63	2
Male	**Sand**	**Kelp**	**Cobble**
Size (mm)	≤100	≤100	≤100
Total n	36	45	48
Infected n	7	11	16
Prevalence	19	24	33
Mean Intensity	43	17	47
Size (mm)	101-130	101-130	101-130
Total n	49	60	39
Infected n	4	11	10
Prevalence	8	18	26
Mean Intensity	47	39	38
Size (mm)	>130	>130	>130
Total n	41	54	48
Infected n	2	2	2
Prevalence	5	4	4
Mean Intensity	38	52	57

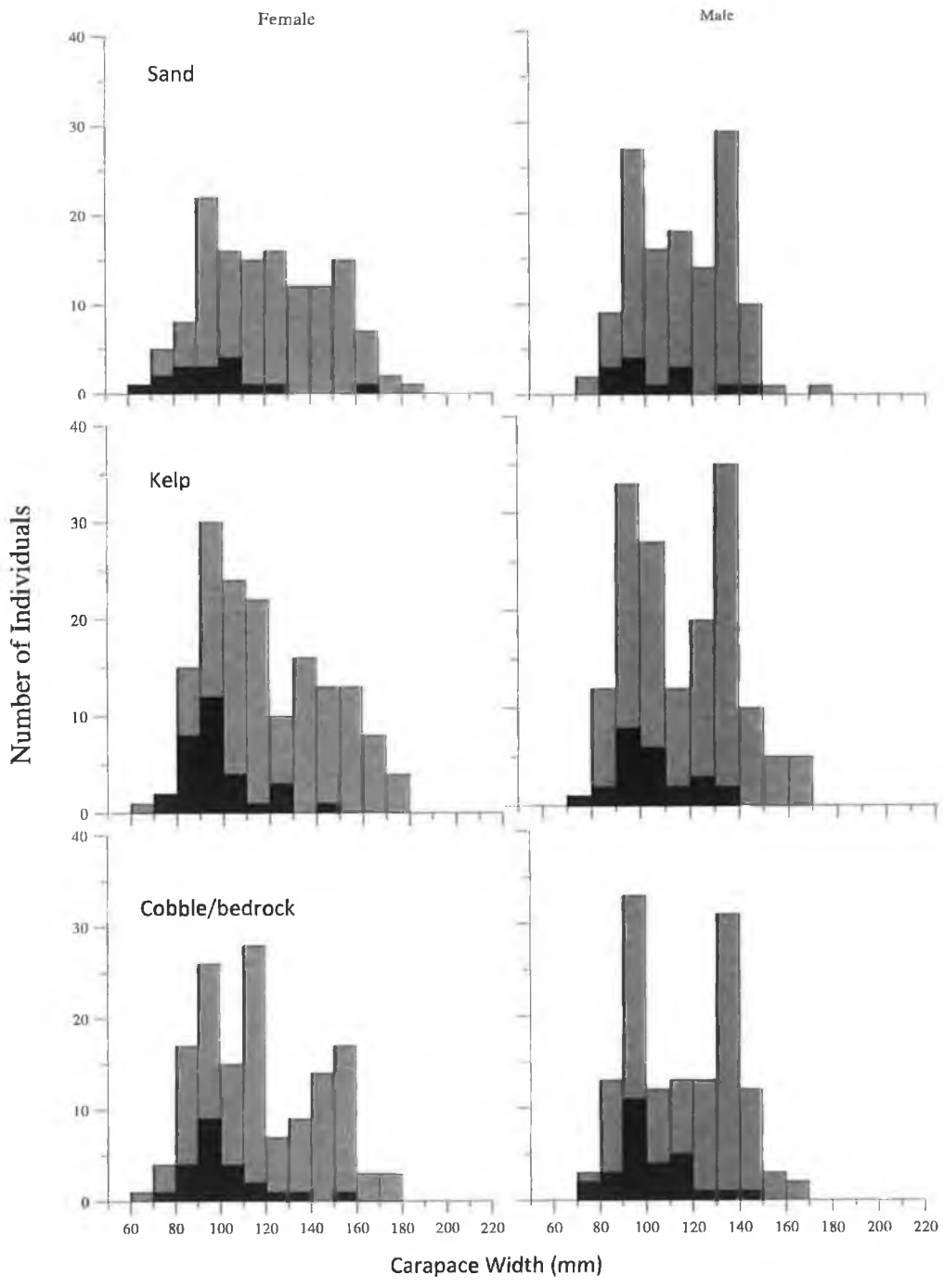


Figure 2.5. Size-frequency distribution of healthy (grey) and *Hematodinium* sp. infected (black) female and male brown crab sampled in Malin Head, Donegal from three habitat types, December 2007.

Comparison of the Q-Q plots revealed pronounced departures of the infection intensity count data from the 45° reference line representing the quantiles of the theoretical normal and Poisson distribution while showing a strong fit to the negative binomial distribution (Figure 2.2). A low AIC value (AIC = 3215.09) was also indicative of the negative binomial model fitting the data well (general linear model based on normal distribution AIC = 3746.313 and generalised linear model based on Poisson distribution AIC = 15161.92). In terms of the main effects driving infection intensity, only month (χ^2 = 153, df = 1, $p < 0.001$) and substrate (χ^2 =11.6, df = 3, $p < 0.05$) explained a significant amount of variation in the parasite counts whereas sex (χ^2 = 0.006, df = 1, $p > 0.05$) and size (χ^2 = 3.11, df = 2, $p > 0.05$) did not. Mean intensity in May was 8 % ± 0.6 % (SE) and increased in December to 39 % ± 3 % (SE). However, a multiple comparison post-hoc test on the negative binomial model of substrate showed that none of the individual substrate comparisons were significantly different but the pebble-cobble comparison (p = 0.07) might be driving significance in the main effects model. There were no statistical interaction between the two main effects, month and substrate (χ^2 = 0.399, df = 2, $p > 0.05$). A box plot summary of *Hematodinium* sp. infection intensity levels by month, sex, size and substrate groupings is provided in Figure 2.6.

During September and November, 72 each of recently moulted and intermoult crabs were sampled. Prevalence of *Hematodinium* sp. was identical (11 %) in recently moulted and intermoult *C. pagurus* in September, however by November prevalence in intermoult crabs (16 %) was higher than recently moulted crabs (9 %).

A total of 19 crabs ranging in size from 18 mm - 81 mm CW were collected during shore based collections. Microscopic examination of haemolymph smears revealed that only the

largest individual of these specimens (81 mm CW) was positive for *Hematodinium* sp. infection (resulting in a prevalence of 5 %). A prevalence of 18 % was recorded from the sample of commercially captured *C. pagurus* surveyed on the same day (n = 60).

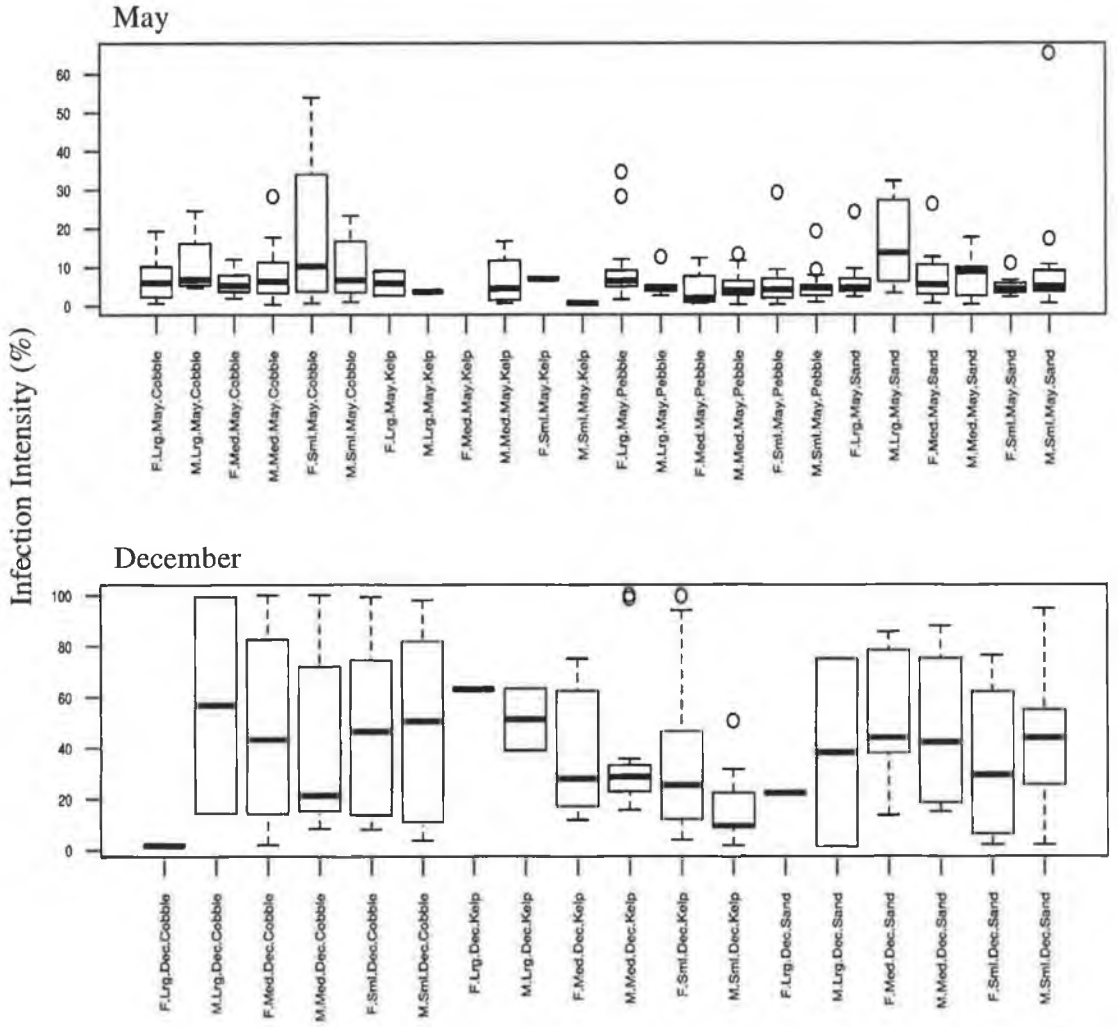


Figure 2.6: Box plot summary of *Hematodinium* sp. infection intensity levels in *Cancer pagurus* from Malin Head, Donegal in May and December 2007. Each box depicts infection intensity by sex (F: female, M: male), size (Lrg: >130mm; Med: 101-130 mm; Sml: ≤ 100 mm) and substrate groupings. The black horizontal bar in each box shows the median of infection intensity for each grouping. The upper and lower boundaries of each box show the upper and lower quartiles respectively. Broken lines with caps indicate minimum and maximum numbers of the data set. Additional points are shown as outliers. Note scale change between May and December

DISCUSSION

The high levels of *Hematodinium* sp. prevalence recorded in this study are similar to those recorded previously at Malin Head since the disease was first recorded there in 2005 and also similar to prevalence values obtained from other geographically removed fished stocks to the southwest and southeast of Ireland (Ní Chualáin et al. 2009). *Hematodinium* sp. prevalence fluctuates throughout the year with little predictability, but it is increases in infection intensity that signal periods when the disease is particularly active at the terminal phase of infection in November and December (Ní Chualáin et al. 2009). Data presented here indicates that monthly or bi-monthly prevalence values obtained from commercial catches of brown crab during the former monitoring programme were likely to be representative of *Hematodinium* sp. distribution and activity regardless of the habitat type fished on or the sex of the individuals sampled. The pooled habitat, sex and season prevalence values of 29, 19 and 10 % obtained in the present study for juvenile, pre-recruit and legal sized crabs respectively constitute an epizootic and in the absence of disease immunity or recovery should represent the percentage of crabs that will be removed from the population through *Hematodinium* sp. induced mortality within the forthcoming year assuming a 12 month lifecycle for the parasite. Mortality studies carried out on Tanner crabs, Norway lobsters and blue crabs infected with *Hematodinium* sp. indicate that infections are generally fatal to the host (Meyers et al. 1987, Eaton et al. 2001, Field et al. 1992, Shields & Squyars 2000). Messick and Shields (2000) have reported 100 % mortality over 35 days in blue crabs naturally infected with the parasite, while infected Norway lobsters are up to 4 times more likely to die than their uninfected counterparts (Field et al. 1992). The potential impact of the parasite on brown crabs becomes particularly apparent however if no immunity can be developed as annual moults are required to progress through the size categories used in the present study. If the 71 % of

individuals that survive the disease in December moult from juvenile to pre-recruit during the following summer, are subjected to a further 19 % mortality in the following December, only 58 % of the original stock will recruit to the fishery during the next growth period. Additionally, the legal-sized category contains individuals from multiple annual cohorts, with larger specimens having recruited to the fishery many years previously; large crabs in the area are thought to be at least 15 years old, having recruited between the ages and 4 – 6 years (Tully et al. 2006). As the first reports of infection only occurred in 2005 at Malin Head, it is possible that the true impact of the parasite on population structure and abundance has yet to be realised as the fished component of the stock at that time had not been subjected to disease induced mortalities at juvenile and pre-recruit stages. If 42 % of stock is removed by disease up to the first time the cohort is subjected to fishing pressure and then to a further 10% per annum, a truncation of population size frequency may currently be taking place.

Size-related variations in prevalence encountered in the present study, were consistent with studies carried out on Norway lobsters *Nephrops norvegicus* and blue crabs *Callinectes. sapidus* (Field et al. 1992, 1998, Messick 1994, Stentiford et al. 2001a, Briggs and McAliskey 2002). The exact cause of the relationship is unclear but the high prevalences observed in the smaller size categories may be explained by the higher moult frequencies of juveniles. It may be that large crabs are more resistant to *Hematodinium* sp. than smaller ones. Very small individuals (< 80 mm CW) collected from the shoreline in the current study were not infected. However, the sample size was small (n = 18). Although disease resistance has been reported for blue crabs (Shields & Squyars 2000) it has not been linked to size. No resistance or immunity has been reported for *Cancer pagurus*. As early benthic stage brown crab are known to recruit into sub-tidal habitats < 25 m deep in Ireland

(Robinson 1999), exposure to the parasite would seem likely for at least some juveniles < 80 mm CW as this overlaps with the depth distribution of the adult component of the population. A concern remains that the impact of the parasite on pre-fisheries recruits may be even worse than predicted here if losses occur in all years from settlement to recruitment. Even if there is some form of refuge for small juveniles, the high infection prevalence in pre-recruits one to two years from recruitment to the fishery represents an infection reservoir and also affords the parasite refuge from removal by commercial fishing. Although some countries, including Ireland, promote management measures that involve the removal of late stage infected individuals to landfill, these may have little or no impact when the landing of sub-legal specimens is not regulated for and conducted at the same time.

A detailed assessment in 2005 suggested a size at 50 % maturity of approximately 113 mm CW for brown crabs in the Malin Head fishery, with most individuals being mature at 130 mm CW (Hayes, unpublished data). As such, individuals in the pre-recruit category used in this study would have consisted of a mixture of mature and immature specimens, therefore contributing to the spawning stock prior to their recruitment to the fishery. Although this pre-recruit component of the stock is not subject to fishing mortality, the results presented suggest that natural mortality is significantly higher than would normally be expected. As each female carries between 1 – 4 million eggs in one clutch (Tully et al. 2006) there may not be an immediate threat in terms of egg limitation; the relatively low proportion of males in the population may suggest that sperm shortage may be more relevant. The levels of infection reported also indicate adjustments may be required in stock assessment methods that utilise estimates of natural mortality values (M). These are commonly used in stock assessment methods for brown crab such as egg production per recruit (EPR), but do

not incorporate disease associated mortalities into their models currently (Tully et al. 2006).

A previous study has shown disease prevalence within the area to be highly variable and unpredictable throughout the year for legal sized individuals, but actual late stage infections and mortalities to be confined to November and December (Ní Chualáin et al. 2009). It is still unclear why such large variations occurred in mixed sex samples drawn randomly from commercial catches, but data from the current study suggest that habitat-type was not a significant contributing factor. The mobile nature of the host species may account for this; brown crabs tagged in Malin Head previously have been shown to undertake temporary and permanent migrations exceeding 200 km within a year, with an average movement rate of 1 km day^{-1} during autumn, winter and spring (Tully et al. 2006). Due to the patchy nature of the benthos in the region, individuals are likely to encounter most habitat types and species assemblages within a relatively short space of time.

In common with findings by Latrouite et al. (1988) the prevalence of *Hematodinium* sp. infection in *Cancer pagurus* showed no association with sex in this study. In *Hematodinium*-infected populations of Scottish *Nephrops norvegicus,* differences in prevalence between the sexes have been attributed to differences in moulting times of males and females (Field et al. 1992, Stentiford et al. 2001a). Moulting in female *N. norvegicus* coincides with the period of peak infection prevalence but precedes the ecdysial season in males allowing soft females to mate with hard - shelled males which may account for the relatively higher prevalence observed in females (Field et al. 1992). Release of dinospores, the putative transmissive stages of *Hematodinium* spp., has also been observed at times when *N. norvegicus* are moulting (Field et al. 1998). If transmission of *Hematodinium* sp. is indeed moult –related, the absence of separate moulting periods in

Irish populations of *C. pagurus* for males and females (Edwards, 1979) might explain the lack of differences in infection parameters observed between sexes in the present study. In Ireland, both male and female *C. pagurus* moult late into autumn (Tully et al. 2006) which coincides with the occurrence of late-stage *Hematodinium* sp. infections and the production of dinospores in December (Ní Chualáin et al. 2009). Compromised host defence mechanisms in recently moulted blue *Callinectes sapidus* and Tanner crabs *Chionoecetes bairdi* are thought to render crabs more susceptible to invading organisms (Messick 1994, Eaton et al. 1991) while Dawe (2002) suggested that penetration of the soft cuticle by motile dinospores leads to infections in recently moulted snow crabs *Chionoecetes opilio*. If the latter suggestion is accepted, exact overlap of moulting and dinospore production may not be required for successful transmission in brown crabs as *C. pagurus* do not become fully hard shelled for an additional 2 months after moulting (Edwards 1979).

Despite strong evidence supporting a moult association with *Hematodinium* sp. infections in other crustaceans, data from the present study failed to support a similar relationship in *Cancer pagurus*. Prevalence in September was identical in recently moulted and intermoult crabs and by November, prevalence was higher in the latter group. This, coupled with the occurrence of light (≤1 % infection intensity), and therefore presumably recent infections 3 mo prior to the production of infective dinospores in December could mean two things; firstly that an additional entry route independent of moulting exists in brown crab hosts or secondly, that dinospores may not be the only transmissive stage in the lifecycle of *Hematodinium sp.* found in *C. pagurus*. Inoculation experiments involving the injection of *Hematodinium* sp vegetative stages into naïve hosts have been successful in establishing new infections in Tanner *Chionoecetes bairdi*, sand *Portunus pelagicus* and blue crabs *Callinectes sapidus* (Meyers et al. 1987, Hudson & Shields 1994, Shields & Squyars 2000)

however this has not been investigated in *Cancer pagurus* to date (see Chapter 4). While cannibalism (Meyers et al. 1987), sexual transmission (Meyers et al. 1996) and ingestion of alternate hosts such as amphipods (Hudson & Shields 1994, Small et al. 2006) have been discussed by others in relation to *Hematodinium* sp. transmission in other hosts, transmission studies have not been attempted in *C. pagurus* (see Chapter 4). Sheppard et al. (2003) were successful in initiating new infections in naïve blue crabs via ingestion of infected conspecifics, yet similar attempts were unsuccessful in Australian sand crabs *Portunus pelagicus* (Hudson & Shields 1994). These studies suggest that transmission routes may vary depending on the host species.

The economic impact of *Hematodinium* sp. in Ireland first centred on the potential for direct consumer dissatisfaction from 'bitter tasting' crabs in the live export market and/or the processing market if product spoiling occurred prior to visible degradation of tissue that would otherwise allow it to be removed during quality control. It now seems that pre-recruitment losses may be a more significant, if less visible, threat to the viability of the stock and fishery. From anecdotal evidence from fishermen and previous monitoring (Ní Chualáin et al. 2009) it appears that the parasite did not impact the fishery in a detectable way prior to 2005. Some fishing practices such as the introduction of infected animals as bait for a gastropod species that is fished on the same ground have been suggested as the mechanism of introduction. If this is the case then the full impact of the disease may not become apparent until the cohorts within the fished stock have all been subjected to its activity.

CHAPTER 3

COMPARISON OF ASSESSMENT METHODS USED TO DIAGNOSE *HEMATODINIUM* SP. INFECTIONS IN *CANCER PAGURUS* L.

ABSTRACT

Endoparasitic dinoflagellates of the genus *Hematodinium* have recently gained attention as significant pathogens of the brown crab *Cancer pagurus* in Ireland. Patent infections, which are characterised by a hyperpigmented carapace and moribund condition, are limited to discrete periods that macroscopic identification is impossible for the majority of the year. This study assessed three alternative methods for diagnosing *Hematodinium* sp. infections in *C. pagurus*. Haemolymph smears; histological sections of gill, heart, midgut, hepatopancreas, muscle and gonad, and a polymerase chain reaction (PCR) assay provided almost equivalent accuracy in gauging infection prevalence regardless of season. Sequences of PCR amplicons from the 18S rRNA gene confirmed the identity of the parasite as belonging to the genus *Hematodinium.* Infection intensity values (< 1 – 87 %) obtained from haemolymph smears underscored infection levels within tissues, 90 % of which contained advanced levels of infection. Alterations to tissues of infected crabs included haemocytopoenia and oedema which caused dilation of the haemal sinuses resulting in pressure necrosis to the connective tissue around the oocytes, myocardial bundles and hepatopancreatic tubules. Claw muscle of infected animals contained the least amount of parasites.

INTRODUCTION

The simplest method for the late-stage detection of *Hematodinium* sp. infection of crustaceans is external assessment of the carapace and arthrodial membranes for signs of

discolouration and changes in opacity. Macroscopic diagnosis has been employed successfully in field-based diagnosis of *Hematodinium* sp. infections in the Norway lobster *Nephrops norvegicus* (Field et al. 1992, Tärnlund 2000, Briggs & McAliskey 2002), Tanner crabs *Chionoecetes bairdi* (Meyers et al. 1987, 1990) and snow crabs C*hionoecetes opilio* (Taylor & Khan 1995, Dawe 2002, Pestal et al. 2003, Shields et al. 2005, 2007). Macroscopic identification has also been used to diagnose infections in brown crabs *Cancer pagurus*, in which it is known as pink crab disease due to a hyperpigmentation of the carapace in patent infections (Stentiford et al. 2002). While it may be the most efficient, cost effective and least invasive diagnostic method available, visual diagnosis can lack sensitivity and provides little quantitative information on the severity of infection within the host. Pleopod staging is a technique used in *Hematodinium* sp. surveys of *N. norvegicus* (Field et al. 1992, Tärnlund 2000, Stentiford et al. 2001b, Briggs & McAliskey 2002) which involves assessment of a pleopod for aggregations of parasites. As the number of dinoflagellates increases with severity in the haemocoel, observers subjectively assign an infection score based on the degree of cell accumulation visible within the pleopod (Field et al. 1992, Field & Appleton 1995). Although the sensitivity of pleopod staging in Scottish *N. norvegicus* is approximately 50 % higher than body colour assessment methods (Stentiford et al. 2001b), its use is limited to *N. norvegicus* and juvenile portunid hosts whose pleopods are sufficiently flattened to allow light penetration by a microscope (Messick 1994).

A more reliable method for diagnosing *Hematodinium* sp. infections is microscopic evaluation of haemolymph smears, this having the additional benefit of being suitable for archiving. The technique requires minimal training and is universally applicable to a variety of crustacean species (for review see Stentiford & Shields 2005). Histological

stains such as Giemsa or haematoxylin and eosin are routinely used to visualise and differentiate between trophont, plasmodial, and dinospore stages of *Hematodinium* sp. (see Meyers et al. 1987, Messick 1994, Wilhelm and Mialhe 1996, Ní Chualáin et al. 2009) While haemolymph smears are somewhat laborious to prepare they provide a clear understanding of the severity of the disease at any one time as both infection prevalence and relative intensity can be determined (Ní Chualáin et al. 2009).

The need for greater sensitivity in *Hematodinium* sp. diagnoses has led to the development of an indirect immunofluorescent antibody technique (IFAT) using polyclonal antibodies derived from cultured vegetative forms of *Hematodinium* sp. isolated from Norway lobsters *Nephrops norvegicus*. The specific fluorescent labelling provided by the IFAT revealed the presence of *Hematodinium* sp. in lobsters previously deemed uninfected by both pleopod staging and haemolymph staining without reacting to host tissues or haemolymph (Field & Appleton 1996). Following the development of the IFAT, other immunodiagnostic techniques have since been developed including a Western blot method (Stentiford et al. 2001b) and an ezyme linked immunosorbent assay (ELISA) (Small et al. 2002); however, there is concern that these techniques may not recognise all *Hematodinium* sp. life stages or those infecting other species (Small et al. 2006).

Polymerase Chain Reaction (PCR) diagnostics to detect *Hematodinium* spp. have been developed for several crustacean host species (Hudson & Adlard 1994, Gruebl et al. 2002, Small et al. 2006, 2007a, 2007b). Primer sets developed by Hudson and Adlard (1994) and Gruebl et al. (2002) using the conserved 18S and 5.8S ribosomal RNA genes are genus specific for *Hematodinium* sp., whereas primer sets developed by Small et al. (2006) using the conserved 18S RNA gene and variable ITS1 region of the RNA gene complex are

species-specific for *Hematodinium* sp. infecting *Nephrops norvegicus, Cancer pagurus, Pagurus bernhardus* and *Chionoecetes opilio.* Ribosomal DNA (rDNA) has frequently been used to distinguish between species of dinoflagellates (Rowan & Powers 1992, Murray et al. 2005). Conserved regions such as the 18S and 5.8S are useful in elucidating higher level phylogenies, while variable regions such as the internal transcribed spacer regions may be used to identify phylogenies in closely related taxa (Hillis & Dixon, 1991).

Of the pathogens and parasites known to infect the brown crab *Cancer pagurus* (for review see Stentiford 2008), pink crab disease caused by *Hematodinium* sp. is the most significant disease to affect *C. pagurus* in Ireland. Prior to its discovery in Ireland in 2004, 'black spot' or shell necrosis was the primary disease encountered by fishers, with affected crabs comprising approximately 4 % of the annual catch between 1996 and 1997 in the northern offshore fishery (Cosgrove 1998). Black lesions or spots on the exoskeletal surface of affected crabs are the result of epicuticular penetration by chitinolytic bacteria and the melanisation of affected regions (Vogan et al. 1999). Such crabs are considered aesthetically unappealing and are discarded by the fishery. Prevalence of *Hematodinium* sp. infection however, has been shown to reach epizootic levels seasonally in brown crab fisheries, raising concern that the disease could have a significant negative impact on the accuracy of any stock assessment exercises and more importantly the sustainability of stocks under fishing pressure.

Recently Ní Chualáin et al. (2009) discussed the importance of continuous monitoring programmes for both prevalence and infection intensity of *Hematodinium* sp. in Irish crustacean fisheries. Although haemolymph smears have been used to monitor *Hematodinium* sp. infections in Irish crustacean fisheries in recent years, the sensitivity of

diagnosis has yet to be evaluated in the most susceptible species recorded to date, *Cancer pagurus*. As a result, infection intensity values based solely on peripheral haemolymph may not reflect infection intensity within the entire crab, account for sub-patent infections or accurately diagnose infected individuals at all. The primary aims of this study were (1) to validate the accuracy of haemolymph smears as a diagnostic for *Hematodinium* sp. in *Cancer pagurus* using a PCR protocol described by Gruebl *et al.* (2002), (2) to investigate the presence of *Hematodinium* sp. in tissues other than the haemolymph and (3) to compare infection intensity values recorded from haemolymph smears with those obtained from representative histological sections. The overall objective was to provide data to Irish fisheries managers that aided in defining the most suitable methods to adopt for ongoing monitoring of the parasite.

METHODS

Cancer pagurus were captured in commercial fishing creels off the coast of Malin Head, in the North of Ireland in September and November 2008 and transported live to the laboratory. Both months were chosen to represent periods of low and high infection intensity respectively, as described by Ní Chualáin et al. (2009). Sex, carapace width (CW), moult status and macroscopic signs of infection were recorded for each crab. Sterile 19 ga. needles on 1 ml syringes were employed to extract 0.7 ml haemolymph from the arthrodial membrane at the junction of the basis and ischium of one of the walking legs. Haemolymph (0.5 ml) was immediately fixed in chilled 4 % neutral - buffered formalsaline; the remaining 0.2 ml was fixed in EtOH for molecular analysis. Smears were prepared by drying the fixed haemolymph on a clean slide, and were subsequently stained with Giemsa (Humason 1979). Smears were examined in random order with an Olympus BX41 compound microscope, without reference to field sampling data.

Crabs were anaesthetised prior to dissection by chilling on ice for 30 min and then pithed. Excisions of the gill, heart, midgut, hepatopancreas, muscle, and gonad were immediately fixed in Davidson's seawater fixative (20 ml formalin, 10 ml glycerol, 10 ml glacial acetic acid, 30 ml 100 % ethanol, 30 ml seawater) for 24 hr and subsequently transferred into 70 % EtOH. Tissues were dehydrated in a series of ethyl alcohol solutions and infiltrated with paraffin. Tissue sections (5 µm thick) were stained with haematoxylin and counterstained with eosin (H&E). Stained sections were analysed using an Olympus BX41 compound microscope and digital images were obtained using an Olympus E330 camera and Olympus Cell* imaging software.

Ethanol - preserved haemolymph samples were shaken to redistribute settled material and a 300 µl aliquot from each was transferred to a new 1.5 ml tube. Tubes were centrifuged for 5 min at 1600 g, the supernatant was then removed and the pellet allowed to air dry to allow evaporation of residual ethanol. Genomic DNA from the pellet was extracted and purified using a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions for animal tissue. DNA concentrations and purity were estimated by measuring the 260/280 optical density ratios using a BioPhotometer (Eppendorf).

The reaction mixture for PCR comprised 1 µl template DNA, 10 µl of GoTaq Green Master Mix (Promega), 0.25 µM of each primer and nuclease free water to a 20 µl final reaction volume. *Hematodinium*-specific primers Hemat-F-1487 (5' –cct ggc tcg ata gag ttg) and Hemat-R-1654 (5'-ggc tgc cgt ccg aat tat tca c) were as described by Gruebl et al. (2002). Thermocycling conditions were as follows: an initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 1 min and extension at 72°C for 1 min with the final cycle incorporating a 10 min extension

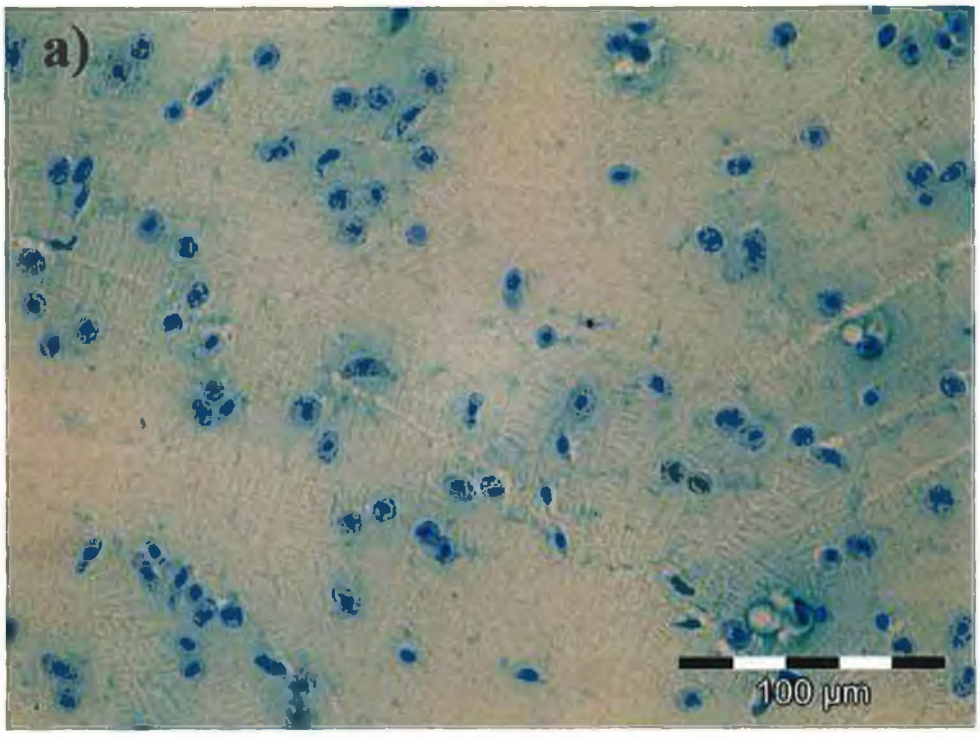
step at 72°C. Amplification reactions were carried out in a PxE thermal cycler (Thermo). Representative amplicons were purified using a QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions and sequenced commercially by Eurofins MWG Operon (Ebersberg, Germany) using the *Hematodinium*- specific forward and reverse primers described above. Sequence searches were performed using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) GenBank database.

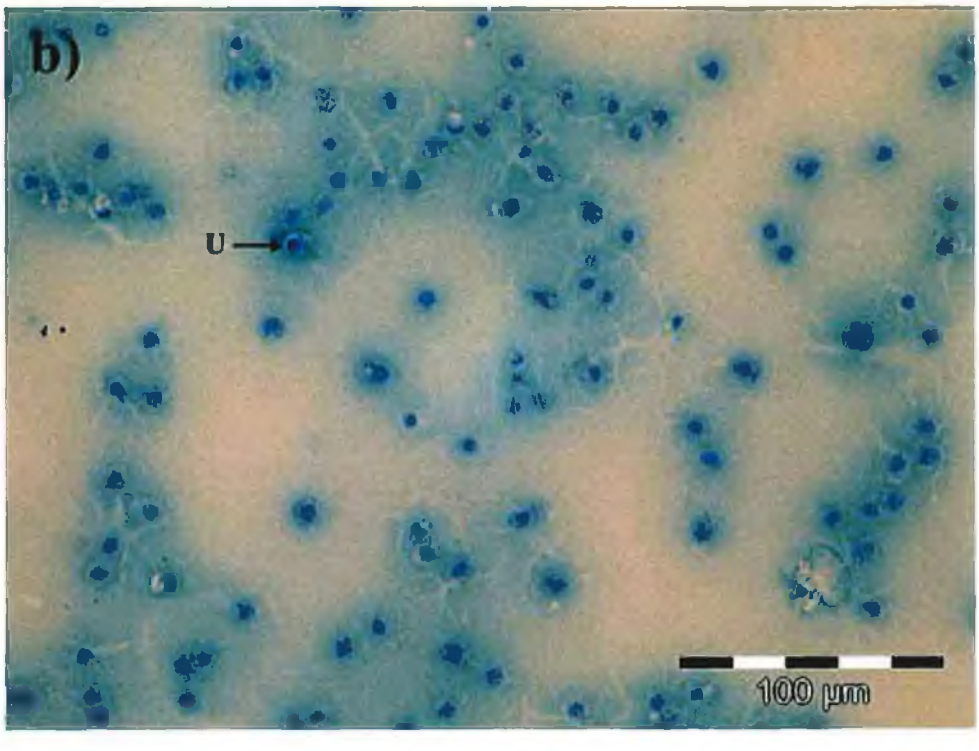
Following amplification, PCR products were electrophoresed on a 2 % (w/v) agarose gel made with 1X Tris-borate EDTA (TBE) buffer with 10 µl of SYBR® Safe per 100 ml TBE. Lanes 1 and 19 typically contained 5 µl 100 bp molecular weight markers (Promega). Gels were visualised and photographed using a DNR Bio-Imaging system. The sensitivity of the PCR assay was determined by amplifying serial dilutions of genomic DNA (100 – 0.1 ng) from the crab with a low infection intensity value (1 %) which was calculated microscopically from haemolymph smears.

Infection prevalence was expressed as a percentage by dividing the number of crabs infected with *Hematodinium* sp. by the number of crabs examined (Bush et al. 1997). Infection intensity of haemolymph smears was defined as the percentage of *Hematodinium* sp. cells counted among haemocytes in a total of 300 cells from 5 fields of view as in Messick & Shields (2000). Mean intensity for each sampling period was the sum of intensities of infected crabs divided by the number of infected crabs (Sheppard et al. 2003). Infection intensity of histological sections as described by Wheeler et al. (2007) was as follows: light infection (1-5 parasites per microscopic field at 200X), moderate infection (5-20 parasites per field), and advanced infection (over 20 parasites per field).

RESULTS

Examination of haemolymph smears revealed that 8 of 76 (11 %) crabs were parasitized by *Hematodinium* sp. in September 2008, none of which presented outwardly visible signs of infection. In all cases smears from infected specimens were dominated by the host's haemocytes and the mean infection intensity was 9 %. In November, 9 of 71 (13 %) crabs were positive for *Hematodinium* sp. infection with a mean infection intensity of 48 %. Three crabs from this sample were severely lethargic and exhibited a pink colouration of the carapace. There was no significant difference in infection intensity between months (Kruskal-Wallace, $p > 0.05$). Extracted haemolymph from highly infected hosts in November was thick and cream-colored as opposed to the translucent form found in healthy individuals. Smears from infected individuals in both months contained mainly uninucleate trophonts with a few dividing cells (Figure 3.1). In light infections, trophonts were distinguishable from host haemocytes by their darkly staining nuclei. Heavy infections were characterised by an increase in bi-nucleate and multinucleate plasmodia accompanied by a severe reduction in host haemocytes. The permanently condensed chromosomes, unique to dinoflagellates were readily observed in *Hematodinium* sp. cells (Figure 3.1d).


a)
100 µm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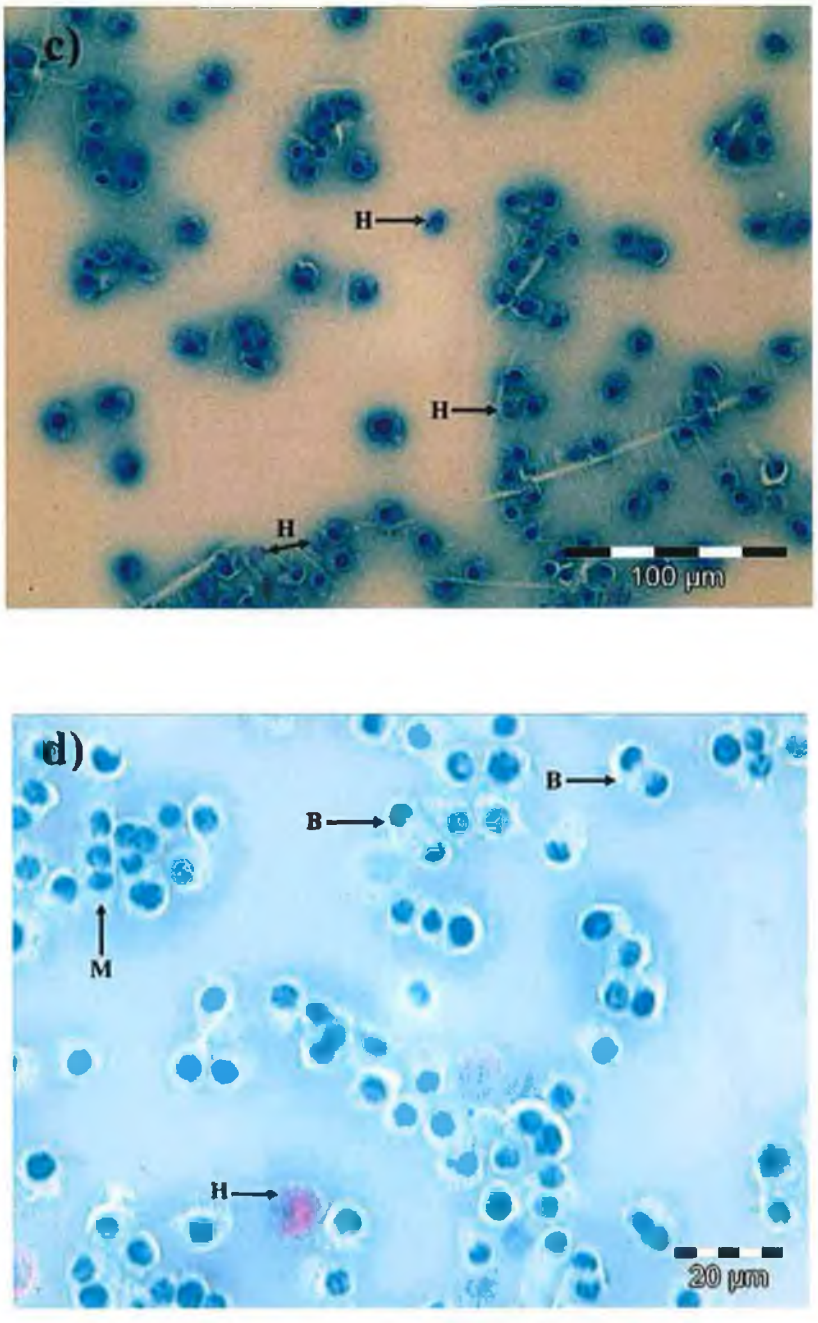

Figure 3.1. Representative life history stages of *Hematodinium* sp. present in the haemolymph of *Cancer pagurus*. **a)** Healthy haemocytes. **b)** Haemocytes of crab with light infection (1 %), note the darkly stained uninucleate trophont (U) surrounded by haemocytes. **c)** Heavy haemolymph infection (75 %) note the scarcity of host haemocytes (H). **d)** Heavy haemolymph infection (87 %), note the binucleate (B) and multinucleated plasmodia (M) and the characteristic condensed chromosomes of dinoflagellates. Host haemocytes (H) are rare.

During dissection, the internal organs of highly infected hosts were yellow in appearance and in severe cases such was the alteration in colour, texture and shape that differentiation between organs was sometimes difficult. Advanced infections (20+ parasites per field) were encountered most frequently in histological sections (Table 3.1). Infections in November were more severe with sheet- like forms of *Hematodinium* sp. containing thousands of parasites in single microscopic fields of view. Vegetative stages of *Hematodinium* sp. occupied the vascular spaces of the gill axis and adjoining filaments and in advanced infections host haemocytes were dominated by vast numbers of plasmodia free in the haemolymph (Figure 3.2). Aggregations of the parasite appeared to separate the myocardial bundles of the heart while relatively few were found in the pericardial lining (Figure 3.3). The spongy connective tissue adjacent to the midgut wall contained masses of plasmodia along its length. Parasites surrounded the gut wall and reserve (RI) cells in particular, but none were found in the contents of the midgut lumen (L) (Figure 3.4). In the hepatopancreas, *Hematodinium* sp. cells were observed between tubules and surrounding RI cells. The haemal sinuses between tubules appeared enlarged compared with healthy crabs presumably from oedema and pressure necrosis from the masses of parasites in the haemolymph. Parasites were not observed within the lumen of hepatopancreatic tubules (Figure 3.5). Muscle tissue was the least affected of the tissues examined, with over 50 % of infected crabs showing light infections in the interstices of muscle blocks, despite having other organs heavily populated with the parasite. In advanced infections the haemal sinuses were dilated causing the muscle blocks to separate (Figure 3.6). Within the ovaries of infected crabs oocytes were separated by aggregates of *Hematodinium* sp. cells but individual oocytes were not penetrated by the parasites. *Hematodinium* sp. infections were not encountered among developing or mature ova rather the parasites were in the haemal sinuses supplying the connective tissues (Figure 3.7).

Table 3.1. Infection intensity values calculated from *Cancer pagurus* haemolymph smears with corresponding tissue infection levels and diagnosis by PCR. *: Initially uninfected but deemed positive for infection following second inspection. +: light infection (1 – 5 parasites per field of view at 200X). ++: Moderate infection (5 – 20 parsites per field of view at 200X). +++: Advanced infection (20 + parasites per field of view at 200X). -: no infection. +?: inconclusive. /: no sample. Hep: Hepatopancreas

Month	Haemolymph Infection Intensity (%)	Gill	Heart	Gut	Hep	Muscle	Gonad	PCR
Sept	-	+?	+?	-	-	-	-	- ve
Sept	0.7	+++	+++	+++	+++	+++	+++	+ ve
Sept	2*	+++	+++	+++	+++	+++	+++	+ ve
Sept	3	+++	+++	+++	+++	+	+++	+ ve
Sept	4	+++	+++	+++	+++	+	+++	+ ve
Sept	5	+++	+++	+++	+++	+	+++	+ ve
Sept	9	+++	+++	+++	+++	+	+++	+ ve
Sept	16	+++	+	+	++	+	++	+ ve
Sept	17	+++	+++	+++	+++	+++	+++	+ ve
Sept	22	+++	+++	+++	+++	+++	+++	+ ve
Nov	1	+++	+++	+++	+++	+++	+++	+ ve
Nov	3	+++	+++	+++	+++	+	+++	+ ve
Nov	5	+++	+++	+++	+++	+	+++	+ ve
Nov	38	+++	+++	+++	+++	+++	+++	+ ve
Nov	59	+++	+++	+++	+++	+++	+++	+ ve
Nov	75	+++	+++	+++	+++	+++	/	+ ve
Nov	83	+++	+++	+++	+++	+++	+++	+ ve
Nov	83	+++	+++	+++	+++	+++	+++	+ ve
Nov	87	+++	+++	+++	+++	+++	+++	+ ve

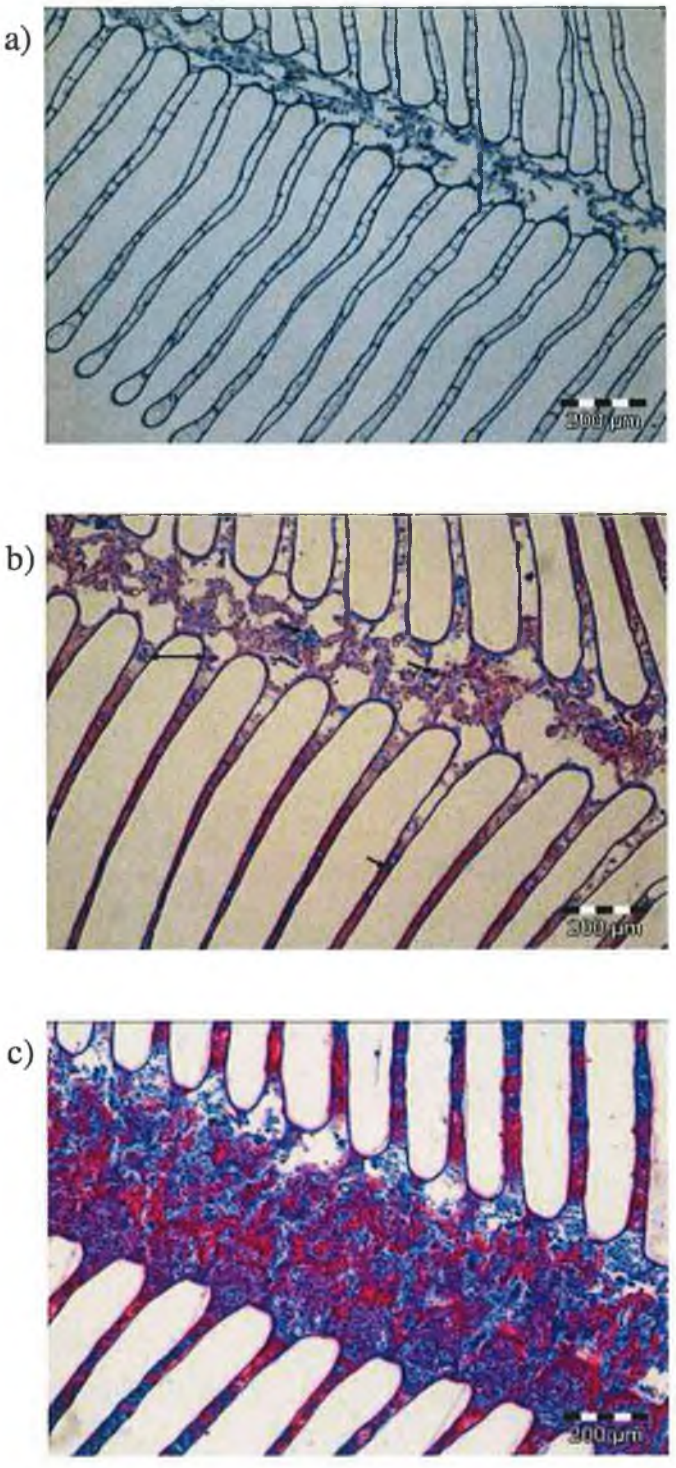

Figure 3.2. Comparative gill tissue from a) uninfected *Cancer pagurus* b) light infection (0.7 %) as calculated from haemolymph smears, note the trophonts in the axis and gill filaments (arrows) c) advanced infection (38 %) as calculated from haemolymph smears.

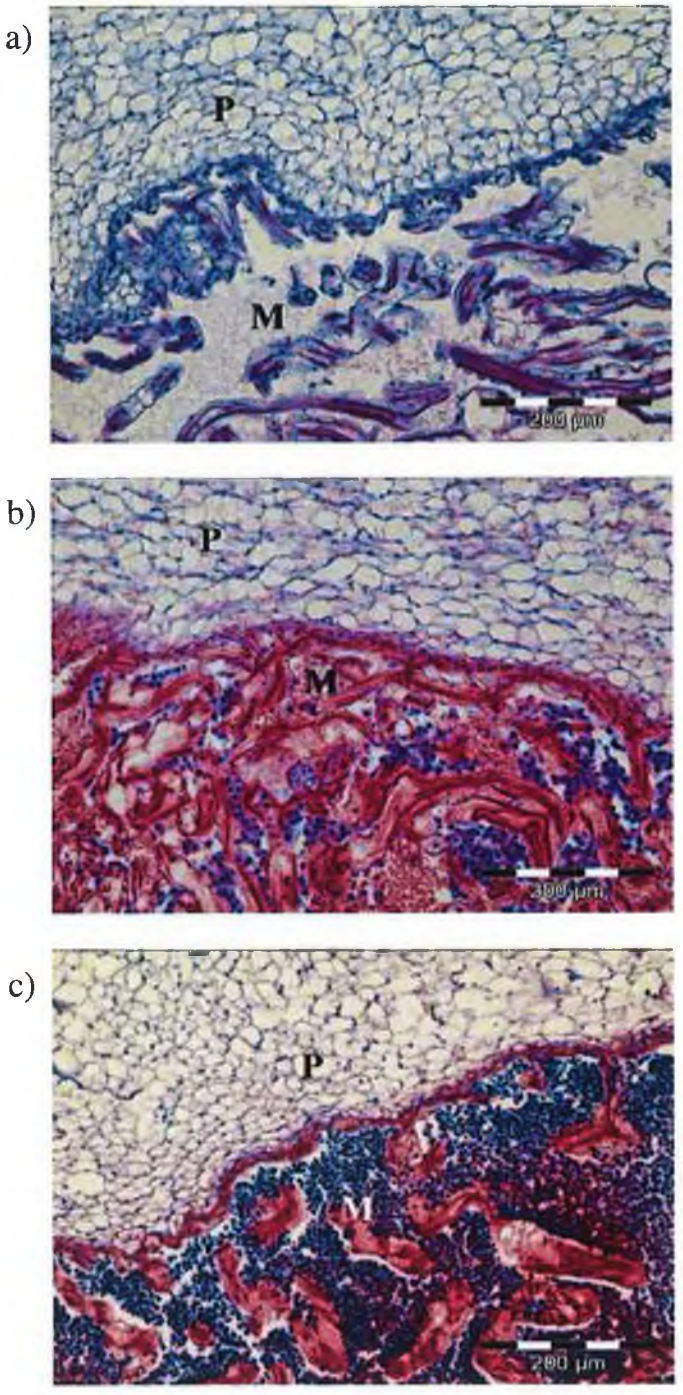

Figure 3.3. Comparative heart tissue from a) uninfected *Cancer pagurus* b) light infection (0.7 %) as calculated from haemolymph smears, c) advanced infection (83 %) as calculated from haemolymph smears, note the trophonts in the myocardial muscle (M) and absence of parasites in the pericardial lining (P) in both light and heavy infections.

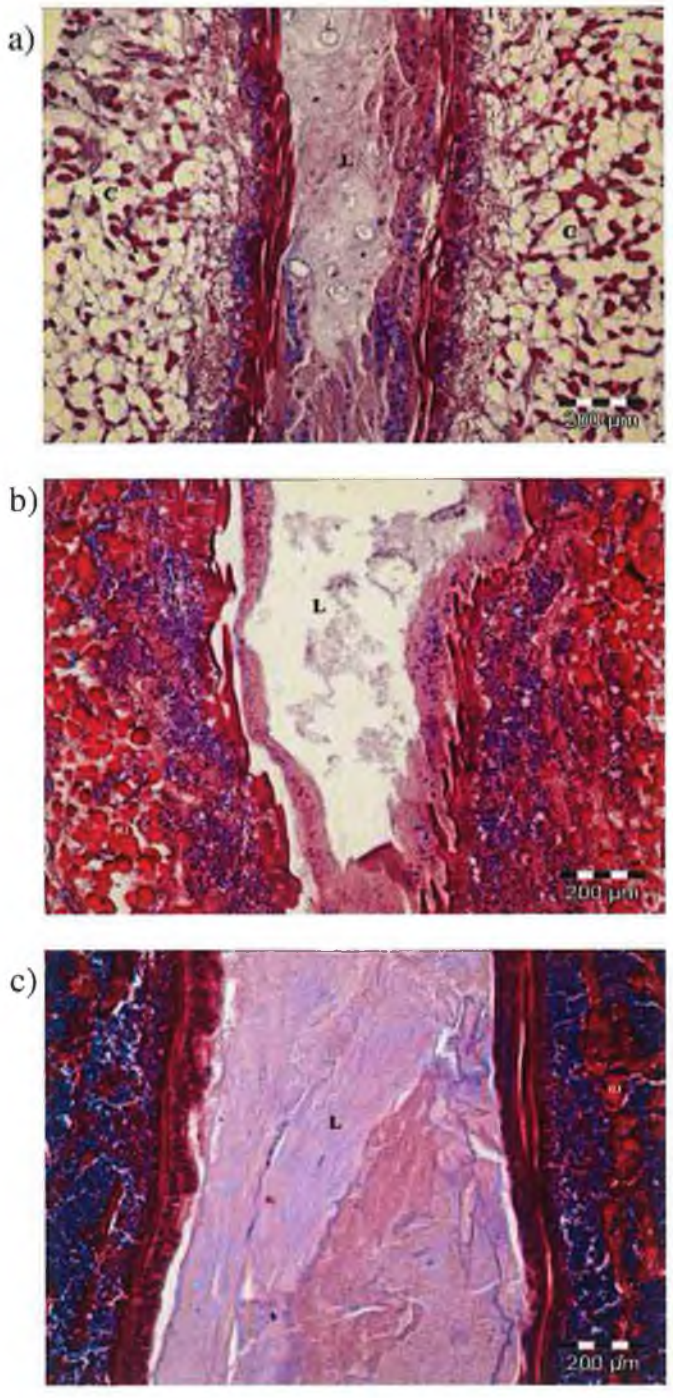

Figure 3.4. Comparative longitudinal midgut section of a) uninfected *Cancer pagurus* b) light infection (0.7 %) as calculated from haemolymph smears, c) advanced infection (83 %) as calculated from haemolymph smears. Note the absence of parasites in the midgut lumen (L) in contrast to the dense aggregations in the connective tissue (C) either side of the gut wall. Note the presence of reserve (RI) cells in infected sections.

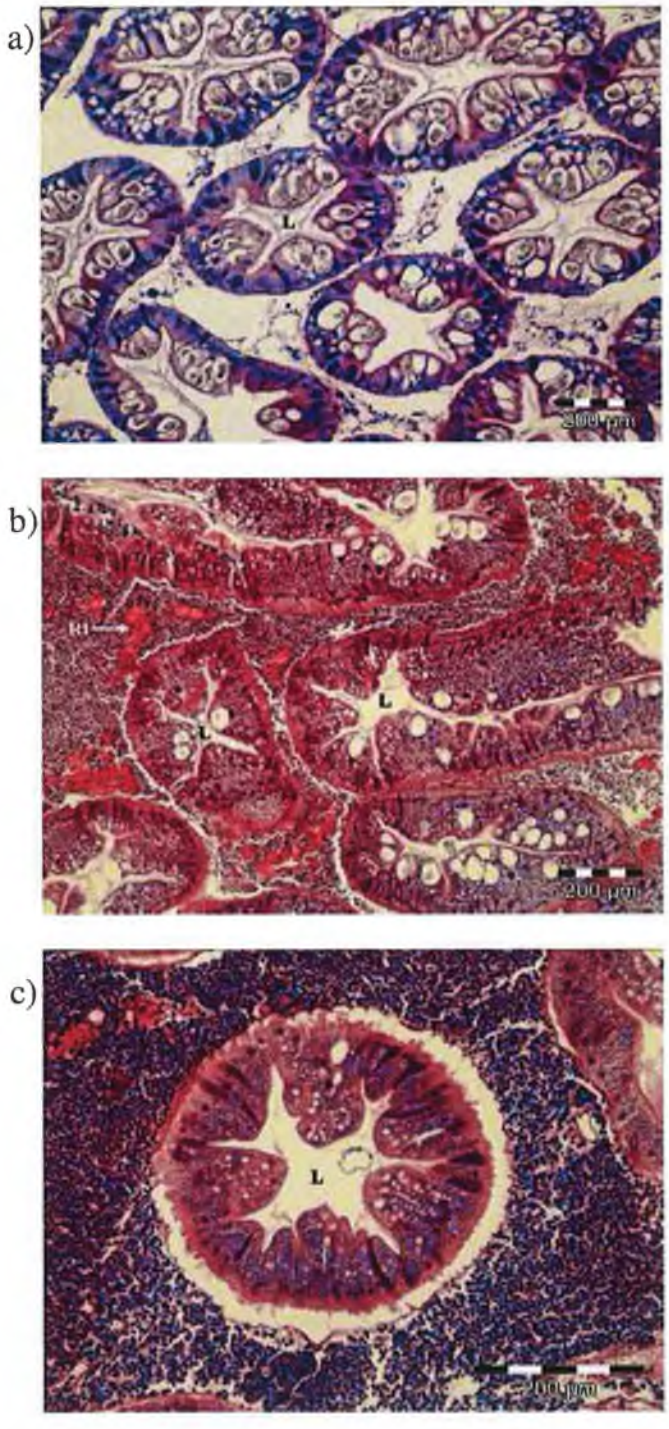

Figure 3.5. Comparative cross section through hepatopancreatic tubules of a) uninfected *Cancer pagurus* b) light infection (1 %) as calculated from haemolymph smears c) advanced infection (83 %) as calculated from haemolymph smears. Note the absence of parasites in the lumen (L) Note the presence of reserve (RI) cells in infected sections.

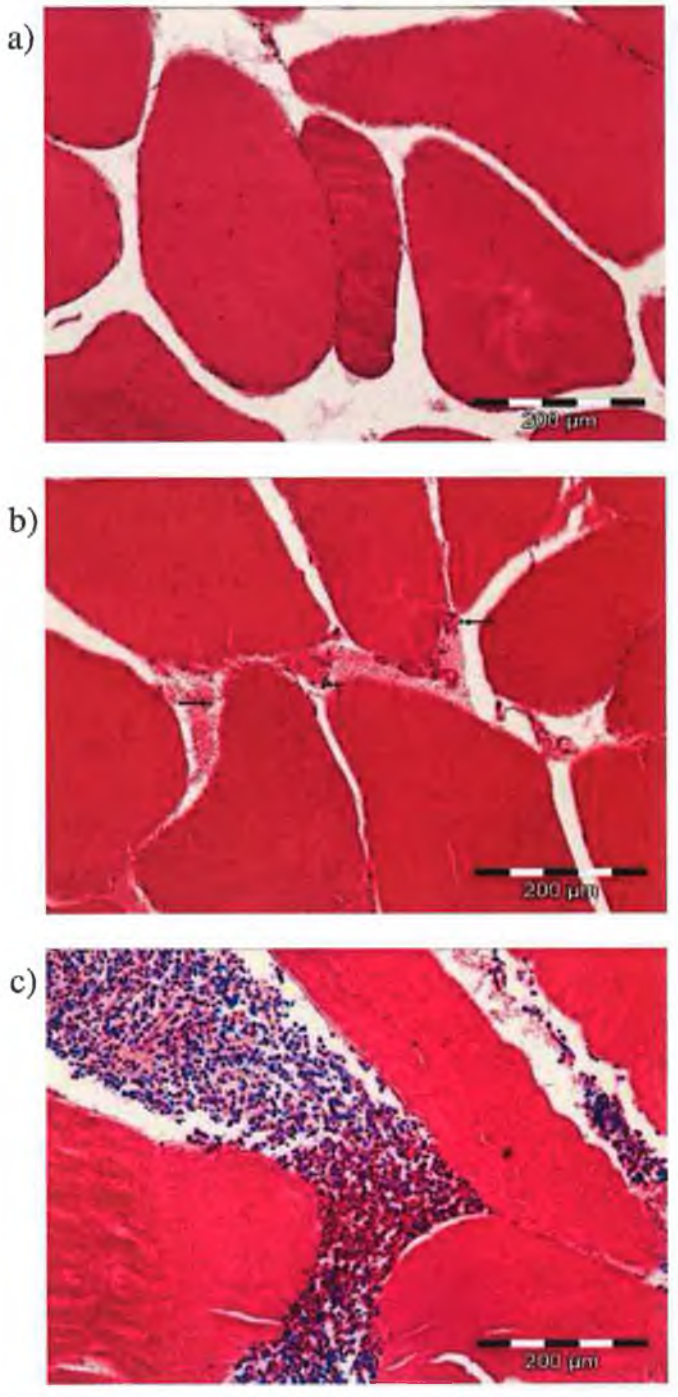

Figure 3.6. Comparative sections of claw muscle a) uninfected *Cancer pagurus* b) light infection (3 %) as calculated from haemolymph smears, note the trophonts present in haemal sinuses between muscle bundles (arrows) c) advanced infection (83 %) as calculated from haemolymph smears. Note the separation of muscle bundles in advanced infections.

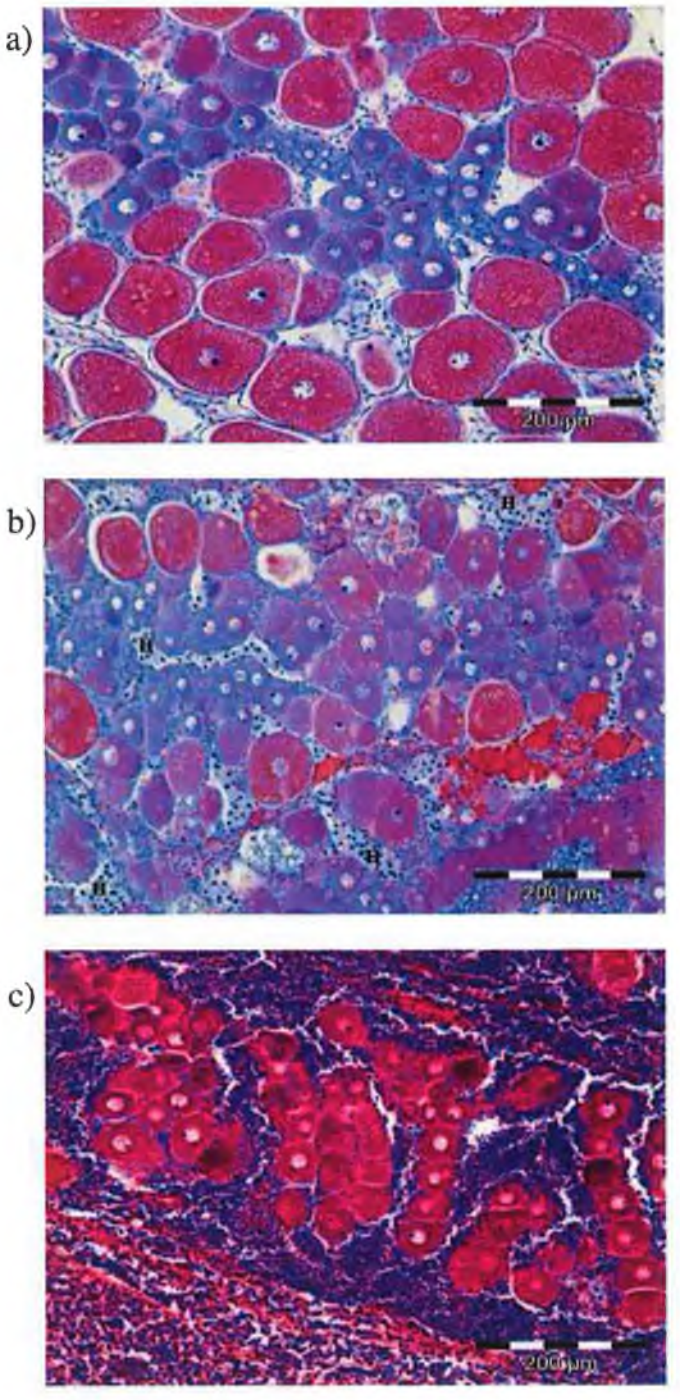

Figure 3.7. Comparative ovary section in female *Cancer pagurus* a) uninfected *Cancer pagurus* b) light infection (2 %) as calculated from haemolymph smears, note *Hematodinium* sp. cells (H) in small aggregations between oocytes c) advanced infection (83 %) as calculated from haemolymph smears. Note the separation of oocytes.

A fragment of the 18S rRNA gene from *Hematodinium* sp. was routinely amplified using primer sequences described by Gruebl et al. (2002). A single 187 - bp amplification product was produced in crabs parasitized by *Hematodinium* sp. while no reaction products were evident in uninfected crabs (Figure 2.8). Representative 18S rRNA gene fragments amplified from infected crabs had a 100 % sequence similarity to comparable gene fragments of *Hematodinium* sp. from a variety of crustacean hosts available in the public sequence database. The sensitivity of the PCR assay was estimated to be 10 ng genomic DNA for crabs with 1 % infection intensity levels.

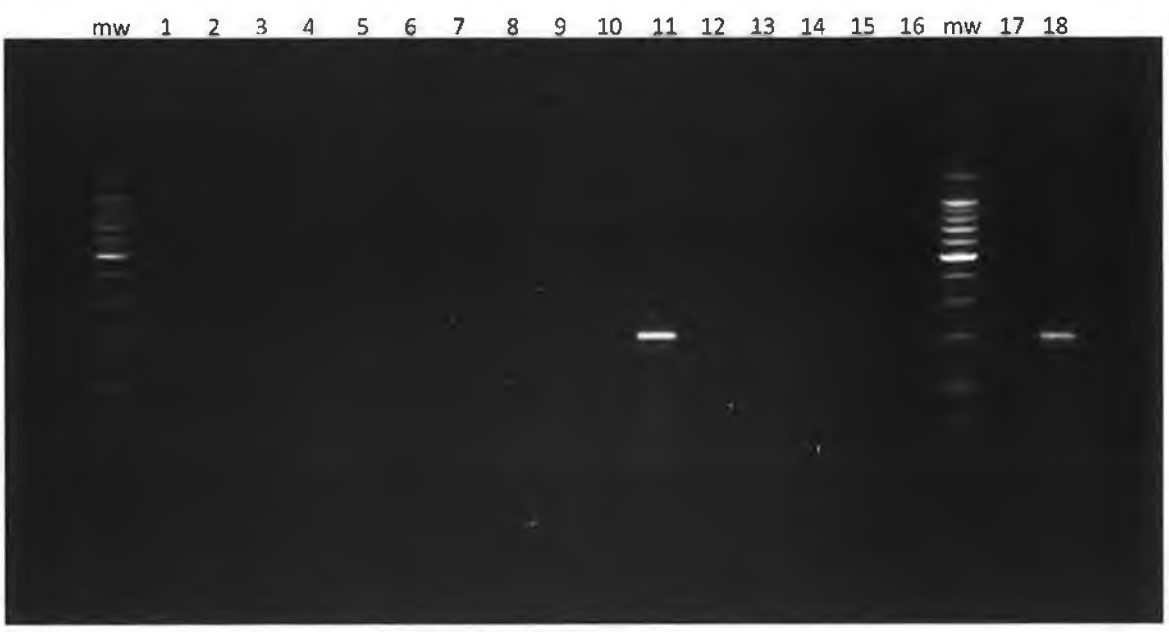

Figure 3.8 Agarose gel showing 187 bp amplification products in lanes 11 and 18 from the haemolymph of crabs infected with *Hematodinium* sp. No amplification product was seen in uninfected crabs (lanes 1-10, 12-16). Lane 17 was a negative control which did not contain genomic DNA. Molecular weight (mw) markers are shown.

All infections diagnosed by haemolymph smears were found to be positive by PCR; conversely, not all infections identified by PCR were detected in haemolymph smears (Table 3.1). One sample from September deemed negative for infection by microscopic examination of the haemolymph produced a strong band when PCR was performed. Upon re-examination, cells from the haemolymph smear were found to be poorly stained and the fixed haemolymph was subsequently re-smeared, stained and examined. On second examination, vegetative stages of the parasite were apparent. Two tissue sections from a second crab in September containing cells resembling *Hematodinium* sp. trophonts were tentatively diagnosed as 'possibly infected' however its corresponding haemolymph smear was devoid of parasites and no diagnostic band was produced in PCR. All crabs from November diagnosed as infected using haemolymph smears contained parasites in all of their tissues and each produced a diagnostic band when PCR was performed. Overall, of the 147 crabs examined for the presence of *Hematodinium* sp., an error of 0.7 % (1 / 147) was associated with the haemolymph smearing technique while 0.7 % (1 / 147) was diagnosed incorrectly using histology. Both errors were resolved using PCR and re-analysis of the one animal in question via cytology showed it to be infected. Infection intensity values obtained from haemolymph smears ranged from 0.7 – 87 % while nearly all (90 %) of the parasitized tissues contained advanced infections. Light infections (< 2 %) in haemolymph smears did not correspond to light infections in tissue sections (Table 3.1).

DISCUSSION

Cytological, histological and molecular data presented in this study showed that detection of *Hematodinium* sp. in brown crabs is possible even at very low infection levels, however each method required specialist knowledge and/or equipment, and had a higher associated economic cost when compared to identification of patent infections. Microscopic

evaluation of haemolymph smears in the present study provided almost equivalent accuracy in infection diagnosis as histology and PCR, but did not require sacrificing animals or as high a processing cost once training was completed. Diagnosing infections by the haemolymph smears and histology methods required considerable training and validation for slide readers due to the resemblance of *Hematodinium* sp. trophonts to host haemocytes. While PCR afforded the removal of subjectivity from analyses when compared to the other methods, it required expensive equipment, processing and provided no information on the life history stage of the pathogen.

Until now examination of tissues other than haemolymph has not been employed in Irish monitoring programmes for *Hematodinium* sp. While advanced infections were present in nearly all of the parasitized tissues examined in the present study, the corresponding haemolymph intensity values ranged from < 1 – 87 %. This would suggest that *Hematodinium* sp. infection within the whole crab can be much more severe than indicated by haemolymph smears alone. Negative alteration of texture and taste has long been associated with macroscopically identifiable infections of *Hematodinium* sp. in *Cancer pagurus* and other host species (Meyers et al. 1987, Latrouite et al. 1988, Field et al. 1992, Ní Chualáin et al. 2009) however taste trials have not been undertaken to date in hosts with latent infections. There is concern that pathological alterations to tissues such as the commercially important hepatopancreas and claw muscle may occur considerably sooner than the development of external signs of infection or possibly even before parasites are microscopically detectable within the haemolymph. This could adversely affect consumer confidence if unpalatable products reached markets, but the socio-economic impact of the parasite is less often considered than the biological and additionally is difficult to quantify at this time.

Pathological alterations to *Cancer pagurus* tissues parasitized by *Hematodinium* sp. were similar to those described by Stentiford et al. (2002). The parasite was present extracellularly as trophont, plasmodial and sheet-like forms within the haemal spaces of all tissues. In advanced infections haemal spaces appeared dilated and infiltration by masses of parasites had caused the separation of individual hepatopancreatic tubules, myocardial muscle bundles and oocytes. In many cases, this condition was accompanied by pronounced haemocytopoenia. In contrast to Stentiford et al. (2002), reserve (RI) cells were often present in large numbers even in advanced sheet-like infections, an indication that infections were probably still progressing. This is supported by Ní Chualáin et al. (2009) who reported the occurrence of patent, late-stage infected, dying or dead 'pink' coloured crabs only very late in the year. The pink colouration associated with late-stage infected crabs is thought to be due to depletion of glycogen which is stored in reserve cells of the host (Stentiford & Shields 2005). There was no evidence to suggest the gut as an infection route as no parasites were found occupying tubule lumens of the hepatopancreas nor were they found in the contents of the midgut lumen. In the present study, the occurrence of aggregations of parasites in the midgut, gill, heart and hepatopancreas sections regardless of haemolymph intensity may support the theory that haemal sinuses in these organs act as loci for the development of infections before they become detectable in the haemolymph (Field & Appleton 1995, 1996, Stentiford et al. 2001c, 2002), although the latency period of *Hematodinium* sp. in this host has yet to be fully elucidated.

Currently in Ireland, advice relating to measures intended to reduce the spread of *Hematodinium* sp. infections or to reporting of associated epizootics is offered to industry on a non-mandatory basis. While management advice warns fishers against discarding infected crabs back into the sea or their use as bait in other fisheries (Tully et al. 2006),

these actions, along with reporting outbreaks, are left solely to the discretion of individual fishermen. In addition, advice currently relates only to patent infections i.e. crabs displaying macroscopic signs of infection. As patent infections are restricted to a short season, diagnosis with this method is virtually impossible for the majority of the year (Ní Chualáin et al. 2009). Removal of infected crabs from fished stocks during earlier stages of infection in an effort to reduce infection prevalence would only be possible with an alternative, more sensitive diagnostic method that also revealed sub-patent infections. In recent years the need for more sensitive diagnoses has led to an expansion in the application of molecular-based diagnoses of fish and shellfish diseases (for review see Cunningham 2002). Primer sets developed by Gruebl et al. (2002) for the detection of *Hematodinium* sp. infections in blue crabs *Callinectes sapidus* consistently amplified a ~ 187 bp fragment of the 18S rRNA gene in infected brown crabs of this study suggesting a strong similarity to *Hematodinium* sp. found in blue crabs. The same primer pair was successfully used by Sheppard et al. (2003) to diagnose *Hematodinium* sp. infections in the blue crab *C. sapidus*, spider crab *Libinia emarginata* and stone crab *Menippe mercenaria.* Those authors concluded that all hosts were infected by the same species of *Hematodinium*. However, the 18S rRNA gene is known to be highly conserved in *Hematodinium* spp. (Hudson & Adlard 1996) and therefore it is more likely they belong to the same genus (Small et al. 2007a). For monitoring purposes a genus specific PCR assay is advantageous in collating prevalence data from brown crabs and other commercially important crustaceans as it remains unknown if different species of *Hematodinium* infect different crustacean hosts or if different crustacean species represent alternate hosts for the same parasite species (Small et al. 2007a).

For the purpose of defining the complexity of future monitoring programmes it would be necessary for managers to consider relative economic cost of various methods, associated efficiency and processing time. While the PCR assay employed in the present study provided increased sensitivity when compared to cytological methods and eliminated any ambiguity associated with histological methods, the overall error associated with the latter methods was relatively low at 1.4 % (2 / 147). This is likely to be considered an acceptable level of error in reporting data to fisheries managers given that *Hematodinium* sp. prevalence in Irish brown crab fisheries can exceed 50 % (Ní Chualáin et al. 2009), although should be remembered that accurate interpretations of haemolymph smears and tissue sections are dependent on highly trained slide readers. It is possible that the merit of the PCR assay may lie in surveys undertaken earlier in the year when infection is much less progressed or for validation in the absence of trained slide readers; however PCR is costlier and also requires significant training to process and evaluate samples.

For ongoing monitoring of the parasite haemolymph smears can be considered accurate and cost effective while simultaneously providing a measure of infection intensity. In terms of practical application, haemolymph collection can be conducted quickly, requires minimal training and can be achieved safely in the field by injection into 'vacutubes' preloaded with formalin. Once fixed, samples can be stored for a number of weeks prior to processing by trained personnel using the haemolymph or PCR methods. In contrast, histological techniques required specialist training to achieve dissection and immediate fixation of tissues that cannot be easily and safely achieved in the field. For commercially harvested species, an economic cost-benefit analysis involving the value of the fishery, the financial cost of the monitoring methodology adopted and the potential increase in biological and monetary returns obtained by disease control and management would be

required to determine the technical complexity and scale (both spatial and temporal) of a monitoring programme.

CHAPTER 4

PRELIMINARY INVESTIGATION INTO *HEMATODINIUM SP.* TRANSMISSION PATHWAYS AND DISEASE PROGRESSION IN *CANCER PAGURUS*

ABSTRACT

Horizontal transmission and disease progression of the parasitic dinoflagellate *Hematodinium* sp. were investigated for the first time in the brown crab *Cancer pagurus*. Naïve *C. pagurus* were fed with conspecific tissue containing trophont stages of the parasite and monitored for up to 77 days for signs of infection in the first experiment. In a second experiment, healthy crabs were held in close proximity to *Hematodinium* sp. infected crabs and monitored for up to 43 days. Possible influences on transmission and disease progression were examined in relation to constant and variable seawater temperature in a third experiment. All attempts to transmit *Hematodinium sp.* were unsuccessful. A long pre-patent period is described with naturally infected crabs surviving for up to 232 days post- capture.

INTRODUCTION

In their original account of the type species, Chatton & Poisson (1931) described *Hematodinium perezi* as a rare parasite of the portunid crabs *Carcinus maenas* and *Liocarcinus depurator*. In the following decades, members of the genus *Hematodinium* have been encountered sporadically in various crustacean species but it was not until the 1980s that *Hematodinium* sp. gained attention as a significant pathogen of economically important crustaceans. Losses due to direct mortality and product spoiling associated with the negative alteration in texture and flavour of meat which occurs in all its hosts in advanced infections have lead to significant economic losses in numerous fisheries

worldwide (Meyers et al. 1987, Eaton et al. 1991, Field et al. 1992, Taylor & Khan 1995, Stentiford et al. 2002, Shields et al. 2005, 2007, Ní Chualáin et al. 2009). Death results from a proliferation of *Hematodinium* sp. cells within the haemolymph that places a severe metabolic load on the affected hosts (Stentiford & Shields 2005). Protein and carbohydrate reserves diminish rapidly during growth of the parasite which leads to host morbidity and eventual death (Stentiford et al. 2000, 2001b, Shields et al. 2003, Stentiford & Shields 2005). In Southeast Alaska, an estimated loss in 1986 of almost US $ 180,000 was attributed to infections caused by *Hematodinium* sp. in the Tanner crab *Chionoecetes bairdi* fishery (Meyers et al. 1987), while epizootics in the blue crab *Callinectes sapidus* fishery are responsible for annual losses of up to US $ 500,000 in Virginia, USA (JD Shields unpubl. data). In France, a near collapse of the velvet crab *Necora puber* fishery during the 1980s was attributed to infections by *Hematodinium sp.* (Wilhelm & Mialhe 1996). Most recently, *Hematodinium* sp. has been implicated as the causative agent of "milky blood disease" in cultured crabs such as the mud crab *Scylla serrata*, (Li et al. 2008) and in the Chinese swimming crab *Portunus trituberculatus* (Xu et al. 2007) and in cultured ridgetail white prawns *Exopalaemon carinicauda* (Xu et al. 2010) in China. Such infections have resulted in up to 100 % crab mortality in affected ponds in Eastern China, where a polyculture pond system for several crab species is in operation (Xu et al. 2007). Detection of *Hematodinium* sp. in Irish crustaceans has been a recent phenomenon but since its discovery in the southwest in 2004, its distribution has been shown to be widespread, with annual epizootics being recorded in all of Ireland's major brown crab *Cancer pagurus* fisheries (Ní Chualáin et al. 2009). In the latter stages of infection, glycogen depletion imparts a cooked appearance to the carapace and consequently it has been termed pink crab disease in this host (Latrouite et al. 1988, Stentiford et al. 2002).

The brown crab *Cancer pagurus* is the one of the most economically important species in the Irish inshore fisheries sector (Tully et al. 2006). Demand for live shellfish in Europe supports an extensive live export industry from Ireland and the United Kingdom for many species including brown crabs. In many cases, live crabs are stockpiled in sea-based holding pots or land-based seawater flow through systems for up to a week in high densities prior to being exported in closed seawater tanks in the back of articulated lorries, often in extremely high densities where crab volume matches that of the water available (Cosgrove 1998). Crabs may be placed into one or more in-water storage facilities and transport tanks prior to reaching the consumer in mainland Europe. Duration from capture to consumer may take anything between 7 – 30 days for crabs caught and exported from Ireland. Mortalities incurred during transport and subsequent storage can result in significant wastage and also reduce economic returns that are passed back to the original seller.

Stresses associated with emersion, physical handling, overcrowding, ammonia toxicity, low levels of dissolved oxygen, temperature and salinity changes can all affect crab survivability at many points along the marketing chain (Paterson & Spanoghe 1997, Schmidtt & Uglow 1997, Chang et al. 1999, Danford & Uglow 2001, Danford et al. 2002) but it is often the initial condition of the crab that determines its ability to survive (Uglow & Hosie 1995). Damaged and soft crabs or crabs displaying signs of shell necrosis are easily recognised and discarded but crabs harbouring parasitic infections like *Hematodinium* sp. are likely to be overlooked during the grading process as infections are not macroscopically identifiable for the majority of the year (Ní Chualáin et al. 2009). As *Hematodinium* sp. prevalence can exceed 50 % in commercial catches (Ní Chualáin et al. 2009), pre-patent infected crabs must therefore comprise a significant proportion of

products destined for European markets, but as yet it is not clear whether the parasite reduces overall crab survivability or whether it has the potential to infect other crabs in storage and transport. While little is currently known about the natural transmission pathways of *Hematodinium* spp., it is widely accepted that host ecdysis plays an important role in its pathogenicity for some species (Meyers et al. 1987, 1990, Eaton et al. 1991, Field et al. 1992, 1998, Messick 1994, Dawe 2002, Shields et al. 2005). However, there is concern that other mechanisms of infection may be possible for intermoult *Cancer pagurus* such as those destined for market, as the occurrence of light ($\leq$ 1 % infection intensity) and therefore presumably new infections is not solely restricted to the moulting season, nor to crabs which have recently moulted (C. Ní Chualáin unpubl. data).

Injection experiments using infected haemolymph as an inoculant have proven useful in examining susceptibility to *Hematodinium* sp. between host species. Meyers et al. (1987) succeeded in inoculating 100 % of experimental Tanner crabs *Chionoecetes bairdi* (n = 5) with the parasite while none of the red king crabs *Paralithodes camtschaticus* (n = 5) developed detectable infections after 155 days. Hudson & Shields (1994) conducted a similar experiment and were able to transmit by injection vegetative stages of *Hematodinium australis* between individuals of the same and different host species. Sand crabs *Portunus pelagicus* (1 out of 3 tested) and mud crabs *Scylla serrata* (2 out of 3 tested) developed infections after being injected with *H. australis* and died 16 days post-inoculation.

It is not known whether the extended period in captivity prior to consumption and the fluctuating conditions associated with storage and transport are significant enough to spoil infected but otherwise marketable crabs. Holding ponds in Ireland and receiving facilities

in Europe often utilise open-water systems that pump directly from the sea, while closed-water systems employed by vivier lorries typically transport crabs at low temperatures (8 – 10 °C) to minimise crab activity and reduce ammonia excretion. Large differences in temperature can, therefore, occur between the transport temperature and that of the importer's ponds (Uglow & Hosie 1995). Proliferation of *Hematodinium* sp. cells in *Cancer pagurus* has previously been associated with changing seawater temperatures where growth of the vegetative stages occurs throughout the warm summer months followed by the production of dinospores as seawater temperatures cool to 8 - 10 ° C in the winter (Ní Chualáin et al. 2009).

The objectives of this study were to explore possible infection routes of *Hematodinium* sp. in *Cancer pagurus* and to monitor disease progression in infected crabs. Cannibalism, cohabitation with infected crabs and fluctuating temperatures, conditions typical of storage and transportation, were examined to see if they contributed to *Hematodinium* sp. transmission in healthy *Cancer pagurus* or if such conditions were capable of exacerbating the disease in crabs already infected with *Hematodinium* sp.

METHODS

Intermoult, legal-sized *Cancer pagurus* (> 130 mm carapace width) were used for the duration of this study to represent commercial crabs destined for market. Crabs were captured in standard baited creel pots from brown crab fisheries known to contain *Hematodinium* sp. infections and transported live to the laboratory. Samples were collected during four separate months- August 2008, January, February and May 2009 due to the difficulty in obtaining infected specimens. Sex, carapace width (CW) and macroscopic signs of infection were recorded for each crab. Crabs were tagged to ensure individual

identification prior to *Hematodinium* sp. diagnosis which was confirmed by microscopic examination of stained haemolymph smears in all experiments. Diagnosis was validated for crabs in experiment 3 using PCR primers developed by Gruebl et al. (2002) which specifically amplify a 187 - bp fragment of the 18S rRNA gene of *Hematodinium*. Haemolymph was withdrawn aseptically from the arthrodial membrane at the juncture of the basis and ischium of one of the walking legs of each crab using a 1 ml syringe equipped with a 19 gauge needle and subsequently fixed in both 4 % neutrally buffered formalsaline for microscopic analysis of smears and 100 % ethanol for molecular diagnosis.

Genomic DNA extraction from ethanol preserved haemolymph samples was conducted as follows: samples were shaken to redistribute settled material and a 300 µl aliquot was centrifuged for 5 min at 1600 g. The supernatant was then removed and the pellet allowed to air dry to allow evaporation of residual ethanol. Genomic DNA from the pellet was extracted and purified using a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions for animal tissue. DNA concentrations and purity were estimated by measuring the 260/280 optical density ratios using a BioPhotometer (Eppendorf). PCR reactions were performed in 20 µl total reaction volume by adding 1 µl template DNA, 10 µl of GoTaq Green Master Mix (Promega), 0.25 µM of each primer and nuclease free water to the final reaction volume. *Hematodinium*-specific primers Hemat-F-1487 (5' –cct ggc tcg ata gag ttg) and Hemat-R-1654 (5'-ggc tgc cgt ccg aat tat tca c) were as described by Gruebl *et al.* (2002). Thermocycling conditions were as follows: an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 1 min and extension at 72 °C for 1 min with the final cycle incorporating a 10 min extension step at 72 °C. Amplification reactions were carried out in

a PxE thermal cycler (Thermo). Following amplification, PCR products were electrophoresed on a 2 % (w/v) agarose gel, stained with SYBR® Safe and viewed under UV illumination.

Haemolymph smears were prepared by air drying the fixed haemolymph on a slide and staining with Giemsa (Humason 1979) so that infection intensity and parasite morphology could be determined. Slides were examined using an Olympus BX41 compound microscope and were defined as positive for infection when at least one clearly identifiable *Hematodinium* sp. cell of any stage (i.e. trophont, plasmodia, dinospores) was observed. Once a slide was defined as positive, a total of 300 cells comprising of parasite cells and haemocytes, was counted from 5 fields of view. The percentage of *Hematodinium* sp. cells in those 300 cells defined the infection intensity of that slide. Mean intensity for initial and final assays was the sum of intensities of infected crabs divided by the number of infected crabs. Slides were blindly read three times to obtain a mean value in order to address any variation in parasite numbers over the slide area. Uninfected crabs and parasitised crabs were housed separately to ensure acclimation prior to treatments without risk of cross infection.

The first experiment was conducted to examine whether *Hematodinium* sp. can be readily transmitted to uninfected brown crabs *Cancer pagurus* through ingestion of infected crab tissue. Crabs were housed in a compartmentalised 500 L recirculating system containing artificial seawater with a salinity of 32. Temperature was maintained at 12 °C using a Seachill TR20 aquarium water chiller. Four uninfected brown crabs were fed approximately 10 g each of infected crab tissue while an identical number of control animals were fed an uninfected ration. Feeding was carried out in separate tanks of

seawater to avoid transference of parasites from infected tissue into the main experimental aquarium. Haemolymph was extracted aseptically from crabs on days 7, 15, 22, 30, 37 and 77 and examined microscopically for evidence of new infections.

A second experiment examined whether *Hematodinium* sp. could be transmitted to uninfected brown crabs through cohabitation with infected crabs. A closed recirculation system identical to the above housed 4 uninfected *Cancer pagurus* in close proximity to 2 infected conspecifics. Tank compartments isolated crabs from each other but allowed seawater to flow between them. Haemolymph was extracted aseptically from crabs on days 14, 22, 29, 37 and 44 and examined microscopically for evidence of new infections.

A third experiment examined the effect of temperature on disease transmission and progression. Crabs were held in two separate 120 L seawater recirculation systems with a salinity of 32, one of which was maintained at a constant 8 °C while seawater temperature in the other was manipulated from 8 to 16 °C and lowered to 8 °C over a period of 42 days (Table 4.1). Temperature manipulations were achieved by placing the 120 L aquarium inside a second tank and adjusting the volume of water in the outer tank which was heated by a Juwel® 200 W aquarium heater. Each system contained 2 naturally infected crabs and 6 uninfected crabs, 2 of which were injected with 250 µL infected haemolymph. Haemolymph was extracted from the 4 naturally infected crabs on days 8, 20, 30, 36 and 42 in order to examine changes in infection intensity. Four uninfected crabs from each aquarium were bled concurrently to determine if *Hematodinium* sp. infections had developed as a result of injection or cohabitation. The remaining two uninfected crabs in each aquarium served as procedural controls for the duration of the experiment and were

not examined for *Hematodinium* sp. infection until the experiment had ended. Cannibalism and fighting between crabs was prevented by placing crabs in separate baskets.

Salinity, pH, dissolved oxygen and temperature were monitored every second day while ammonia (mg/L NH_3), nitrite (mg/L NO_2^-) and nitrate (mg/L NO_3^-) concentrations were determined spectrophotometrically by the salicylate method (Hach # 8155), ferrous sulphate method (Hach # 8153) and cadmium reduction method (Hach # 8039) respectively using a Hach DR-2800 colorimeter on a weekly basis. Crabs were fed mussels *Mytilus edulis* weekly and partial water changes were conducted approximately every 10 days to remove waste and to maintain seawater quality.

RESULTS

Ingestion of *Hematodinium* sp. - infected *Cancer pagurus* tissue, cohabitation with infected *C. pagurus* and direct injection of *Hematodinium* sp. trophonts were unsuccessful in initiating new *Hematodinium* sp. infections in naïve brown crabs held in captivity (Table 4.1). Brown crabs experimentally fed with *Hematodinium* sp. infected crab tissue failed to develop infections over a 77 day period. Two crabs which had been fed infected tissue died at 3 and 11 d, while 2 control crabs died at 34 d, however examination of their haemolymph revealed no evidence of *Hematodinium* sp. infection.

Disease transmission also failed to occur through cohabitation of uninfected brown crabs with parasitised crabs. With the exception of one uninfected crab which died at 34 d, survivorship of infected and uninfected crabs was 100% after 44 d. Mean infection intensity increased slightly from 3 ± 0.7 % (SE) to 8 % ± 0.3 % (SE) from days 0 - 44

however there was considerable fluctuation in one crab where infection intensity peaked at 19 % after 22 d (Figure 4.1).

Seawater temperature manipulations had no effect on disease transmission. New infections could not be transmitted to crabs by injection of *Hematodinium* sp. cells or by cohabitation with infected crabs regardless of constant or changing seawater temperature. Infection status was examined by the haemolymph method and verified by PCR when the trial ended. Survivorship of uninfected experimental crabs was 100 % over 42 d while one naturally infected crab (crab 4) held continuously at 8 ° C died at 22 d. *Hematodinium* sp. infection intensity at time of death had increased to 66 % (Figure 4.2b). Mean intensity of naturally infected crabs exposed to changing seawater temperatures and those held continuously at 8 ° C from the initial to final haemolymph assay is shown in Figure 4.3. While trial 3 was conducted over 42 days the overall time between crab capture and the trial beginning was considerably longer (Figure 4.2). In total, infected crabs survived 232, 127, 211 and 199 days post capture in the laboratory during which time only the vegetative form (trophonts) of the parasite was encountered.

Table 4.1. *Hematodinium* sp. transmission trials in *Cancer pagurus*

Trial	Temp °C	Treatment	Survival (d)	New Infections
1	12	Ingestion of infected tissue	3-77	0/4
2	12	Cohabitation with infected *C. pagurus*	34-43	0/4
3a	8	Cohabitation with infected *C. pagurus*	42	0/2
		Injection with infected haemolymph	42	0/2
3b	8 -16 -8	Cohabitation with infected *C. pagurus*	42	0/2
		Injection with infected haemolymph	42	0/2

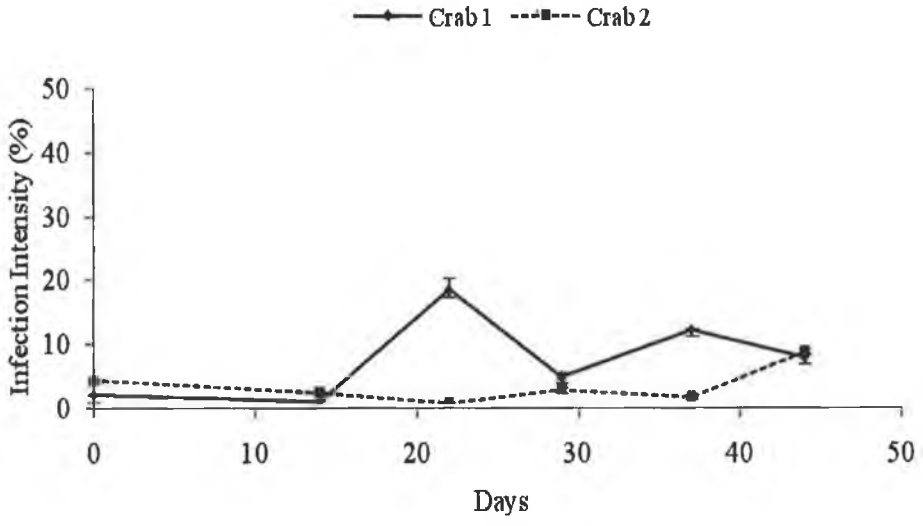

Figure 4.1. Trial 2: Mean infection intensity* of two *Hematodinium* sp. infected *Cancer pagurus* over 44 days at 12 °C. *Mean intensity for this trial refers to the mean infection intensity of each crab, obtained when the slides were read 3 times.

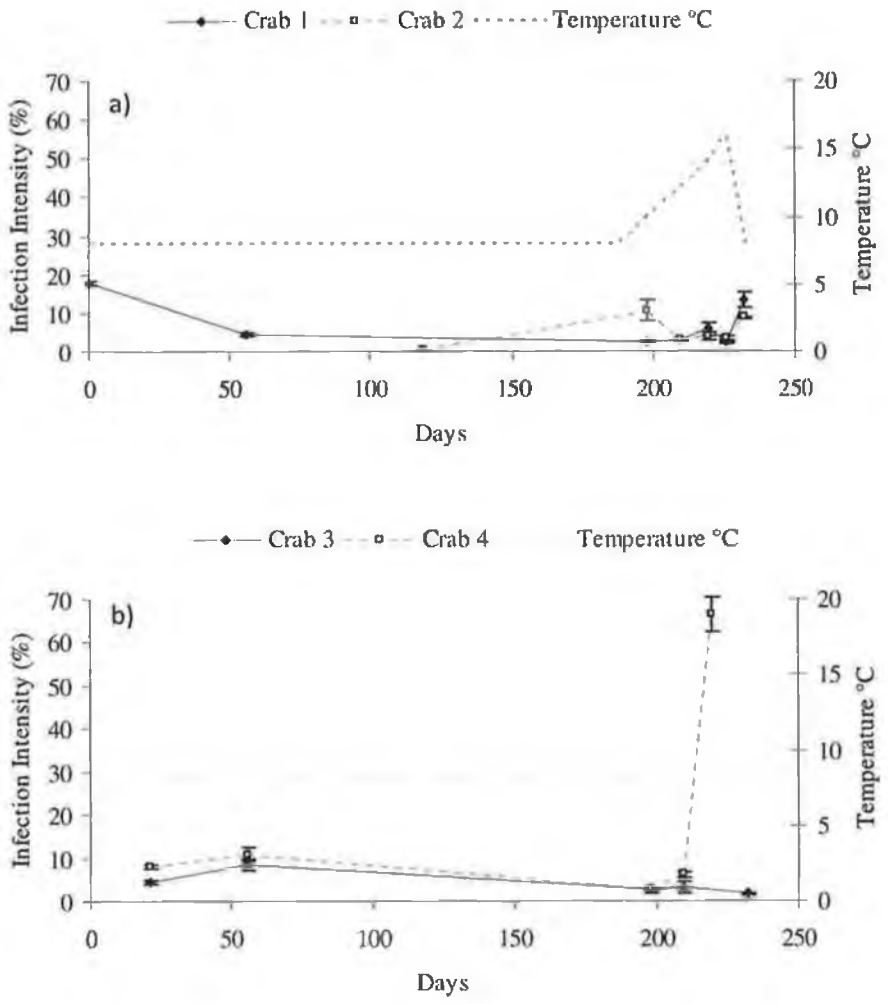

Figure 4.2. Trial 3: Mean infection intensity* (± SE) of naturally infected *Cancer pagurus* exposed to a) changing seawater temperature and b) constant seawater temperature. *Mean intensity for this trial refers to the mean infection intensity of each crab, obtained when the slides were read 3 times.

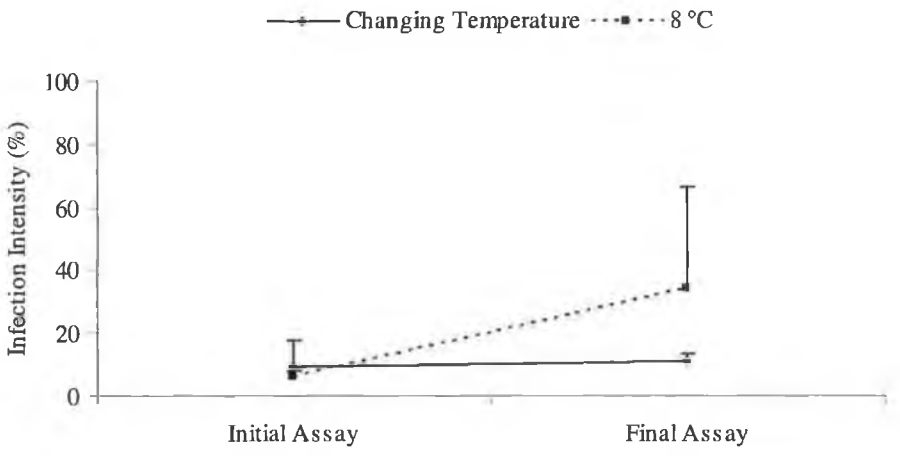

Figure 4.3. Trial 3: Mean intensity (+ SE) change over time of *Hematodinium* sp. infections in crabs exposed to changing seawater temperatures (n=2) and those held continuously at 8 ° C (n=2).

DISCUSSION

This is the first time *Hematodinium* sp. transmission and development have been examined in the brown crab host, *Cancer pagurus* and while transmission experiments proved unsuccessful, two key findings arose nonetheless: firstly that infected crabs can survive for lengthy periods and secondly that *Hematodinium* sp. infection can progress to fatality in individual crabs without apparent triggers. The apparent increase in infection intensity (from 8 % to 66 %) over time for one naturally infected crab held continually at 8 ° in trial 3 may indicate an alternative physiological factor influencing disease progression along with or independently of temperature. The long latency period of *Hematodinium* sp. in the present study (up to 232 days post capture) appears to more closely resemble that of naturally infected Alaskan Tanner crabs *Chionoecetes bairdi* which can survive for up to 158 days in captivity (Meyers et al. 1987). However, it greatly exceeds that for naturally infected snow crabs *Chionoecetes opilio* which are purportedly infected by the same species of *Hematodinium* as *Cancer pagurus* (Small et al. 2007b). Median time to death of

naturally infected snow crabs is approximately 60 days (Shields et al. 2005) and may reflect differences in host susceptibility to the parasite.

While dinospores are considered the primary infective stage of parasitic dinoflagellates (Coats 1999), the occurrence of *Hematodinium* sp. dinospores in Irish *Cancer pagurus* is limited to a short period in the winter months and does not explain the presence of new infections at other times. Trophonts and plasmodia are the most frequently encountered stages of *Hematodinium* sp. throughout the year and therefore the most likely stages to pose a transmission risk during storage and transportation. Inoculation experiments involving other crab species have shown that vegetative stages (trophonts) of *Hematodinium* spp. are capable of establishing new infections when injected into naïve Tanner crabs *Chionoecetes bairdi* (Meyers et al. 1987), snow crabs *Chionoecetes opilio* (Shields et al. 2005), blue crabs *Callinectes sapidus* (Shields & Squyars 2000) and sand crabs *Portunus pelagicus* (Hudson & Shields 1994). In Tanner crabs, new infections are detectable in the haemolymph as early as 25 days post injection (Eaton et al. 1991), whereas infections in blue crabs are detectable after 6 days (Messick and Shields 2000). While direct injection may not fully reflect a real-life transmission pathway, it nevertheless demonstrates the potential susceptibility of a species. Its relevance in the present study applies in the context of crabs damaged or injured during storage and/ or transportation. Even the slightest damage to a crab's exoskeleton through rough handling or overcrowding could become a site of haemolymph loss or point of infection by opportunistic life stages of the parasite. In particular, injuries to *Hematodinium* sp. infected crabs which may be lethal in their own right could potentially expose the remaining consignment to infection if infective stages of the parasite enter the storage seawater. Meyers et al. (1987) have

reported that vegetative stages of *Hematodinium* sp. ex. *C. bairdi* can survive for 5 d or more in seawater; however they do not comment on their infectivity after this time.

Recently Walker et al. (2009) were successful in transmitting *Hematodinium* sp. infections to naïve *Callinectes sapidus* through ingestion of conspecific tissue infiltrated with *Hematodinium* sp. trophonts. Within 6 h of feeding, single and bi-nucleate forms of the parasite were present in the connective tissue of the hepatopancreas and after 16 h *Hematodinium* sp. was detectable in the haemolymph. In the present study, the inability to initiate new infections through the use of trophonts (in tissue for feeding or in haemolymph for injecting) might suggest that either an additional *in vivo* life stage exists outside of what has currently been described or that the *Hematodinium* sp. relies on an intermediate host to be successful in completing its lifecycle. It may be that the pre-patent period of *Hematodinium* sp. infecting *Cancer pagurus* is much longer than described for other species. Equally, it is also possible that the naïve crabs used for the duration of these experiments were resistant to *Hematodinium* sp. infections. Space limitation and a paucity of naturally infected crabs resulted in small sample sizes for each of the three experiments in the present study and only commercial-sized crabs were used. Such small sample sizes have, however, resulted in demonstrating transmission success in other studies (Meyers et al. 1987, Hudson & Shields 1994). Recent studies have shown considerably higher *Hematodinium* sp. prevalences in juvenile *C. pagurus* than in commercial-sized crabs (Ní Chualáin et al. 2009) and could indicate that the latter are less susceptible to infection. If adult crabs are less susceptible, it could explain why none of the transmission experiments succeeded. Further refinements in future *C. pagurus* transmission studies should include the use of juvenile crabs which would also allow for a greater number of replicates in the experimental design.

Temperature appears to have a strong effect on *Hematodinium* sp. proliferation in other crustacean species. Trials carried out on *Hematodinium* sp. infected blue crabs *Callinectes sapidus* showed a significant increase in mean infection intensity in infected crabs held at 9 – 15 °C with mean intensity decreasing below 9 °C (Messick et al. 1999), while *Hematodinium* sp. cultured from Norway lobsters *Nephrops norvegicus* appears to undergo a series of developmental changes at 8 °C (Appleton & Vickerman 1998). Temperature fluctuations which are commonplace during the harvest, storage and transportation processes of brown crabs did not alter infection intensity nor did it induce dinospore production or sporulation in parasitised crabs in the present study. Hyperpigmented carapace, milky haemolymph and lethargy, signs which typify late-stage infections of *Hematodinium* sp. in *C. pagurus*, were not induced in naturally infected crabs through seawater temperature manipulations despite strong evidence linking temperature with *Hematodinium* sp. disease progression (Ní Chualáin et al. 2009). In the present study, the alternating increases and decreases in infection intensity observed in naturally infected crabs in trials 2 and 3 might be explained by a migration of the parasites to other organs of the host. Histological studies of infected *Nephrops norvegicus* in Scotland have revealed a possible predilection of the parasite towards organs such as the hepatopancreas and the midgut (Field & Appleton, 1995, 1996, Stentiford et al. 2001c) and it is possible that parasites in the present study were exhibiting a similar behaviour.

Post-capture mortalities in *C. pagurus* associated with late-stage infections by *Hematodinium* sp. have previously been reported from Ireland and the United Kingdom (Ní Chualáin et al. 2009, Stentiford et al. 2002). Although parasitised crabs that do not exhibit outward signs of infection likely represent a significant proportion of crabs destined for the European market it would appear that no additional losses occur from

Hematodinium sp. infected *Cancer pagurus* after they enter the marketing chain. Sub-patent infected crabs survived for periods well beyond typical storage and transportation times during which time external signs of infection did not develop. Whilst positive from an aesthetics point of view, it remains unknown as to what stage the quality of infected crabs deteriorates after initial *Hematodinium* sp. acquisition and if this occurs prior to the onset of external signs of infection. Should poor texture and flavour be associated with pre-patent infections, consumer confidence in Irish crab products could be diminished as it is currently impossible to identify such crabs during the conventional grading process. However, as crabs with pre-patent *Hematodinium* sp. infections are presumably processed in local factories within 48 hr of landing, and no product spoiling due to *Hematodinium* sp. has been reported, it seems unlikely that infection is detectable on the palate at this stage of development.

Until *Hematodinium* sp. ex. *C. pagurus* can be successfully cultured in the laboratory, much about its pathogenicity remains unknown. Juvenile and pre- recruit *Cancer pagurus* appear to be more susceptible to *Hematodinium* sp. infections compared to commercial sized crabs (Ní Chualáin et al. 2009), but until the natural transmission pathways and infective stages of *Hematodinium* sp. are elucidated it is difficult to offer any preventative or remedial advice in managing its distribution. It remains unknown if current storage and transport conditions compound infection or transmission and further investigation is warranted.

GENERAL DISCUSSION

The aim of this study was to examine the recent emergence of *Hematodinium* sp. in Irish crustacean fisheries and to provide the first epizootiological data on pink crab disease in Ireland. As with any emerging animal disease, many infection parameters needed to be defined but early research efforts focused on distribution, host range and temporal patterns of *Hematodinium* sp. in Ireland. Data presented in Chapter 1 of this thesis revealed that the parasite was limited, in the main, to the brown crab *Cancer pagurus* but also highlighted that it was widespread in the country's three largest fisheries for this species. Neither spider crabs *Maja brachydactela* nor lobsters *Homarus gammarus* were diagnosed with *Hematodinium* sp. infections throughout the course of sampling and virtually all (99.8 %) velvet crabs *Necora puber* were free from infection. Given Ireland's proximity to the Clyde Sea area in Scotland where prevalence values of *Hematodinium* sp. in *N. puber* have been reported to be in excess of 30 % (Hamilton et al. 2009) it is somewhat surprising that *N. puber* in Ireland have not been similarly affected when the parasite is clearly present. Such is *N. puber*'s susceptibility to *Hematodinium* sp. that a near collapse of the French fishery in the 1980s was attributed to the parasite (Wilhelm & Boulo 1988, Wilhelm & Mialhe 1996). In addition, while *Hematodinium* sp. is well established in majid hosts such as snow and Tanner crabs *Chionoecetes opilio* and *C. bairdi* from Alaska and Newfoundland (Meyers et al. 1987, 1990, Eaton et al. 1991, Love et al. 1993, Meyers et al. 1996, Pestal et al. 2003, Shields et al. 2005) the confamilial *M. brachydactela* in Ireland appeared to remain unaffected despite Small et al. (2007) proposing that *C. pagurus* and *C. opilio* were infected by the same species of *Hematodinium* based on sequence similarity of the first internal transcribed spacer region (ITS1) of the ribosomal RNA complex. Whether differences exist between host susceptibility to the same *Hematodinium* species or if different species/clades of *Hematodinium* infect different hosts remains unknown but may

explain the presence/absence pattern of *Hematodinium* sp. infection in the four sympatric crustacean species studied here.

Prevalence levels observed for the brown crab *Cancer pagurus* during this study exceeded those recorded for most other host species worldwide. Although differences in biology, physiology and the environment may have contributed to this, it was also probably a result of the sensitivity of the main diagnostic method employed. In earlier surveys of Norway lobsters *Nephrops norvegicus* (Field et al. 1992; Tärnlund 2000; Briggs and McAliskey, 2002), Tanner crabs *Chionoecetes bairdi* (Meyers et al. 1987, 1990) and snow crabs *Chionoecetes opilio* (Taylor and Khan, 1995; Dawe, 2002), macroscopic evaluation of the carapace was primarily used to identify *Hematodinium* sp. infections and is currently still employed in surveys of *C. opilio* (Shield et al. 2005, Shields et al. 2007) despite being approximately 50 % less sensitive than microscopic analysis of haemolymph smears (Pestal et al. 2003). *Hematodinium* sp. prevalence in the present study failed to follow the seasonal trends characteristic of most *Hematodinium* sp. hosts worldwide ((Newman & Johnson 1975, Meyers et al. 1990, Eaton et al. 1991, Field et al. 1992, Love et al. 1993, Messick 1994, Field et al. 1998, Stentiford et al. 2001a, Sheppard et al. 2003) but the use of microscopic examination of smears did allow the detection of periodicity of infection intensity. Had macroscopic identification been solely employed in the present study, prevalence would certainly have been incorrectly interpreted as distinctly seasonal due to the presence of crabs with patent infections only in late November and December. Chapter 3 of this thesis has shown that careful consideration of sampling methodologies is required to ensure that efficient and effective monitoring is achieved. There is certainly a 'trade-off' in terms of the skill levels, equipment and costs associated with the detection, quantification and seasonal profiling of infection levels, and these may alter over time as

understanding of the progression of the disease becomes clearer. In the case of Irish fisheries, the author would recommend a procedure by which fishermen were informed of the ways in which to identify and centrally report disease outbreaks at any time of year, but for sampling resources to be increased in the months leading up to Oct – Jan annually when reports from industry are received. Such surveys would prove useful as an advanced warning system for impending epizootics. A concerted sampling effort of all size classes may then result in a more accurate assessment of the impact of the disease (when compared to the variable and unpredictable prevalence parameter) inter and intra-annually. Traditional PCR techniques may have value in the periodic monitoring of other species for presence/absence of the parasite and in validation of microscopically examined smears and histology, but until real-time PCR methods have been developed and proven for quantifying infection intensity for Irish species then haemolymph smears would appear to be the most functional method

Data presented in Chapter 2 provided perhaps the clearest indication of the threat *Hematodinium* sp. poses to brown crab populations and fisheries. If crabs are unable to develop some form of resistance or recovery, the parasite's potential to remove large numbers of sub-legal sized crabs from the population before recruiting to the fishery is of great concern. The exact relationship between host susceptibility and size is unclear but may be connected to the moulting process (Meyers et al. 1987, 1990, Eaton et al. 1991, Field et al. 1992, 1998, Messick 1994, Dawe 2002, Shields et al. 2005, 2007). Equally disconcerting, is the possibility that the full effects of *Hematodinium* sp. on the *C. pagurus* population of Malin Head are yet to be realised. Initial reports of pink crab disease began in 2005 and given the readily identifiable colouration and behaviour of crabs in the terminal stages of pink crab disease, it is unlikely that fishermen failed to notice such crabs

in their catches before this time. Therefore it is likely that *Hematodinium* sp. infections in the Malin region are a recent occurrence and it is now, 4 - 5 years later that the various cohorts are being exposed to successive years of *Hematodinium* sp. infection-related losses that population structure may be beginning to be significantly altered. Although no current data are available to verify this theory, it is not unreasonable to suggest that fisheries recruitment will be significantly diminished due to disproportionate pre-recruit natural mortality and few individuals will survive to larger sizes causing population truncation, particularly if other density-dependent processes have a lesser regulatory effect on numbers. When combined, these factors would quickly lead to reduced economic viability for the fishery and fishing communities.

Prevalence data obtained throughout the course of this study may be considered apparent or representative only for commercially-captured *Cancer pagurus.* Samples were obtained using fishing pots that allowed escapement of individuals < 80 mm CW and as such juvenile crabs were under-represented in the sampling regime. Biases in prevalence between sample collection methods have been investigated in trapped and trawled *Necora puber* (Wilhelm & Boulo 1988, Wilhelm & Mialhe 1996) and *Chionoecetes opilio* (Pestal et al. 2003, Shields et al. 2005). In both cases trawl samples had significantly higher prevalences compared to trap samples, most likely due to non-selective catching of healthy and parasitised crabs (Stentiford & Shields 2005). For *Cancer pagurus,* any possible sources of bias can only be addressed with a parallel study employing less selective gear than fishing pots.

Parasitic dinoflagellates of the genus *Hematodinium* should be considered not only the most significant pathogens affecting stocks of Irish brown crabs *Cancer pagurus* but also the most serious natural threat to the fishing industry which relies upon it. Soon after the initial monitoring began, it became apparent that the parasite was impacting in an unprecedented manner. Until 2004 when reports of pink, dying crabs began to emerge from the southwest of Ireland, black spot disease was the primary disease of *Cancer pagurus* encountered by fishermen nationwide. Cosgrove (1998) reported that approximately 4 % of the northern offshore fishery's annual *C. pagurus* catch was discarded due to shell necrosis between 1996 and 1997. By comparison however, infections by *Hematodinium* sp. have been dramatically higher with prevalence levels exceeding 50 % in commercial catches (Ní Chualáin et al. 2009). While crabs displaying either black spot disease or pink crab disease are rejected by the fishery during the onboard grading process, the most important difference between the two diseases is a crab's ability to recover from black spot shell necrosis through moulting; infections by *Hematodinium* sp. appear to be terminal within 12 months. *Hematodinium* sp. infected crab would also appear to have a much greater potential to spread through sporulation of dinospores.

It is difficult to offer the brown crab industry remedial management advice when so little is known about the natural transmission pathways of *Hematodinium* sp. in *Cancer pagurus.* De-clawing of crabs, the use of unmarketable crabs as bait in other fisheries, the discarding of diseased crabs during the grading process may or may not contribute to the spread of the disease. Transmission trials undertaken in Chapter 4 failed to infect naive *C. pagurus* through ingestion of infected tissue but cannibalism should not however be immediately discounted as an infection route; it may simply be an indication that life history stages other than vegetative trophonts are responsible for dispersal of the parasite. Caution is

advisable however and crabs displaying signs of pink crab disease should be retained and transported to an onshore waste facility even if such crabs only represent a fraction of infected crabs. Until further data becomes available in relation to the transmission routes and pathogenicity of *Hematodium* sp. within Ireland, national management responses will probably be focussed around reducing the impact of losses on local communities rather than within the host populations themselves.

The eradication of any parasite is difficult, but within the marine environment where broad scale directed attacks on the scale that would be necessary to impact *Hematodium* sp. are not feasible; this task becomes impossible. Disease outbreaks causing mass mortalities of marine systems including Caribbean sea urchins (Lessios 1988), phocine distemper virus of the harbour seal (Heide-Jorgensen et al. 1992), pilchard mortalities (Jones et al. 1997) have appeared to increase in recent years (Harvell et al. 1999, Hayes et al. 2001). Climate warming, pollution, harvesting and introduced species may be important factors in these emerging diseases (Lafferty et al. 2004). The global distribution of *Hematodinium* sp. would suggest that it is a highly successful pathogen that is adaptable to host and environment. Almost all of the attention directed toward the study of *Hematodinium* sp. has been centred around host species where commercial losses have been recorded, but it is likely many other species of no economic value may be also be affected. The ecological or ecosystem impacts of *Hematodinium* sp. remain as yet undetermined, but could nevertheless be significant.

REFERENCES

Akaike H (1983) Information measure and model selection. Bull Int Stat Inst 50 (1): 277-291

Anderson RM, May RM (1979) Population biology of infectious diseases: Part 1. Nature 280: 361-367

Anderson RM, May RM (1981) The population dynamics of microparasites and their invertebrate hosts. Philos Trans R Soc Lond B 291: 451-524

Anon (1999) Bord Iascaigh Mhara, Irish inshore fisheries sector – review and recommendations, Dublin 1999: 1-73

Anon (2005) Bord Iascaigh Mhara, Managing Ireland's inshore fisheries, the management framework for shellfisheries- committee structures, functions and process. Dublin 2005:1-16

Appleton PL, Vickerman K (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

Beldomenico PM, Begon M (2010) Disease spread, susceptibility and infection intensity: vicious circles? Tr Ecol Evol 25 (1): 21-27

Bhaud Y, Guillebault D, Lennon JF, Defacque H, Soyer-Gobillard MO and Moreau H (2000) Morphology and behaviour of dinoflagellate chromosomes during the cell cycle and mitosis. J. Cell Sci. 113: 1231–1239

Bonami JR, Hasson KW, Mari J, Poulos PT, Lightner DV (1997) Taura syndrome of marine penaeid shrimp: characterization of the viral agent. J Gen Virol 78: 313-319

Briggs RP, McAliskey M (2002) The prevalence of *Hematodinium* in *Nephrops norvegicus* from the western Irish Sea. J Mar Biol Assoc UK 82: 427-433

Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997) Parasitology meets ecology in its own terms: Margolis et al. revisited. J. Parasitol. 83: 575-583

Cachon J, Cachon M (1987) Parasitic dinoflagellates. In: Taylor FJR (ed) The biology of dinoflagellates. Blackwell Scientific Publications, p 571-610

Chang ES, Chang SA, Keller R, Reddy PS, Snyder MJ, Spees JL (1999) Quantification of stress in lobsters: Hyperglycemic hormone, stress proteins and gene expression. Am Zool 39: 487-495

Chantanachookin C, Boonyaratpalin S, Kasornchandra J, Direkbusarakom S, Usanee Ekpanithanpong U, Supamataya K, Sriurairatana S, Flegel TW (1993) Histology and ultrastructure reveal a new granulosis-type virus in *Penaeus monodon* affected yellow - head disease. Dis Aquat Org 17: 145-157

Chatton E, Poisson R (1931) Sur l'existence, dans le sang des crabs, de peridiniens parasites: *Hematodinium perezi* n.g., n. sp. (Syndinidae). CR Sceances Soc Biol Paris 105: 553-557

Chou HY, Huang CY, Wang CH, Chiang HC, Lo CF (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 23:165-173

Coats DW (1999) Parasitic lifestyles of marine dinoflagellates. J Eukaryot Microbiol 46: 402-409

Cosgrove R (1998) A survey of the Donegal edible crab (*Cancer pagurus* L.) fishery. M.Sc. Thesis, Trinity College Dublin

Cunnigham, CO (2002). Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture, 206: 19-55

Danford AR, Uglow RF (2001) Physiological responses of blue crabs (*Callinectes* sp.) to procedures used in the soft crab fishery in La Laguna de Terminos, Mexico. Marketing and Shipping live aquatic products. Alaska Sea Grant Report AK-SG-01-03, Alaska Sea Grant Program, University of Alaska, Fairbanks, AK, p 1-8

Danford AR, Hagerman L, Uglow RF (2002) Effects of emersion and elevated haemolymph ammonia on haemocyanin oxygen affinity of *Cancer pagurus.* Mar Biol 141: 1019-1027

Dawe E (2002) Trends in the prevalence of bitter crab disease caused by *Hematodinium* sp.in snow crab (*Chionoecetes opilio*) through out the Newfoundland and Labrador continental shelf, 385-400. In: Crabs in cold water regions: biology, management, and economics. Alaska Sea Grant Report AK-SG-02-01, Alaska Sea Grant Program, University of Alaska, Fairbanks, AK, p 385-400

Eaton WD, Love DC, Botelho C, Meyers TR, Imamura K, Koeneman T (1991) Preliminary results on the seasonality and life cycle of the parasitic dinoflagellate causing Bitter Crab Disease in Alaskan Tanner crabs (*Chionoecetes bairdi*). J Invertebr Pathol 57: 426-434

Edwards E (1979) The edible crab and its fishery in British waters. Fishing News Books Ltd. Farnham Surrey

FAO (2005) Review of the State of the World Marine Fishery Resources. Food and Agriculture Organisation of the United Nations, Rome

FAO (2008) The State of the World Fisheries and Aquaculture (SOFIA). Food and Agriculture Organisation of the United Nations, Rome

Field RH, Appleton PL (1995) A *Hematodinium*-like 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

Field RH, Hills JM, Atkinson RJA, Magill S, Shanks AM (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

Food and Agriculture Organisation (FAO), FAOSTAT. Available at http://faostat.fao.org/

Frischer ME, Lee RF, Sheppard MA, Mauer A, Rambow F, Neumann M, Brofft JE, Wizenmann T, Danforth JM (2006) Evidence for a free-living life stage of the blue crab parasitic dinoflagelate, *Hematodinium* sp. Harmful Algae 5: 548-557

Gaines G, Elbrächter M (1987) Heterotrophic nutrition. In: Taylor FJR (ed) The biology of dinoflagellates. Blackwell Scientific Publications, p 224-268

Gajadhar AA, Marquardt WC, Hall R, Gunderson J, Ariztia-Carmona EV, Sogin ML (1991) Ribosomal RNA sequences of Sarcocystis muris, Theileria annulata and Crypthecodinium cohnii reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. Mol Biochem Parasitol 45: 147-154

Gruebl T, Frischer ME, Sheppard M, Neumann M, Maurer AN, Lee RF (2002) Development of an 18S rRNA gene-targeted PCR based diagnostic for the blue crab parasite *Hematodinium* sp. Dis Aquat Org 49: 61-70

Hackett JD, Anderson DM, Erdner DL, Bhattacharya D (2004) Dinoflagellates: A remarkable evolutionary experiment. Am J Bot 91: 1523-1534

Hamilton KM, Shaw PW, Morritt D (2009) Prevalence and seasonality of *Hematodinium* (Alveolata: Syndinea) in a Scottish crustacean community. ICES J Mar Sci 66: 1837–1845

Harvell D, Mitchell CE, Ward JR, Altizer S, Dobson A, et al. (2002). Climate warming and disease risks for terrestrial and marine biota. Science 296:2158-62

Hayes ML, Bonaventura J, Mitchell TP, Pros- pero JM, Shinn EA et al. (2001). How are climate and marine biological outbreaks functionally linked? Hydrobiolgia 460:213- 20

Heide-Jorgensen MP, Harkonen T (1992). Epizootiology of the seal disease in the Eastern North Sea. J. Appl. Ecol. 29:99-107

Hillis DM, Dixon MT (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. Q Rev Biol 66: 411-453

Hudson DA, Adlard RD (1994) PCR-techniques applied to *Hematodinium* spp and *Hematodinium*-like dinoflagellates in decapod crustaceans. Dis Aquat Org 20:203-206

Hudson DA, Adlard RD (1996) Nucleotide sequence determination of the partial SSU rDNA gene and ITS1 region of *Hematodinium* cf *perezi* and *Hematodinium*-like dinoflagellates. Dis Aquat Org 24:55-60

Hudson DA, Shields JD (1994) *Hematodinium australis* n. sp., a parasitic dinoflagellate of the sand crab *Portunus pelagicus* from Moreton Bay, Australia. Dis Aquat Org 19: 109-119

Humason GL (1979) Animal tissue techniques, 4th edn. WH Freeman and Company, San Francisco

Johnson PT (1983) Diseases caused by viruses, rickettsiae, bacteria and fungi. In: Provenzano AJ (ed) The biology of crustacea, Vol 6. Pathobiology. Academic Press, New York p 1-78

Jones JB, Hyatt AD, Hine PM, Whitting- ton RJ, Griffin DA, Bax NJ (1997). Special topic review: Australasian pilchard mortali- ties. World J. Microb. Biot. 13:383-92

Kinne O (1990) Comments on diseases of crustacea. In: Kinne O (ed) Diseases of marine animals,Vol III. Biologische Anstalt Helgoland, Hamburg. p 4-14

Lafferty KD, Porter JW, Ford SE (2004) Are diseases increasing in the ocean? Annu Rev Ecol Evol Syst 35:31-54

Lessios HA (1988). Mass mortality of *Diadema antillarum* in the Caribbean: What have we learned? Annu. Rev. Ecol. Syst. 19:371- 93

Latrouite D, Morizur Y, Noël P, Chagot D, Wilhelm G (1988) Mortalite du *tourteau Cancer pagurus* provoquee par le dinoflagellate parasite: *Hematodinium* sp. Cons Int Explor Mer, CM 1988/K:32

Levandowsky M, Kaneta P (1987) Behaviour in dinoflagellates. In: Taylor FJR (ed) The biology of dinoflagellates. Blackwell Scientific Publications, p 360-398

Li YY, Xia XA, Wu QY, Liu WH, Lin YS (2008) Infection with *Hematodinium* sp. in mud crabs *Scylla serrata* cultured in low salinity water in southern China. Dis Aquat Org 82: 145-150

Lightner DV, Redman RM, Hasson KW, Pantoja CR (1995) Taura syndrome in *Penaeus vannamei* (Crustacea: Decapoda): gross signs, histopathology and ultrastructure. Dis Aquat Org 21: 53-59

Love DC, Rice SD, Moles DA, Eaton WD (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

Mayo MA (2002) A summary of taxonomic changes recently approved by ICTV. ArchVirol 147: 1655-1656

Messick GA (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 GA, Jordan SJ, Van Heukelem WF (1999) Salinity and temperature effects on *Hematodinium* sp in the blue crab *Callinectes sapidus.* J Shellfish Res 18: 657-662

Messick GA, Shields JD (2000) Epizootiology of the parasitic dinoflagellate *Hematodinium* sp in the American blue crab *Callinectes sapidus.* Dis Aquat Org 43: 139-152

Meyers TR (1990) Diseases of crustacea- diseases caused by protistans and metazoans. In: Kinne O (ed) Diseases of marine animals,Vol III. Biologische Anstalt Helgoland, Hamburg, p 350-389

Meyers TR, Koeneman TM, Bothelho 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

Meyers TR, Botelho C, Koeneman TM, Short S, Imamura K (1990) Distribution of bitter crab dinoflagellate syndrome in southeast Alaskan tanner crabs, *Chionoecetes bairdi.* Dis Aquat Org 9: 37-43

Meyers TR, Morado JF, Sparks AK, Bishop GH, Pearson T, Urban D, Jackson D (1996) Distribution of bitter crab syndrome in tanner crabs (*Chionoecetes bairdi, C. opilio*) from the Gulf of Alaska and the Bering Sea. Dis Aquat Org 26:221-227

Mouritsen KN, Jensen KT (1997) Parasite transmission between soft-bottom invertebrates: temperature mediated infection rates and mortality in *Corophium volutator*. Mar Ecol Prog Ser 151: 123-134

Munn CB (2004) Marine eukaryotic microbes. In: Marine microbiology: ecology and applications. Bios Scientific Publishers Ltd, p 125-136

Munn CB (2006) Viruses as pathogens of marine organisms- from bacteria to whales. J Mar Biol Ass UK 86: 453-467

Murray S, Flø Jørgensen M, Ho SYW, Patterson DJ, Jermiin LS (2005) Improving the analysis of dinoflagellate phylogeny based on rDNA. Protist 156: 269 - 286

Newman MW, Johnson CA (1975) A disease of blue crabs (*Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp. J Parasitol 63: 554-557

Ní Chualáin C, Hayes M, Allen B, Robinson M (2009) *Hematodinium* sp in Irish *Cancer pagurus* fisheries: infection intensity as a potential fisheries management tool. Dis Aquat Org 83:59-66

Nunan LM, Poulos BT, Lightner DV (1998) The detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) in imported commodity shrimp. Aqualculture 160: 19-30

OIE 2006 Manual of diagnostic tests for aquatic animals 2006. OIE, Paris. 469 pp

Patterson BD, Spanoghe PT (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

Pestal GP, Taylor DM, Hoenig JM, Shields JD, Pickavance R (2003) Monitoring the presence of the lethal parasite *Hematodinium* sp. in snow crabs from Newfoundland. Dis Aquat Org 53: 67-75

Pile AJ, Lipcius RN, Van Montfrans J, Orth RJ (1996) Density-dependent settler-recruit-juvenile relationships in blue crabs. Ecol Monogr 66: 277-300

Power JH, Moser EB (1999) Linear model analysis of net catch data using the negative binomial distribution. Can J Fish Aquat Sci 56: 191-200

Rizzo PJ (1987) Biochemistry of the dinoflagellate nucleus. In: Taylor FJR (ed) The biology of dinoflagellates. Blackwell Scientific Publications, p 143-173

Rizzo PJ (2003) Those amazing dinoflagellate chromosomes. Cell Res 13: 215-217

Robinson M (1999) Community structure and recruitment of decapods in shallow sub-littoral habitats. Ph.D Thesis, University of Dublin

Roughgarden J, Gaines S, Possingham H (1988) Recruitment dynamics in complex life cycles. Science 241: 1460-1466

Rowan R, Powers DA (1992) Ribosomal RNA sequences and the diversity of symbiotic dinoflagellates (zooxanthellae). Proc Natl Acad Sci USA 89: 3639-3643

Sánchez-Martínez JG, Aguirre-Guzmán G, Mejía-Ruíz H (2007) White spot syndrome virus in cultured shrimp: a review. Aquac Res 38: 1339-1354

Schmitt ASC, Uglow RF (1997) Haemolymph constituent levels and ammonia efflux rates of *Nephrops norvegicus* during emersion. Mar Biol 127: 403-410

Sheppard M, Walker A, Frischer ME, Lee RF (2003) Histopathology and prevalence of the parasitic dinoflagellate *Hematodinium* sp, in crabs (*Callinectes sapidus, Callinectes similis, Neopanope sayi, Libinia emarginata, Menippe mercenaria*) from a Georgia estuary. J Shellfish Res 22: 873-880

Shields JD (1994) The parasitic dinoflagellates of marine crustaceans. Annu Rev Fish Dis 4: 241-271

Shields JD, Squyars CM (2000) Mortality and hematology of blue crabs, *Callinectes sapidus*, experimentally infected with the parasitic dinoflagellate *Hematodinium perezi.* Fish Bull 98: 139-152

Shields JD, Scanlon C, Volety A (2003) Aspects of the pathophysiology of blue crabs, *Callinectes sapidus,* infected with the parasitic dinoflagellate *Hematodinium perezi.* Bull Mar Sci 72: 519-535

Shields JD, Taylor DM, Sutton SG, O'Keefe PO, Collins PW, Ings DW, Pardy AL (2005) Epizootiology of bitter crab disease (*Hematodinium* sp.) in snow crabs, *Chionoecetes opilio*, from Newfoundland, Canada. Dis Aquat Org 64: 253-264

Shields JD, Taylor DM, O'Keefe PG, Colbourne E, Hynick E (2007) Epidemiological determinants in outbreaks of bitter crab disease (*Hematodinium* sp.) in snow crabs, *Chionoecetes opilio* from Newfoundland, Canada. Dis Aquat Org 77: 61-72

Small HJ, Wilson S, Neil DM, Hagan P, Coombs GH (2002) Detection of the parasitic dinoflagellate *Hematodinium* in the Norway lobster *Nephrops norvegicus* by ELISA. Dis Aquat Org 52: 175-177

Small HJ, Neil DM, Taylor AC, Atkinson RJA, Coombs GH (2006) Molecular detection of *Hematodinium* spp. in Norway lobster *Nephrops norvegicus* and other crustaceans. Dis Aquat Org 69:185-195

Small HJ, Shields JD, Hudson KL, Reece KS (2007a) Molecular detection of *Hematodinium* sp. infecting the blue crab *Callinectes sapidus*. J Shellfish Res 26: 131-139

Small HJ, Shields JD, Moss, JA, Reece KS (2007b) Conservation in the first internal transcribed spacer region (ITS1) in *Hematodinium* species infecting crustacean hosts found in the UK and Newfoundland. Dis Aquat Org 75:251-258

Stentiford GD (2008) Diseases of the European edible crab (*Cancer pagurus*): a review. ICES J Mar Sci 65: 1578-1592

Stentiford GD, Shields JD (2005) A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans. Dis Aquat Org 66: 47-70

Stentiford GD, Neil DM, Coombs GH (2000) Alterations in the biochemistry and ultrastructure of the deep abdominal flexor muscle of the Norway lobster *Nephrops norvegicus* during infection by a parasitic dinoflagellate of the genus *Hematodinium*. Dis Aquat Org 42:133-141

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, Chang ES, Chang SA, Neil DM (2001b) Carbohydrate dynamics and the crustacean hyperglycaemic hormone (CHH): effects of parasitic infection in Norway lobster *Nephrops norvegicus*. Gen Comp Endocrinol 121: 13-22

Stentiford GD, Neil DM, Coombs GH (2001c) Development and application of an immunoassay diagnostic tecnnique 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) Infection by a *Hematodinium*-like parasitic dinoflagellate causes Pink Crab Disease (PCD) in the edible crab *Cancer pagurus*. J Invertebr Pathol 79: 179-191

Stentiford GD, Bonami JR, Alday-Sanz V (2009) A critical review of susceptibility of crustaceans to Taura syndrome, yellowhead disease and white spot disease and implications of inclusion of these diseases in European legislation. Aquaculture 291: 1-17

Tärnlund S (2000) A comparison of two methods for identifying and assessing the parasitic dinoflagellate *Hematodinium* sp. in Norway lobster (*Nephrops norvegicus*). MSc thesis, University of Götenborg

Taylor AC, Field RH, Parslow-Williams PJ (1996) The effects of *Hematodinium* sp.-infection on aspects of the respiratory physiology of the Norway lobster, *Nephrops norvegicus* (L.). J Exp Mar Biol Ecol 207: 217-228

Taylor DM, Khan RA (1995) Observations on the occurrence of *Hematodinium* sp. (Dinoflagellata: Syndinidae): the causative agent of Bitter Crab Disease in the Newfoundland snow crab (*Chionoecetes opilio*). J Invertebr Pathol 65: 283-288

Tully O, Robinson M, O'Keeffe E, Cosgrove R, Doyle O, Lehane B (2006) The Brown Crab (*Cancer pagurus* L.) Fishery: Analysis of the resource in 2004-2005. Bord Iascaigh Mhara, Fish Res Ser: No. 4

Uglow RF, Hosie DA (1995) The live marketing of Irish brown crab: an investigation of the effects of current procedures on the quality of delivered product. A study by the University of Hull commissioned by An Bord Iascaigh Mhara. Unpublished

Unestam T, Weiss DW (1970) The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: Responses to infection by a susceptible and a resistant species. J Gen Microbiol 66: 77-90

Vickerman K, Coombs GH (1999) Protozoan paradigms for cell biology. J Cell. Sci 112: 2797-2798

Vogan CL, Llewellyn P, Rowley AF (1999) Epidemiology and dynamics of shell disease in the edible crab *Cancer pagurus*: a preliminary study of Langland Bay, Swansea UK. Dis Aquat Org 35: 81-87

Walker AN,Lee RF, Frischer ME (2009) Transmission of the parasitic dinoflagellate *Hematodinium* sp. infection in blue crabs *Callinectes sapidus* by cannibalism. Dis Aquat Org 85: 193-197

Wheeler K, Shields JD, Taylor DM (2007) Pathology of *Hematodinium* infections in snow crabs (*Chionoecetes opilio*) from Newfoundland, Canada. J Invertebr Pathol 95:93-100

Wilhelm G, Boulo V (1988) Infection de l'etrille *Liocarcinus puber* (L.) par un dinoflagelle parasite: *Hematodinium* sp. Cons Int Explor Mer Ser CM, K: 41: 1-10

Wilhelm G, Mialhe E (1996) Dinoflagellate infection associated with the decline of *Necora puber* crab populations in France. Dis Aquat Org 26: 213-219

Xu WJ, Shi H, Xu HX, Hamish S (2007) Preliminary study on the *Hematodinium* infection in cultured *Portunus trituberculatus.* Acta Hydrobiol Sini 31: 637-642

Xu WJ, Xie J, Shi H, Li C (2010) *Hematodinium* infections in cultured ridgetail white prawns, Exopalaemon carinicauda, in eastern China. Aquaculture 300: 25-31