OCCURRENCE, HISTOPATHOLOGY AND FINE STRUCTURAL STUDIES ON HEMATODINIUM SP. (DINOFLAGELLIDA: SYNDINIDAE) PARASITIZING THE SNOW CRAB (CHIONOECETES OPILIO)

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OCCURRENCE, HISTOPATHOLOGY AND FINE STRUCTURAL STUDIES ON *HEMATODINIUM* SP. (DINOFLAGELLIDA :SYNDINIDAE) PARASITIZING THE SNOW CRAB (*CHIONOECETES OPILIO*)

by

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Abstract

Snow crabs exhibiting macroscopic evidence of *Hematodinium* sp. infection were collected off the east coast of Newfoundland during the period 1993 to 1996. Infected crabs were collected over a large area but prevalence of infection was low. Pathology of the internal organs in affected crabs was observed in association with the developmental stages of *Hematodinium* sp. The parasite invaded hemal spaces causing disruption of normal tissue morphology, and ultimately mortality of the host. *Hematodinium* sp. developed through three distinct morphological stages during the progress of infection: vegetative, multinucleate prespore and dinospore. The latter is the final stage within the crab. These stages of the parasite developed synchronously and sequentially within any one crab, but might differ between individual crabs. The ultrastructure of these stages was examined using electron microscopy, which revealed the following characteristics in all developmental stages: the presence of many trichocysts, mitochondria with tubular cristae and vacuoles with lipid-like material. In the dinospore stage, flagella and micropores were observed in addition to the other common features. The morphological characteristics of *Hematodinium* sp. described in this investigation conform to previous descriptions of *Hematodinium* spp. reported from a variety of crabs inhabiting different geographic regions.

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TABLE OF CONTENTS

List of Tables

List of Figures

INTRODUCTION

1.1 Purpose of Study

Snow crabs (*Chionoecetes opilio*) exhibiting macroscopic signs of infection of *Hematodinium* sp. were first noted in 1990 (Taylor and Khan, 1994) during the annual Department of Fisheries and Oceans (D.F.O.) stock assessment surveys. Previous to this record snow crabs in Newfoundland waters were considered to be relatively free from naturally occurring pathogens or parasites (Bratty et al., 1985).

The purpose of this study is to document the morphology of the life history stages of the parasite *Hematodinium* sp. occurring in the snow crab, to compare the morphological findings with those published in the literature and compare the species differentiation between the various descriptions in the literature and the species described in this report. Also to note the prevalence of infection in snow crab populations of the east coast of Newfoundland.

1.2 Review of parasitism as a life history strategy

The interdependence of organisms is widely acknowledged, the complexity of the interrelationships being many and varied. Interactions between species may be injurious, neutral or beneficial to one or both. Within two – species interactions, parasitism has been interpreted as obligatory for the parasite and detrimental to the host (Read, 1970). The success of a host – parasite relationship is dependent on a balance of continuing success for both species. The development of a parasite within a host involves competition for body resources, including nutritional requirements and physical space. The relationship between *Hematodinium* sp. and snow crab is more injurious, apparently resulting in the death of the crab host once the parasite *Hematodinium* sp. has completed its development (Field et al., 1992).

A description of any host-parasite relationship involves an understanding of both the parasite and host biology and the interactions between the two. Therefore the biology of the *Hematodinium* sp. and the biology of the snow crab are considered independently then together.

1.3 Overview of the Order Dinoflagellida

Hematodinium spp. are protozoans assigned to the order Dinoflagellida. Taxonomically the genus has been classified in the kingdom Protista, sub kingdom Protozoa, phylum Sarcomastigophora, subphylum Mastigophora. The order Dinoflagellida is characterized by the following features: two heterodynamic flagellae, inserted apically or laterally, one flagella ribbon-shaped with paraxial rod and single row of fine hairs, other smooth or with two rows of stiffer hairs; chloroplasts typically golden brown or green; storage products of starch and fat; cells flattened or of complex symmetry with transverse and ventral grooves and often possessing an armor of cellulosic plates: nucleus unique among eukaryotes in having chromosomes that consist primarily of nonprecomplexed DNA; mitosis intranuclear; coccoid unicells, colonies, and filaments; sexual reproduction present (Levine et al., 1980). A typical dinoflagellate has two flagella within the girdle and sulcus. Operation of these flagella results in a characteristic spiraling movement through the water.

Within the order Dinoflagellida there are approximately 130 genera and 1200 species exhibiting a wide divergence in morphology, nutrition and habitat. Dinoflagellates may be either autotrophic or heterotrophic and most are important members of the phytoplankton in marine and freshwater ecosystems. Autotrophic dinoflagellates form the base of complex food webs and perform the initial transfer of light energy into food energy. These dinoflagellates are responsible for seasonal "blooms", when a rapid increase in individuals is stimulated by environmental conditions such as photoperiod and

temperature. Species that are heterotrophic, can be holozoic, parasitic or symbiotic. *Hematodinium* spp. are parasitic (Chatton and Poisson, 1930).

1.4 Literature review of *Hematodinium* spp.

The genus *Hematodinium* has two described species *H. perezi* (Chatton and Poisson, 1930) and *H. australis* (Hudson and Shields, 1994), both of which occur exclusively within crustacean hosts. *Hematodinium perezi* has been recorded in a variety of hosts from diverse locations including, *Carcinus maenas* from France, (Chatton and Poisson, 1930), benthic amphipods from the continental shelf of the northeastern United States (Johnson, 1986), *Cancer borealis* and *Ovalipes ocellatus* in the mid Atlantic Bight (MacLean and Rudell, 1978). *Hematodinium australis* has been described from several hosts including *Portunus pelagicus* (Hudson and Shields, 1994) and *Trapezia aerolata* (Hudson et al.,1993) from Australia. Other records describing the parasite as *Hematodinium* sp. or *Hematodinium* –like have been made from several hosts including Norway lobster (*Nephrops norvegicus*) from the west coast of Scotland (Field et al., 1992), Alaskan tanner crab (*Chionoecetes bairdi*) from the Sullivan Island area of southeast Alaska (Meyers et al., 1987), snow crab (*Chionoecetes opilio*) in Newfoundland (Taylor and Khan, 1994), *Pandulus platyceros* from both British Columbia (Bower et al., 1993) and Alaska (Meyers et al.,1994). This wide diversity of hosts and geographic occurrence suggests a cosmopolitan distribution for the genus. *Hematodinium* spp. have only been observed in a parasitic relationship and there is no record of a free living form.

The first description of *Hematodinium perezi* was made from an organism isolated from *Carcinus maenas* and *Portunus depurator* (Chatton and Poisson, 1930). They described a parasitic protozoan that lives in the hemolymph of crabs and divides by amitosis. The characteristics they observed include plasmodial forms with dikaryon-type

nuclei containing five chromosomes arranged in V-shapes, apparent absence of a nuclear membrane and continual state of mitotic activity of the nucleus, a type of mitosis referred to as dinomitosis. Chatton and Poisson (1930) considered the parasite to be rare, as few of the crabs they examined were infected. They were unable to document the complete life cycle of *Hematodinium perezi* and did not describe a flagellated stage. Their classification was based on the similarities between the genera *Hematodinium* and *Syndinium* . The latter genus is a parasite of copepods for which a free living flagellated stage was known.

In Alaska *Hematodinium* sp. infections in Tanner crabs *Chionoecetes bairdi* are known to cause "Bitter Crab Disease" or "Bitter Crab Syndrome " (Meyers et al., 1996), which causes a bitter chalky taste to be imparted to the crab meat. As the Tanner crab fishery is of major economic importance in Alaska the occurrence and spread of *Hematodinium* sp. within the Tanner crab population has major economic implications. When the parasite was first identified in the crab populations of Alaska in 1985 and 1986 (Meyers, 1987), *Hematodinium* sp. infected 11.4% of the crab harvested during a February sampling period. Bitter Crab Syndrome recently affected up to one third of the commercial areas fished with infection rates varying from 5% to 95% (Meyers et al., 1996). Researchers were soon able to identify infected crabs by their macroscopic appearance including a pink carapace and milky-white hemolymph. When examined microscopically, the hemolymph of infected crabs contained a round to ovoid unicellular organism and as well multinucleate aggregates and binucleate forms. The nuclei of the cells were eccentric with distinct margins which frequently exhibited the dikaryon type morphology. Nucleoli were not visible within the nucleus. The cytoplasm was non-staining, vesicular and sometimes exhibited a thin amorphous border. Subsequent studies (Love et al., 1993) revealed that the parasite occurred in three forms; a nonmotile vegetative stage (the replicative form) and two motile dinospore forms. The two spore types include a larger slow moving biflagellated dinospore and a smaller fast moving dinospore or

microspore. These two dinospore types might imply a sexual cycle but the authors did not provide any other supporting evidence for sexual reproduction. Love et al. (1993) also noted a significant relationship between month and prevalence and intensity of the disease. The intensity of the infection was also related to shell condition as recently molted crabs showed greater levels of infection.

Hematodinium perezi was reported to infect blue crabs (*Callinectes sapidus*) from coastal bays of Virginia and Maryland, USA (Messick, 1994). Several morphological forms of the parasite were observed, including uninucleate cells with dikaryon type nucleus, dividing cells and multinucleate cells having prominent chromatin and no apparent nuclear membrane. Some multinucleate cells exhibited amoeboid movement when examined live. Other *Hematodinium perezi* had hyperchromatic, dense nuclei. Flagellated dinospores were not observed in this study. Messick (1994) found a seasonal trend to infections and observed the highest prevalence during August. This pattern in prevalence is consistent with observations by Newman and Johnson (1985) who note that infected crabs were recorded at all times of the year except during late winter - early spring.

Taylor and Khan (1995) reported the first occurrence of Bitter Crab Disease of snow crab *Chionoecetes opilio* in Atlantic Canada when they isolated *Hematodinium* sp. from material collected during 1990 annual crab surveys. At that time infections were low in prevalence and patchy in distribution. Taylor and Khan (1995) observed three stages of *Hematodinium* sp. which were similar to those described by Meyers et al. (1987). A vegetative, nonmotile, uninucleate stage was predominant in 71% of the infections. Uni – and multinucleate stages of the parasite were noted, including dinospores that resemble the microspores found by Meyers et al., (1987). The infected crabs had new-hard shells indicating a molt within the previous two years. Infected crabs were captured during April and May but not during the October sampling period, suggesting seasonality in infection rates as reported by other authors.

Shields (1992) reported the finding of *Hematodinium* sp. during a survey of parasites and symbionts of *Portunus pelagicus* in Moreton Bay, Eastern Australia. Hudson and Shields (1994) subsequently described this as *Hematodinium australis* , a new species, based on the following criteria: size of the vegetative stage, morphology of the plasmodium, appearance of the chromatin, life history parameters, geographic location and host. *Hematodinium australis* is distinguished from *Hematodinium perezi* by its larger vegetative size, ovoid as opposed to vermiform plasmodia, and presence of trichocysts in the vegetative stages. The authors summarized and justified their naming of a new species with a tabular comparison of all previous descriptions.

Identification of Crustacea harboring infections of *Hematodinium* spp. has been based on the macroscopic characteristics, including a 'cooked' appearance of the living host and the presence of milky white hemolymph. Confirmation of the macroscopic diagnosis was made by microscopic examination of the hemolymph smears stained with either Leishman's, Giemsa or Diff Quick stains. Infections of *Hematodinium* sp. in *Nephrops norvegicus* have been detected by observation of the parasite visible within the hemolymph through the transparent cuticle of the pleopods (Field et al., 1992 and Field and Appleton, 1995) The pleopod examination method has also been used successfully in *Callinectes sapidus* (Messick, 1994). An indirect fluorescent antibody technique has been developed to identify *Hematodinium* spp. infections in *Nephrops norvegicus* (Field and Appleton, 1996). The antibody test is based on a polyclonal antibody derived from rabbits immunized with a mixture of vegetative forms of *Hematodinium* sp. from *in vitro* cultures. The indirect antibody technique has been used successfully on both hemolymph and tissue samples, providing correct diagnosis of infected hosts that were not recognized previously using visual methods.

Polymerase chain reaction techniques have also been developed to detect infections of *Hematodinium* sp. in crustaceans (Hudson and Adlard, 1994). The DNA was extracted from a variety of infected host - parasite combinations, including *Hematodinium* sp. infecting *Nephrops norvegicus, Callinectes sapidus, Chionoecetes bairdi, Chionoecetes opilio*, and *Hematodinium australis* infecting *Portunus pelagicus* . The primers used in the study were not specific to the *Hematodinium* spp. The authors state that a positive test should be treated as diagnostic for non-host DNA and suggest that infection by *Hematodinium* sp. can be confirmed by microscopic examination.

1.5 Development of *Hematodinium* sp. in the crustacean host

Hematodinium sp. occurs in the hemolymph of a crustacean host and develops through a series of stages. The trophont or vegetative stage develops into a multinucleate plasmodium, which in turn divides by a process called dinomitosis to form dinospores. While the complete life cycle and mechanism of transmission between hosts is unknown, a long latency period punctuated by shorter intense periods of spore release and host mortality seem typical of the *Hematodinium* spp. infections (Love et al., 1993).

1.6 Review of *Chionoecetes opilio* biology

The snow crab (*Chionoecetes opilio*), a host to the *Hematodinium* sp., is distributed in the northwestern Atlantic from West Greenland southwards to the Atlantic coast of Canada and into the Gulf of Maine. *C. opilio* also has a Pacific distribution in the shallow waters of the Bering Strait and around the Aleutian Islands. In Newfoundland waters, the crustacean is common in most regions. The snow crab shows distinct sexual dimorphism: the males are considerably larger than the females. The carapace width of sexually mature females ranges in size from 47–95 mm while in sexually mature males it is usually >95 mm.

There is no single crab species whose reproduction is sufficiently well understood to serve as a model for brachyuran crabs. Several aspects of the reproductive biology of *C. opilio* have been investigated. Sexual maturity in females occurs following a terminal molt, at which time the abdomen undergoes a large allometric shift in growth relative to carapace size. This is considered to be a structural adaptation to facilitate egg brooding. There appears to be a direct relationship between fecundity and carapace width. Estimates of the number of eggs carried by a single female range from 12,000–128,000; smaller females bear fewer eggs. Mature females include primaparous (first time) and multiparous (repeat) spawners. In primaparous females the development of the ovaries is restricted by the premolt size. Consequently, first time spawners produce fewer eggs than multiparous females. Primaparous females devote energy simultaneously to gonadal and somatic growth, with unknown effects on the broods' survivorship (Sainte-Marie, 1993).

Mating and courtship in primaparous females occur in the spring when a male locates a female that is ready to undergo terminal molt. The male will then carry the female around in a precopulatory embrace for approximately one week during which time he drives away other intruding males (Comeau et al., 1992). The male assists the female at ecdysis (molt) which is followed by copulation. Males do not release the females for approximately eight hours after copulation, possibly a strategy to ensure the success of the mating. In multiparous females, the males will carry the females in the precopulatory embrace for several weeks and copulation occurs soon after the previous years larvae are released (Hooper, 1986, Comeau et al, 1992). The female is released soon after copulation. In both primaparous and multiparous females, egg extrusion occurs within 24 hours after copulation.

Egg development occurs outside the body where the eggs are attached to hairy pleopods located under the abdomen. The period of egg development to their release takes

approximately one year. During this period the eggs change color from bright orange to black. Egg loss during this developmental period can be substantial, due to parasitism, predation, disease or insufficient vitelline reserves. Hatching begins twelve months after fertilization.

The eggs hatch into a prezoeal stage which is about 3 mm long, and within one hour prezoeal molt to a zoea (zoe I) stage. The zoe 1 then pass through a second zoeal stage (zoe II) before molting to the megalopa stage (Motoh, 1982). The larvae are released into the water column, rise to the surface and are dispersed at this point by the prevailing water currents. The time spent as a pelagic larval stage is not known with certainty. Zoe I larvae were caught during vertical tows during the months May through July, whereas no second stage zoeal larvae or megalopa larvae were captured during the same period (Comeau et al., 1992).

Dispersal of the pelagic crab larvae (as with most marine organisms that exhibit this life history strategy) is dependent on several factors, including length of larval life, prevailing water currents and the larval behavioral responses to environmental variables such as temperature, salinity and light intensity. The pelagic crab larvae exhibit stage specific behavioral patterns that will influence their position in the water column and ultimately their ability to return to suitable habitat and recruit to the adult population. Mortality of pelagic developmental stages is thought to be high, due to predation and effects of adverse environmental conditions. Despite the hazards associated with a pelagic mode of dispersal, mechanisms exist for the return of dispersed larvae to restock a resident population. Larval survivorship and recruitment to adult populations have important implications for the maintenance of population stability, geographical patterns of distribution and community structure (Sastry, 1983).

The megalopa larva undergoes a molt to produce a juvenile crab that has a carapace width of approximately 2 mm. The juvenile crab then assumes the benthic habit and grows through a series of molts; the frequency of which decreases with age. These molts tend to be more frequent in young crabs, and length of intermolt periods increases with age. There is much debate as to whether *C. opilio* undergoes a terminal molt or continues to molt at increasingly long time periods. In females, the literature agrees that a terminal molt to maturity occurs. In males the point is open to debate (Dawe et al., 1991). It has been noted that males undergo a terminal molt associated with the development of large chelae. Large chelae are associated with reproductive success as males so endowed are more successful in capturing and holding the female during copulation (Dawe et al., 1991).

Observations of shell condition indicate that snow crabs in Conception Bay, Newfoundland molt from April to June (Miller and O'Keefe, 1981). In this soft–shelled condition the animals are vulnerable to predation and seek protection by burying in the bottom sediment. The new shell is formed directly beneath the old. The old shell splits along the posterior edge of the carapace and the crab backs out of the old shell. The new shell then rapidly hydrates and hardens with time. At each molt the snow crab can gain up to 60% of its mass and 20% in width. Shell condition has been assigned numbers 1-3 depending on the coloration, hardness, and presence of ectocommensals; this is an aid to determine molting period and suitability for harvest (Taylor et al., 1993).

1.7 Diet of *Chionoecetes opilio*

Snow crabs are omnivorous and higher rates of feeding activity has been observed at night (Bailey, 1990). Snow crab prey consists primarily of benthic invertebrates including often species with low mobility such as sedentary polychaetes and mollusks (Wieczorek and Hooper, 1995). The snow crabs exhibit selective feeding behavior as demonstrated by a comparison of prey availability compared to stomach contents. The

foraging behavior of snow crab is a combination of opportunistic feeding on readily available prey species combined with selective feeding in the case of less abundant species. (Wieczorek and Hooper, 1995)

1.8 Circulatory system of *Chionoecetes opilio*

The circulatory (hemocoelomic) system in the snow crab consists of a heart and sinusoids. The heart is a large pentagonal chamber dorsally located in the thorax above the respiratory organs. The walls are thickened by several layers of crossing muscle strands. Arteries leave the heart penetrating the pericardial membrane and after a varying number of branching processes deliver the hemolymph to sinusoids or hemal spaces that bathe the tissues. The hemolymph surrounds the tissues where exchange of nutrients and waste occurs, then a network of venous sinuses drain the hemolymph to a ventral branchopericardial sinus across the gills and back to the heart.

The hemolymph consists of a cellular component (hemocytes) and a matrix of protein, calcium and other dissolved components. Three types of hemocytes are generally recognized (Johnson, 1980); hyaline, semi-granular and granulocytes recognized on the basis of morphology. They are thought to form a continuous differentiation series with many intermediate forms. The hemocyte series show a regularly decreasing nucleo-cytoplasmic ratio with an increasing number of cytoplasmic granules (Johnson, 1980). The hemocytes function as part of the defense mechanism rapidly forming a dense clot as a result of amputation or cuticle disruption. There is evidence that hemopoetic production varies with the molt cycle.

1.9 Significance of *Hematodinium* sp. infections

The commercial snow crab fishery is of major economic importance to the province of Newfoundland and Labrador. The significance of the industry has been elevated since

the moratorium on the cod fishery. A disease that affects the crab stocks could be biologically, economically and socially important. Therefore a general survey of the distribution of infected crabs along with a morphological study to compare the species of *Hematodinium* sp. infecting snow crabs in the northwest Atlantic to other species occurring in different hosts was considered to be a project of practical application.

The preceding review of the biology of the snow crab and literature review of *Hematodinium* sp. was the basis for my own research on the host – parasite interaction occurring in the northwest Atlantic.

MATERIALS AND METHODS

2.1 Collection of *Chionoecetes opilio*

Snow crabs were collected from four sample sites around Newfoundland (Fig.1) Conception Bay, Bonavista Bay, White Bay and off St. John's. Only male crabs were examined because females have a patchy distribution and are too small to be retained by traps using the commercial-sized mesh

The sample collections in Conception Bay (47° 32' 35' N 53° 05'99' W) during April of 1993, to 1996 were performed using the M.V. Karl and Jackie II owned and operated by the Ocean Science Center. Crabs were captured using standard commercial Japanese conical traps with 13.1cm diameter mesh. All traps were baited with previously frozen short finned squid (*Illex illecebrosus*) and Atlantic mackerel (*Scomber scombrus*), set in long-line fleets of twelve then left for a two day soak period before being hauled. This sample site was chosen because of previous capture of infected snow crabs.

The Bonavista Bay, White Bay, St. John's and the September Conception Bay samples were collected in co-operation with the Department of Fisheries and Oceans, St. John's using the vessel M.V. Shamook. The crabs were captured using standard Japanese commercial crab traps set in long line fleets consisting of eleven 13.1cm. mesh and one 3.1 cm mesh traps baited with previously frozen short finned squid (*Illex illecebrosus*) and Atlantic mackerel (*Scomber scombrus*). Sites were stratified by depth and randomly selected. Sets were left for a 24 -48 hour soak period then hauled. In June 1996, in addition to the trap sample, a sample was obtained off St. John's using a #36 shrimp trawl modified by addition of a half inch "tickler " chain across the mouth of the trawl.

The total number of crab captured in each set was enumerated. At each site the infected crabs were identified macroscopically, isolated and held for examination. From the

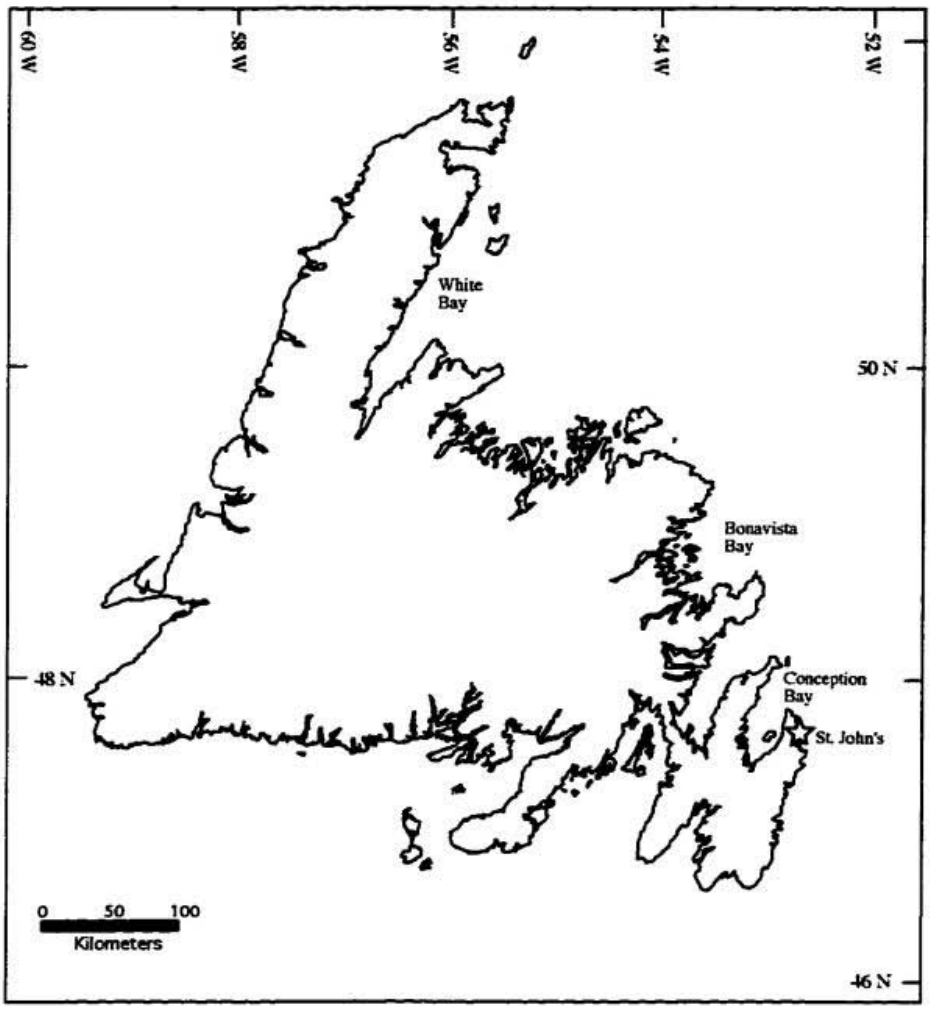

Figure 1. Map of Newfoundland showing sampling sites.

remaining macroscopically uninfected crabs between 25 to 100 specimens with new–hard shells and carapace width >95 mm were chosen and bled through the intersegmental membranes at the base of the left coxa of the third cheliciped using a heparinized 3 cc. syringe and 22 gauge needle. A hemolymph smear was made and an additional drop of hemolymph was examined as a wet preparation for the presence of *Hematodinium*. sp. The smears were then fixed in methanol and stained either with Diff Quick™ (Baxter Scientific) or Giemsa (5 mls Giemsa in 95 mls phosphate buffer pH. 7.6) for further microscopic examination.

2.2 Necropsy of *Chionoecetes opilio*

Crabs exhibiting macroscopic signs of *Hematodinium sp*. infection were transported alive to laboratory facilities in chilled sea water and isolated in 60 liter tanks supplied with running sea water maintained at 1.0^0 C in the dark. Crabs were fed thawed, previously frozen herring (*Clupea harengus harengus*) to satiation twice weekly. Samples of hemolymph were examined, as wet preparations and Giemsa stained smears, daily for one week, thence weekly for eight weeks, to determine the course of the infection.

Crabs that were unable to be transported to holding facilities (due to lack of holding facilities at sea and distance) were necropsied. These crabs were bled and the hemolymph preserved in Karnovsky fixative (see Appendix 1). Tissues including, gill, gonad, hepatopancreas and muscle were excised for histological examination then fixed in 10% neutral buffered formalin A representative sample of all tissues was taken from macroscopically normal crabs of comparable size and shell hardness.

2.3 Preparation of samples for histological observations

Preparation of samples for wax histology involved fixation of tissues from euthanized crab in 10% neutral buffered formalin pH 7.4. Preserved samples were then

placed in cassettes for processing on a Fisher Tissuematon™ using a conventional processing schedule (see Appendix 2), then placed under vacuum for 30 minutes at (25 psi). Samples were then embedded in Surgipath™ blue ribbon wax (Fisher Scientific). Six µm. sections were cut from embedded tissues using an American Optical 812 rotary microtome with disposable blades. Sections were mounted on albuminized slides, heat - fixed for an hour then stained with haematoxylin and eosin (see Appendix 3). Sections were subsequently examined with a Leitz™ light microscope. Representative sections were photographed through a Zeiss™ axiovert 35 microscope with attached camera (Contax 167 MT) using Kodak™ 100 film with a Kodak 81A filter for daylight film with a tungsten light source.

2.4 Samples for scanning electron microscopy

Hemolymph samples from *Hematodinium* sp. infected crabs were fixed in Karnovsky fixative prepared in sea water.The samples were then osmicated, dehydrated in graded alcohol, then filtered on to Anopore 0.2 µm membrane (McKenzie et al., 1992). The membranes were mounted on to an aluminum stub, sputter coated with gold and examined in a Hitachi S570 scanning electron microscope with an accelerating voltage of 20 KV. Images were recorded on Polaroid 665 positive / negative film.

2.5 Preparation of samples for transmission electron microscopy

Hemolymph and tissue samples from infected and control crabs were fixed in Karnovsky fixative prepared in sea water. Tissues were left for 48 hours then transferred to 1M cacodylate buffer until processing. The tissues were then osmicated in 1% osmium tetroxide in 1M cacodylate buffer for 1 hour, rinsed in buffer and dehydrated through a series of ethanol's 70%-100%, infiltrated with Spurr ™ resin and embedded in Spurr ™ resin polymerized at 70^0 C overnight (Spurr, 1969). The resulting blocks were cut on a

Reichart™ OMU2 microtome using a glass knife for the 0.5 µm sections and a Diatome™ diamond knife for the thin sections. Thick (0.5 µm) sections were stained with 1% toluidine blue in 1% boric acid for general architecture. From examination of the 0.5 µm sections, representative areas were chosen for thin sectioning. Thin sections were cut at 70 – 80 nm (gold on the interference scale) mounted on 300 mesh copper grids, air dried and then stained with Reynolds lead citrate stain for 2 minutes. Stained grids were examined in a Phillips 300 transmission electron microscope using an accelerating voltage of 60 KV. Images were captured on Kodak™ Electron Microscope Film 4489, 8.3 x 10.2 cm sheets. The film was developed in Kodak™ D19 developer diluted 1:1 with water for 4 minutes, rinsed in water for 1 minute then fixed in Kodak™ Rapid Fix for 4 minutes. The images were printed on Kodak™ polycontrast paper and developed using a stabilization process followed by fixation in Kodak™ rapid fix for 5 minutes.

RESULTS

3.1 Macroscopic identification of *Hematodinium* sp. infections

Live snow crabs are identified macroscopically as positive for *Hematodinium* sp. infection by a characteristic "cooked" coloration. The ventral portions of the limbs are an opaque white color, rather than the translucent whitish-gray color characteristic of normal crabs. Dorsally the carapace is pinkish as opposed to the normal orange - tan color. The diseased crabs display signs of morbidity including drooping limbs and mouth parts. When the carapace is disrupted the hemolymph is milky white in color rather than the normal translucent gray. The infection was confirmed microscopically by presence of *Hematodinium* sp. in the hemolymph.

There were no microscopically identified infections that were not previously identified macroscopically. (Table 1 and Table 2). All infected crabs were male except for three females which were taken in a sample of ninety eight snow crab caught in White Bay in September 1995.

3.2 Prevalence of *Hematodinium* sp.infections at sites sampled

Conception Bay was sampled at the same site during April in four consecutive years. The percentage of snow crab obtained at this site that exhibited macroscopic signs of infection ranged from a high of 3.75 % (5 infected of 135 crabs caught) in 1993 to a low of 0.57% in 1995 (2 infected of 347 crabs caught) (Table 1).

Collections off St. John's were comprised of different sites sampled over a period from 1994 to 1996 (Table 2). The sites off St. John's were sampled for three years in late May or early June of 1994, 1995 and 1996. The prevalence of infected crabs ranged from a low of 0.004% in 1996 to a high of 0.05% in 1994.

Conception Bay was sampled in late September to early October for three consecutive years, 1994 to 1996. In 1994 no infected crabs were found; in 1995 one and in 1996, seven (0.05%) were recorded. The sample location sites of the fall collections did not correspond to the spring collection sites.

Bonavista Bay was sampled for two years in late August to early September in the years 1995 and 1996. The prevalence was 0.05% in 1995 and 0.03% in 1996.

White Bay was sampled only in September of 1995. It had a prevalence of 1.8% (98 infected of 5,297 crabs). Ninety five of these infected crabs were male, and three were female. These were the only infected females identified in the study.

3.3 Captive monitoring of infection

In April 1994, four infected snow crabs and six uninfected snow crab were held in captivity, in separate tanks, to monitor the progress of infection. The crabs were bled every other day for two weeks then weekly until June 1994. The hemolymph was examined both as a wet preparation and giemsa stained smear. The *Hematodinium* sp.examined from hemolymph samples developed from a vegetative uninucleate stage through to a multinucleate plasmodium and then into the dinospore stage. The crabs were held successfully until July 1994. The experiment was concluded because a water flow failure resulted in the death of all the captive crabs, both infected and control. Additional attempts to convey infected live crabs back to the laboratory failed, possibly because the crabs were already stressed due to the parasite infection.

Table 1 Prevalence (%) of *Hematodinium* sp. infection in snow crab sampled during April in Conception Bay, from 1993 to 1996.

Date	Location	number infected identified macroscopically	Number of crab examined microscopically *	Total number of crabs caught	Percent infected
Apr.-93	Conception Bay	5	5	135	3.70%
Apr.-94	Conception Bay	4	4	126	3.17%
Apr.-95	Conception Bay	2	102	347	0.57%
Apr.-96	Conception Bay	1	26	82	1.20%

* either hemolymph and/or tissue examination

Table 2. Prevalence (%) of *Hematodinium* sp. infection in snow crab sampled from the northwest Atlantic, 1994 to 1996

(Data supplied by Department of Fisheries and Oceans)

Date	Location	Number infected identified macroscopically	Number of crab examined microscopically *	Total number of crabs caught	Percent infected
May-94	St. John's	6	106	10785	0.05%
Oct.-94	Conception Bay	0	100	20751	0.00%
Jun.-95	St. John's	5	105	22963	0.02%
Aug.-95	Bonavista Bay	6	6	11,769	0.05%
Sept.-95	Conception Bay	1	101	23773	0.00%
Sept.-95	White Bay	98#	21	5,297	1.8%
Jun.-96	St. John's	1	61	22,323	0.004%
Sept.-96	Bonavista Bay	2	2	5,784	0.03%
Oct. -96	Conception Bay	7	0	12,308	0.05%

3 Female

* either hemolymph and/or tissue examination.

3.4 Hemolymph smears

The uninfected hemolymph of the snow crab contains two types of hemocytes, hyaline cells and granulocytes. In Giemsa stained smears, the hyaline cells (Figure 2) averaged from 5 to 9 µm in diameter with scanty basophilic cytoplasm. The nucleus was 4 to 7 µm in diameter and granular with a rim of chromatin. The granulocytes are larger in diameter, 7 to 12 µm with a round to irregular nucleus. The cytoplasm of granulocytes contain variable numbers of refractile granules, depending on the age of the cell (Johnson, 1980). The normal hemolymph clots rapidly forming aggregations of cells. None of the hemolymph smears from apparently uninfected crabs examined show evidence of *Hematodinium* sp. infection.

Infected hemolymph contained extremely high concentrations of parasites. The normal hemocyte population is either displaced or masked by the number of parasites. Hemolymph from infected snow crab did not clot. The hemolymph smears, when stained with Giemsa showed three distinct morphological forms of the parasite: spherical vegetative, plasmodial (multinucleate), and pyriform dinospores. Hemolymph smears from individual crab contained only a single form.

The vegetative cells are spherical, non motile and contain an eccentric nucleus and vesicular cytoplasm (Figure 3). The plasmodial stage is multinucleate with the number of nuclei ranging from one to eight (figure 4). The shape of the plasmodial stage varies depending on the number of nuclei present. The dinospore is pyriform in shape and motile with two flagella. The dinospore nucleus is shifted to one end with distinct cytoplasmic granulation present (Figure 4).

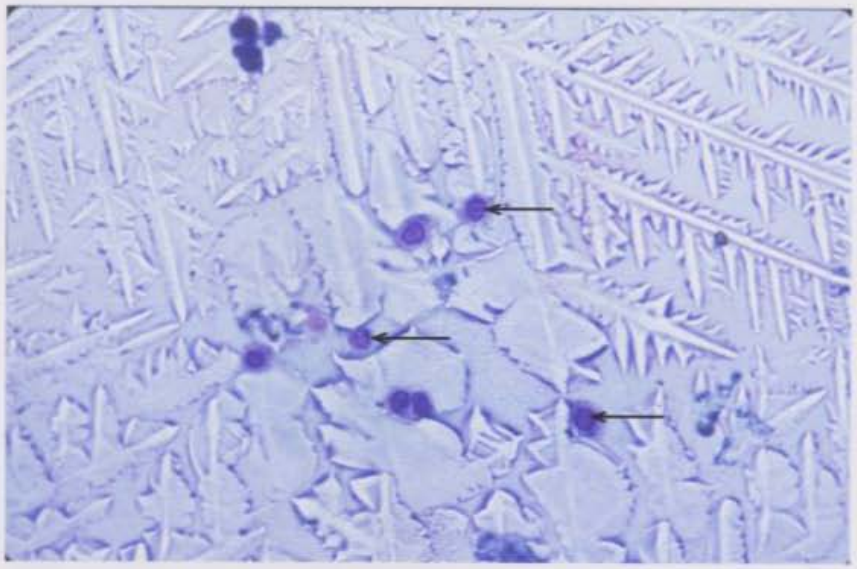

Figure 2. Giemsa-stained smear of normal *Chionoectes opilio* hyaline cells (arrows). Magnification X100.

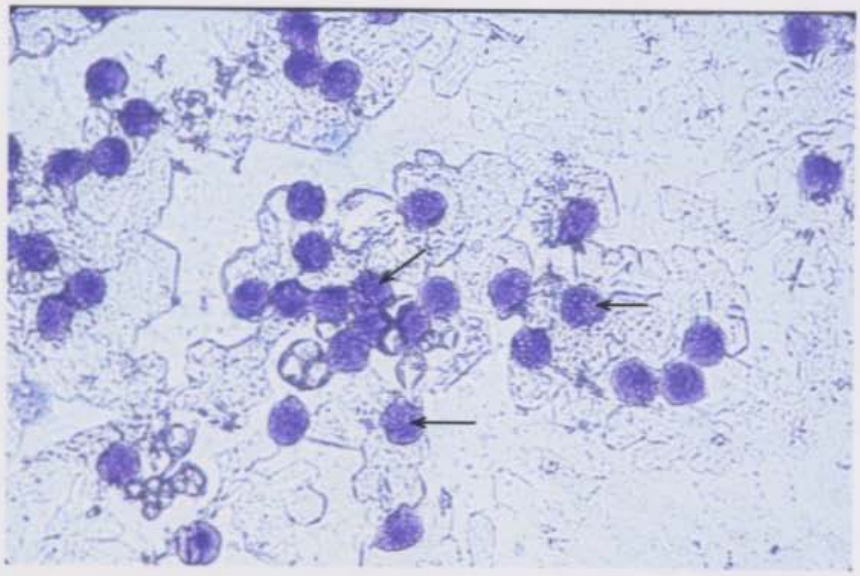

Figure 3. Giemsa - stained smear of vegetative form (arrows) of *Hematodinium sp.* in *Chionoecetes opilio* hemolymph. Magnification X100 .

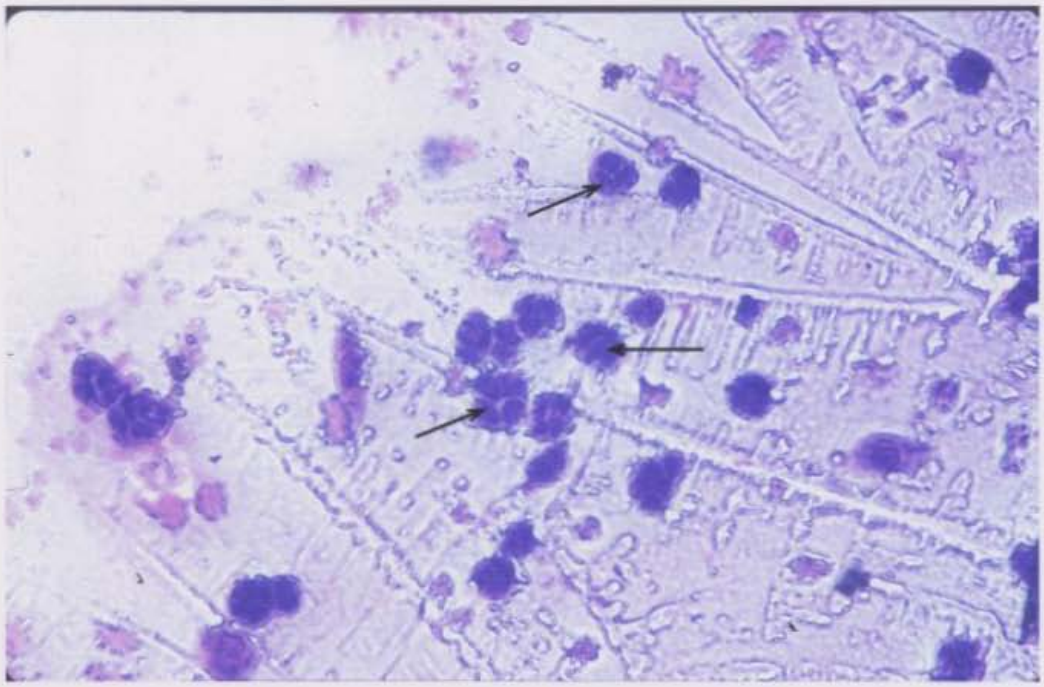

Figure 4. Giemsa - stained smear of multinucleate stage of *Hematodinium* sp. (arrows) Magnification X100.

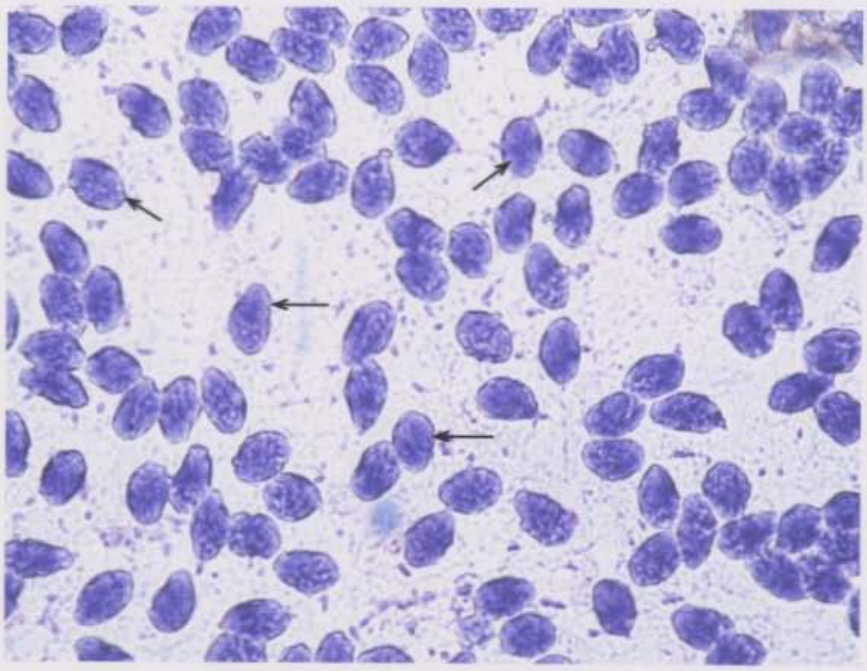

Figure 5. Giemsa - stained smear of the dinospores (arrows) of *Hematodinium sp.* in *Chionoecetes opilio* hemolymph. Magnification X100.

3.5 Histology of infected and uninfected tissues from *Chionocetes opilio*

The histology of selected organs was examined to determine the effect of the *Hematodinium* sp. on the snow crab host tissues. The tissues examined include gill, muscle, hepatopancreas and testis.

3.5.1 Gill histology

The snow crab gills function in osmotic control, respiration and excretion. Structurally, each gill consists of a central axis bearing serially paired leaf-like lamellae that are flattened sacs. The epithelial cells along the flattened surface of the lamellae are squamous (functioning for gaseous transport) with many columnar supporting trabecular cells joining the two lamella surfaces. There are hemocytes present within the hemal channel of the gill. The uninfected gill exhibits normal morphology (Figure 6). The gill filament from a *Hematodinium* sp. infected crab is distended with disruption of the epithelium and cross linkages of trabecular cells. The hemocytes are displaced by the large numbers of the parasite. The parasite is restricted to the hemal spaces within the gill and is seldom found outside the hemal spaces unless obvious rupture of the filament had occurred (Figure 7).

3.5.2 Muscle histology

The uninfected striated muscle contains tightly packed muscle bundles containing fibers with the hemal spaces containing connective tissue and hemocytes (Figure 8). Striated muscle has peripherally located nuclei. Three types of striations are present in the muscle: A, I and Z bands. The Z banding is not always visible under light microscopy. The infected muscle shows grossly distended hemal spaces, disrupting the connective tissue and replacing the hemocytes with *Hematodinium* sp. The muscle fibers themselves are not as tightly packed as in the uninfected crab (Figure 9). This disruption of the

musculature is expressed in the crab as drooping limbs and mouth parts with lethargy at the time of capture.

3.5.3 Hepatopancreas histology

The hepatopancreas consists of a series of blind tubules that are separated by hemal sinuses. These tubules act in the absorption of nutrients, lipid storage and production of digestive enzymes. In uninfected crab, the tubules are packed fairly close together and the interstitial spaces contain hemocytes and connective tissue. The tubule epithelium is composed of four cell types: E- cells, R-cells, F- cells and B-cells. E-cells are undifferentiated cells that form a mitotic center at the blind end of the tubule. R-cells are found proximal to the E-cells and are both absorptive and storage cells. B-cells produce and secrete digestive enzymes. F-cells are thought to be the precursors of the B-cells (Figure 10).

In the *Hematodinium* sp. infected crab hepatopancreas, the entire hemal space between the tubules is filled with the parasite. The connective tissue appears ruptured and no hemocytes are evident. There appears to be an increased vacuolation of the B-cells. *Hematodinium* sp. is absent from the lumen of the tubules (Figure 11).

3.5.4. Testis histology

The testis is a tubular organ consisting of a central seminiferous duct surrounded by testicular lobes. The entire testis is enveloped in a thin fibrous capsule with extensions of the capsule separating the lobules. Lobes may be tightly bound by connective tissue while others are separated by hemal spaces. The uninfected testis shows normal histology with the hemal spaces containing hemocytes (Figure 12). The infected testis shows normal testis histology but the hemal spaces are invaded by the *Hematodinium* sp. parasite (Figure 13).

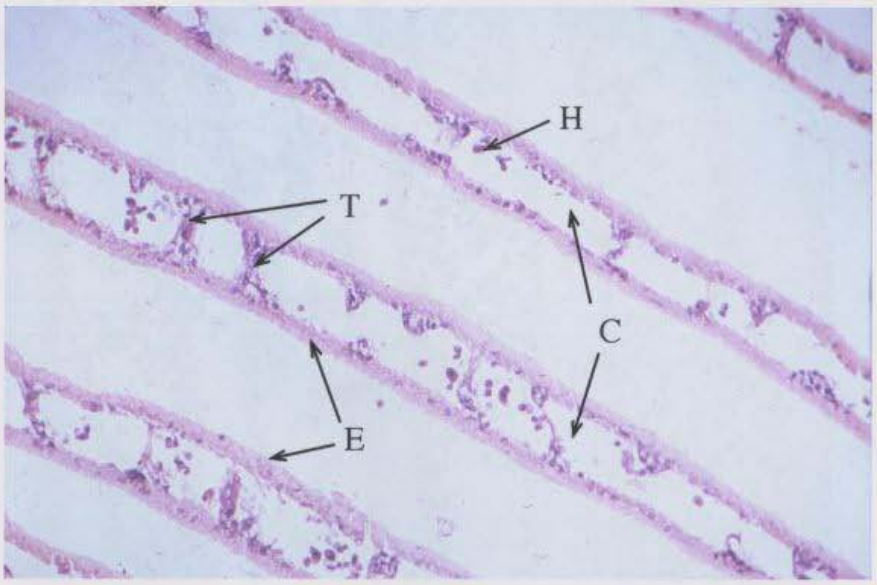

Figure 6. Light micrograph of uninfected *Chionoecetes opilio* gill lamellae showing: hemal channel (C), epithelial cells (E), trabecular cells (T) and hemocytes (H). Haematoxylin and eosin stain. Magnification X40 .

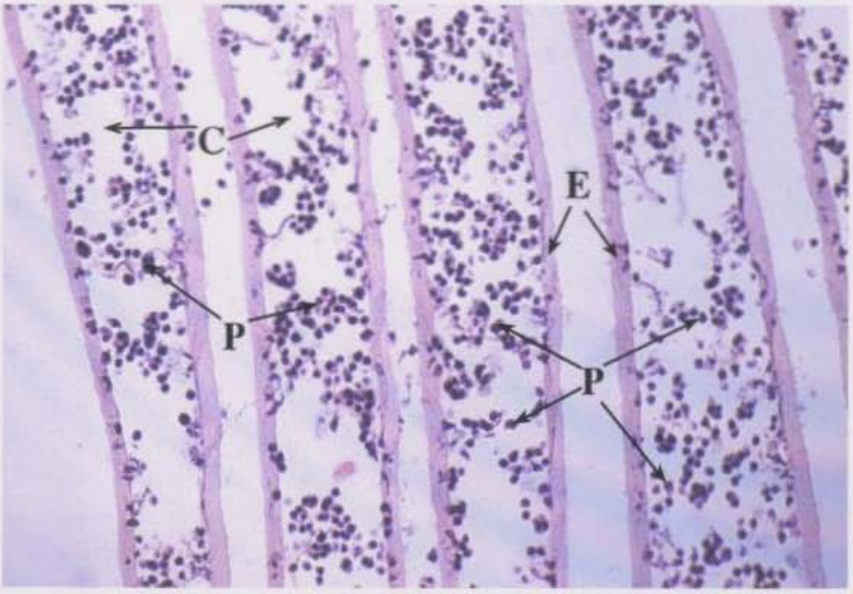

Figure 7. Light micrograph of *Chionoectes opilio* gill lamellae infected with *Hematodinium* sp. showing, epithelium (E), hemal channels (C), *Hematodinium* sp. (P). Haematoxylin and eosin stain. Magnification X 40.

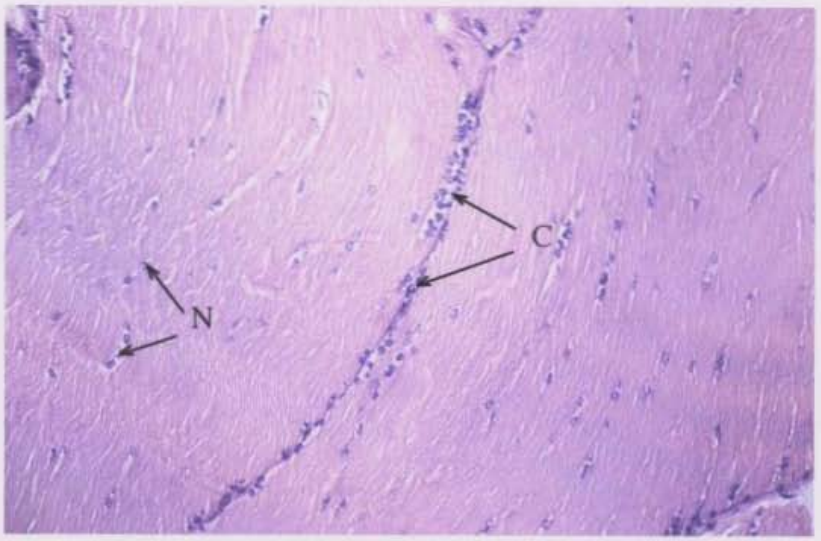

Figure 8. Light micrograph of uninfected*Chionoecetes opilio* skeletal muscle showing connective tissue and hemocytes (C), muscle cell nuclei (N). Haematoxylin and eosin stain. Magnification X40.

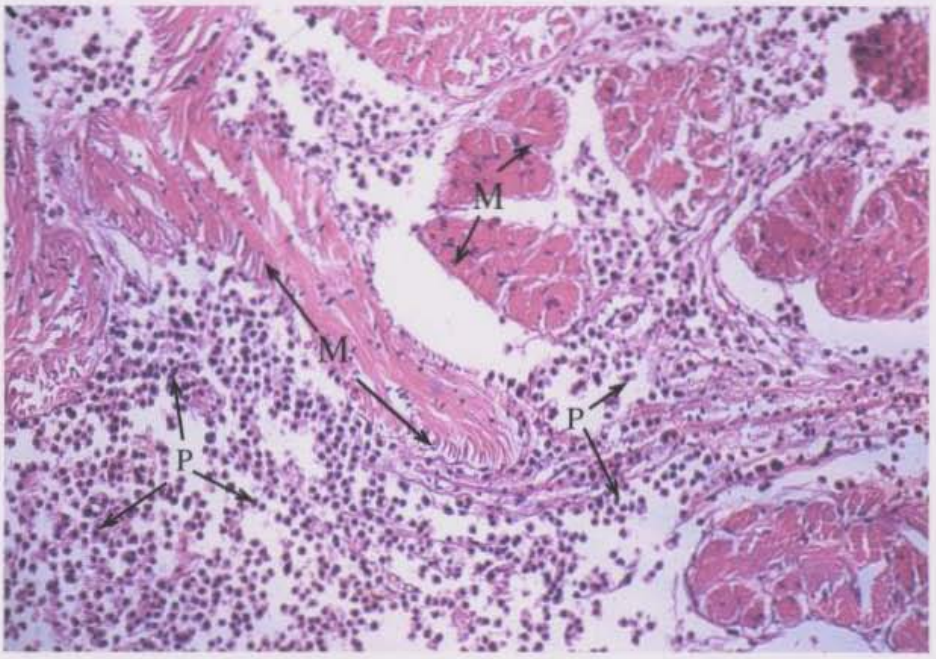

Figure 9. Light micrograph of *Chionocetes opilio* skeletal muscle infected with *Hematodinium* sp. vegetative stage, showing muscle fibres (M) *Hematodinium* sp. (P). haematoxylin and eosin stain. Magnification X40.

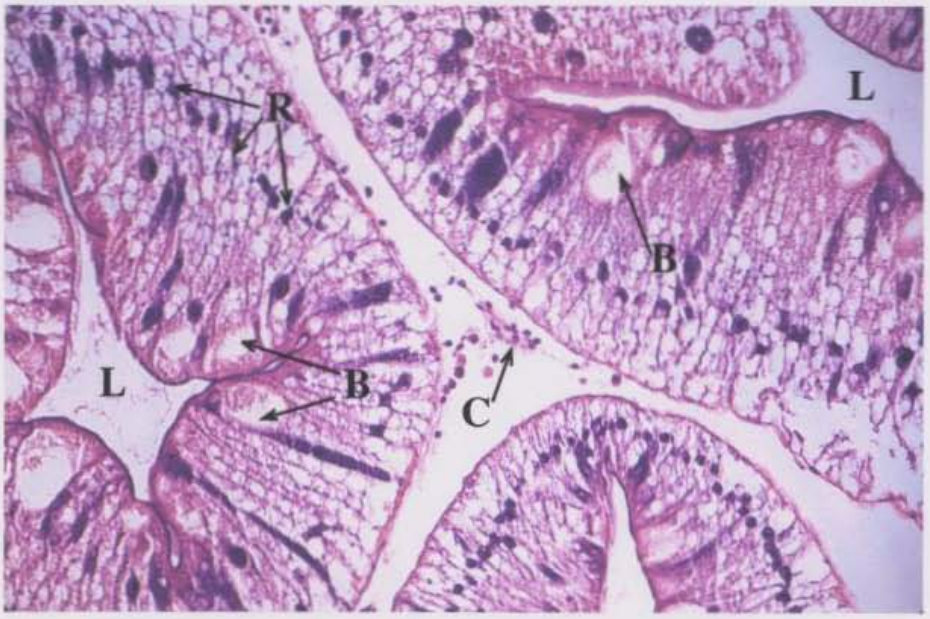

Figure 10. Light micrograph of uninfected *Chionoectes opilio* hepatopancreas showing tubule lumen (L), R-cell (R), B-cell (B), connective tissue and hemocyte in interstitial space (C), Haematoxylin and eosin stain. Magnification X40.

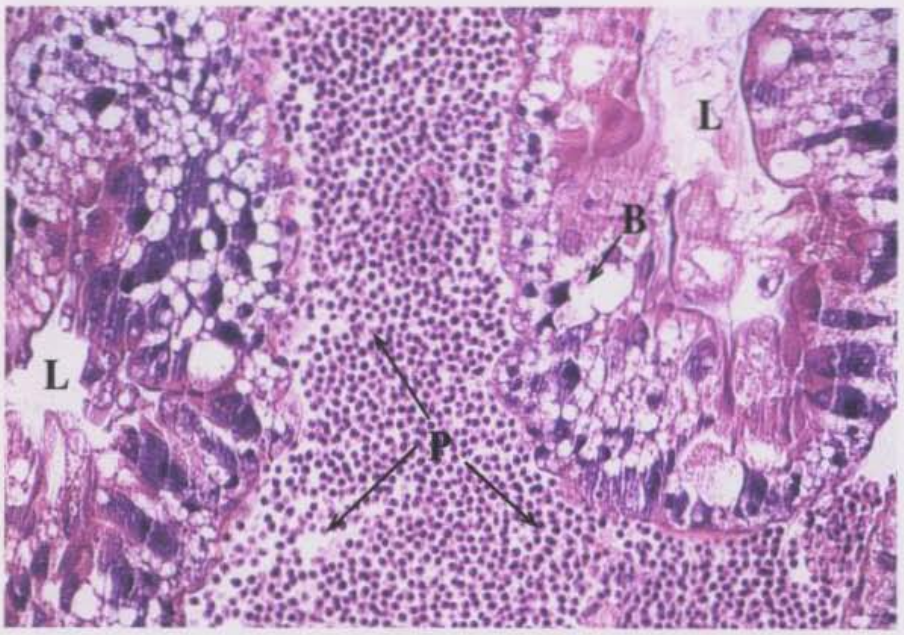

Figure 11. Light micrograph of *Chionoecetes opilio* hepatopancreas infected with *Hematodinium sp.* showing tubule lumen (L), B-cell (B), *Hematodinium sp.* (P). Haematoxylin and eosin stain. Magnification X40.

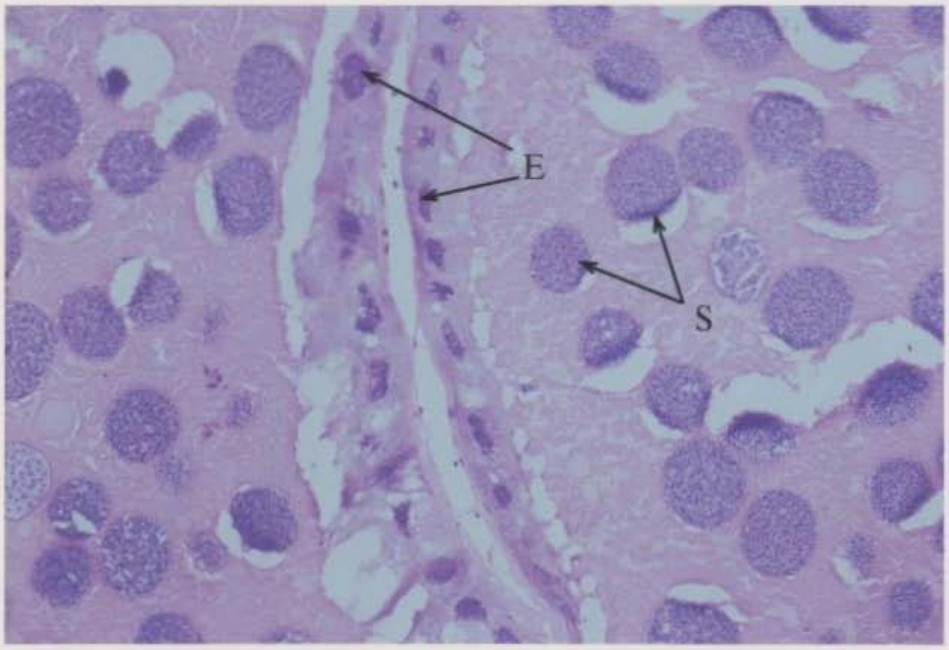

Figure 12. Light micrograph of uninfected *Chionoecetes opilio* testis showing, epithelial cells (E), spermatophores (S). Haematoxylin and eosin stain. Magnification X40.

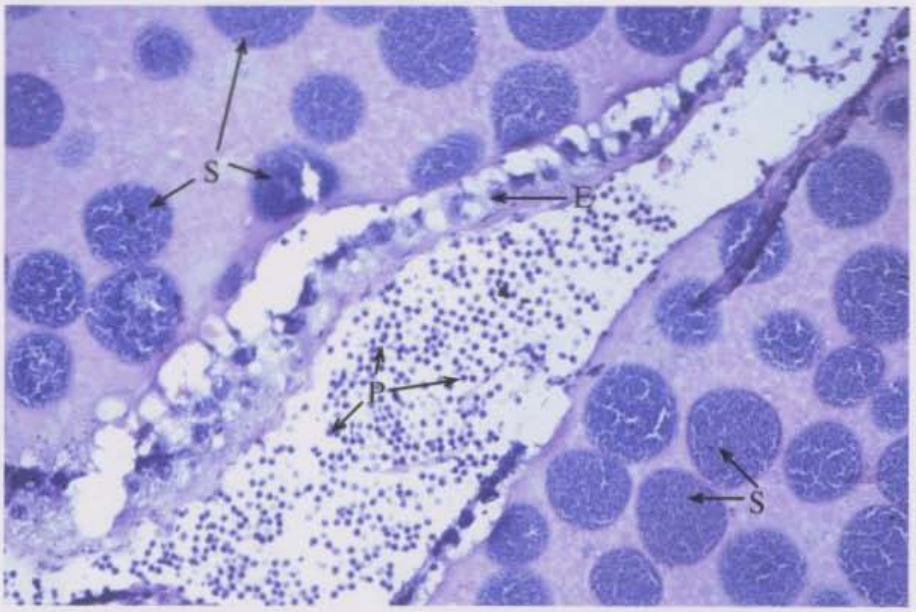

Figure 13. Light micrograph of *Chionoecetes opilio* testis infected with *Hematodinium* sp. showing, epithelial cells (E), spermatophores (S) and *Hematodinium* sp. (P). Haematoxylin and eosin stain. Magnification X40

3.6 Scanning electron microscopy of *Hematodinium* sp.

3.6.1 Scanning electron microscopy of vegetative cells

The vegetative cells of *Hematodinium* sp. were observed to have smooth surfaces with no obvious thecal plate arrangement or surface architecture. The cells appear uniformly spherical and average 9 µm in diameter (n=20) (Figure 14).

3.6.2. Scanning electron microscopy of dinospores

The dinospores of *Hematodinium* sp. are elongate with two flagella. There is no obvious surface architecture or thecal plate arrangement. The dinospores are uniform in size and shape, averaging 8 µm in length and 3 µm width (n=20) (Figure 15). A longitudinal flagella extends posteriorally and a transverse flagella, inserted above the longitudinal flagella, extends around the circumference of the cell (Figure 16).

3.7 Transmission electron microscopic studies of *Hematodinium* sp.

3.7.1 Transmission electron microscopy of vegetative cells

In the vegetative stage of *Hematodinium* sp. the nucleus contains condensed chromatin. The cytoplasm contains mitochondria with tubular cristae (Figure 18), trichocysts and numerous large lipid vacuoles (Figure 17). Trichocysts are found in all developmental stages of the *Hematodinium* sp. The exact function of trichocysts is unknown (Spector, 1984) although several hypotheses have been proposed including toxin discharge and secretion. A longitudinal section of a trichocyst is shown (Figure 18).

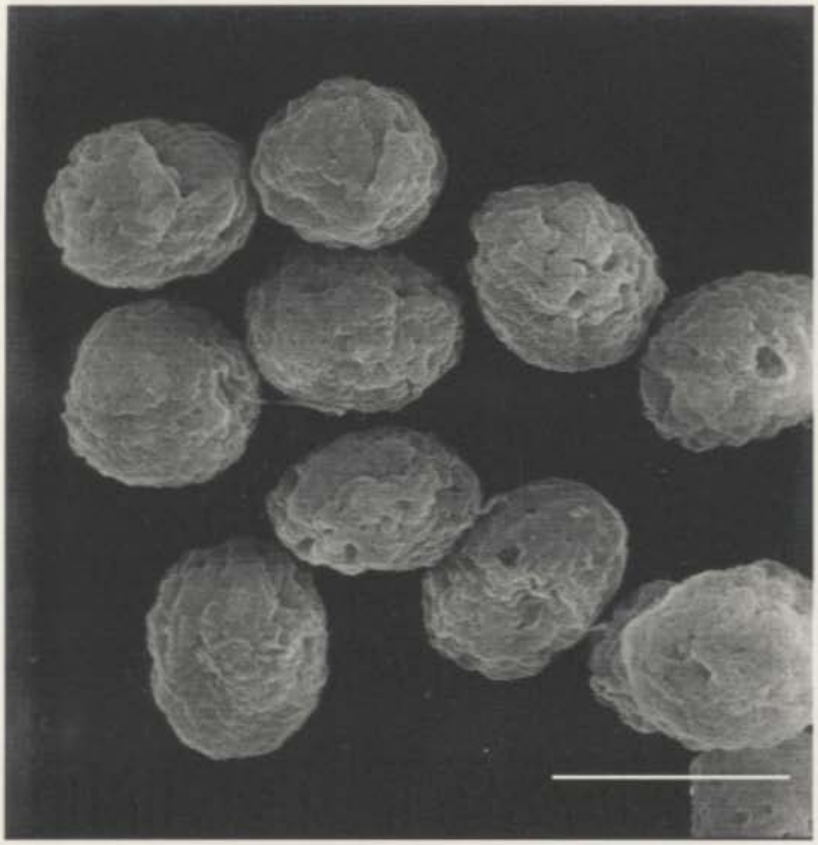

Figure 14. Scanning electron micrograph of the vegetative stage of *Hematodinium* sp. occurring in *Chionoecetes opilio*. Bar = 10 µm.

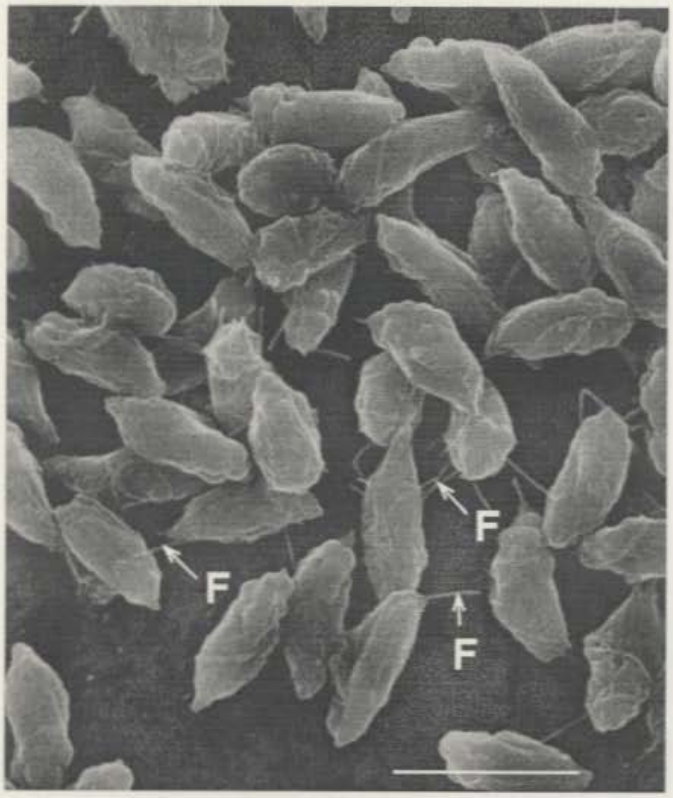

Figure 15. Scanning electron micrograph of the dinospore stage of *Hematodinium* sp. showing the pyriform shape and flagella (F) Bar = 10 µm .

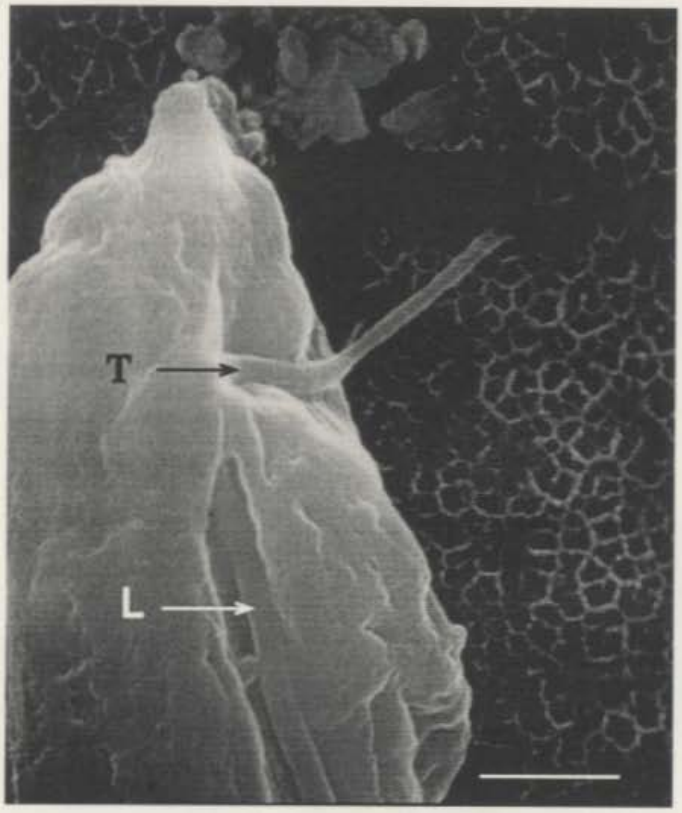

Figure 16. Scanning electron micrograph showing the insertion of the transverse (T) and longtitudinal (L) flagella in the dinospore of *Hematodinium* sp. occurring in *Chionoectes opilio*. Bar = 1 µm.

3.7.2. Transmission Electron Microscopy of multinucleate plasmodia

The multinucleate plasmodium is the rapidly dividing developmental stage between the vegetative cell and the dinospore. The size and shape varies depending on the number of nuclei present, which range from one to eight (Figure 19). The transmission electron microscopic detail (Figure 20) reveals nuclei with condensed chromatin. The cytoplasm contains vacuoles, trichocysts and mitochondria.

3.7.3 Transmission Electron Microscopy of dinospore

The dinospore is elongate with vacuoles polarized towards the apical end (Figure 21). The nucleus contains condensed chromatin with the nucleus shifted to the posterior end. The cytoplasm contains two flagellae, which are composed of a 9+2 axoneme arrangement, connected by a striated root (Figure 22). The cytoplasm also contains numerous trichocysts, located towards the periphery of the cell and mitochondria with tubular cristae The organism is surrounded by an amphesial alveolus. There are micropores associated with the dinospore stage (Figure 23).

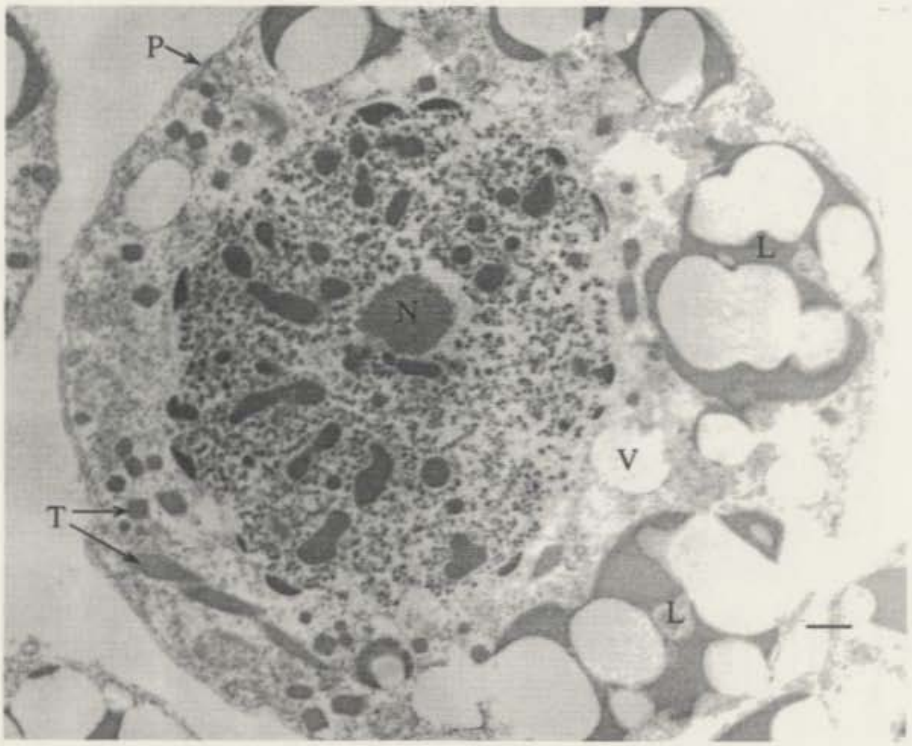

Figure 17. Transmission electron micrograph of the vegetative stage of *Hematodinium* sp. showing, lipid droplets (L), vacuole (V), trichocyst (T), nucleus with condensed chromatin (N) and pellicle (P). Bar = 1μm.

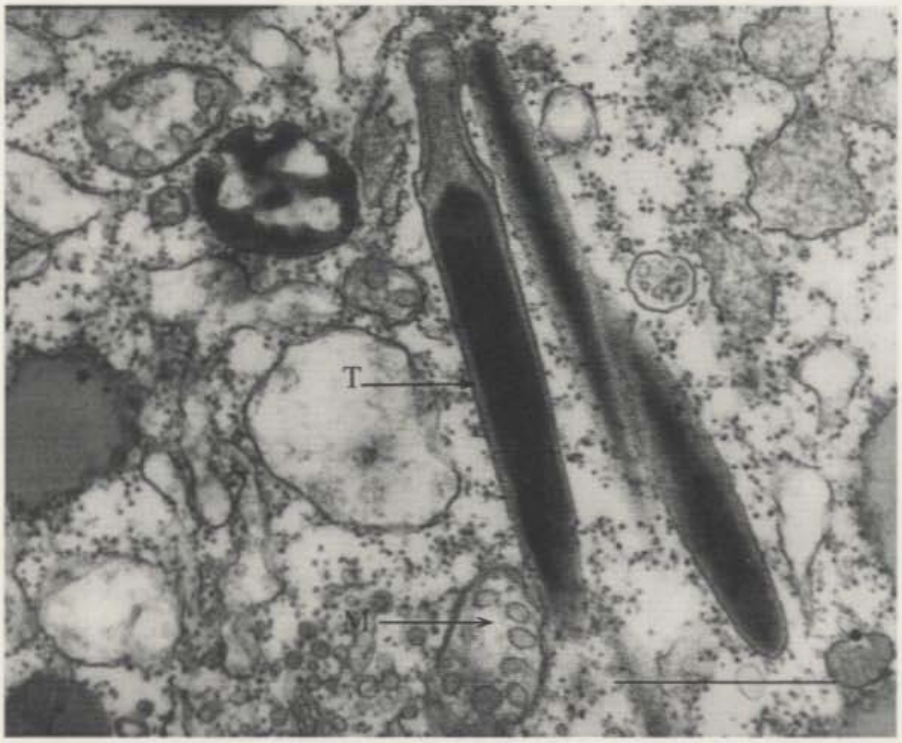

Figure 18. Transmission electron micrograph of a longtitudinal section of a trichocyst (T) in a vegetative stage of *Hematodinium* sp. mitochondrion (M). Bar = 1μm.

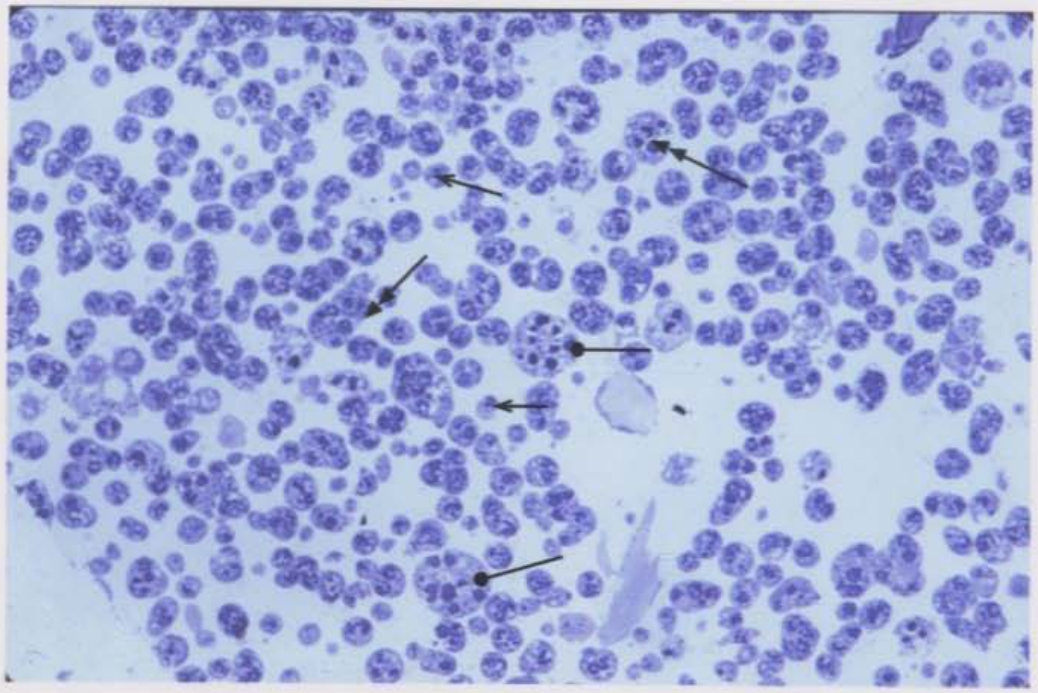

Figure 19. Light micrograph of multinucleate stages of *Hematodinium* sp. showing the varying number of nuclei, one (single arrow), four (double–headed arrow) and eight (round–headed arrow). 0.5 μm section, toluidine blue stain. Magnification X100.

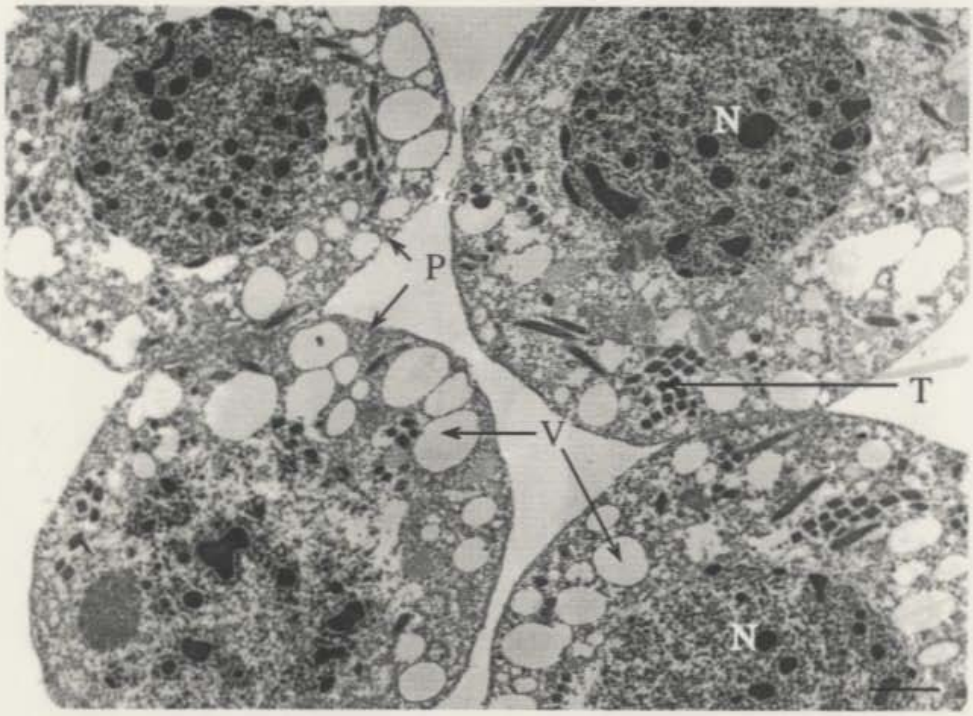

Figure 20. Transmission electron micrograph of a late multinucleate stage of *Hematodinium* sp. showing , vacuole (V), nucleus (N), trichocyst (T), and pellicle (P). Bar = 1µm.

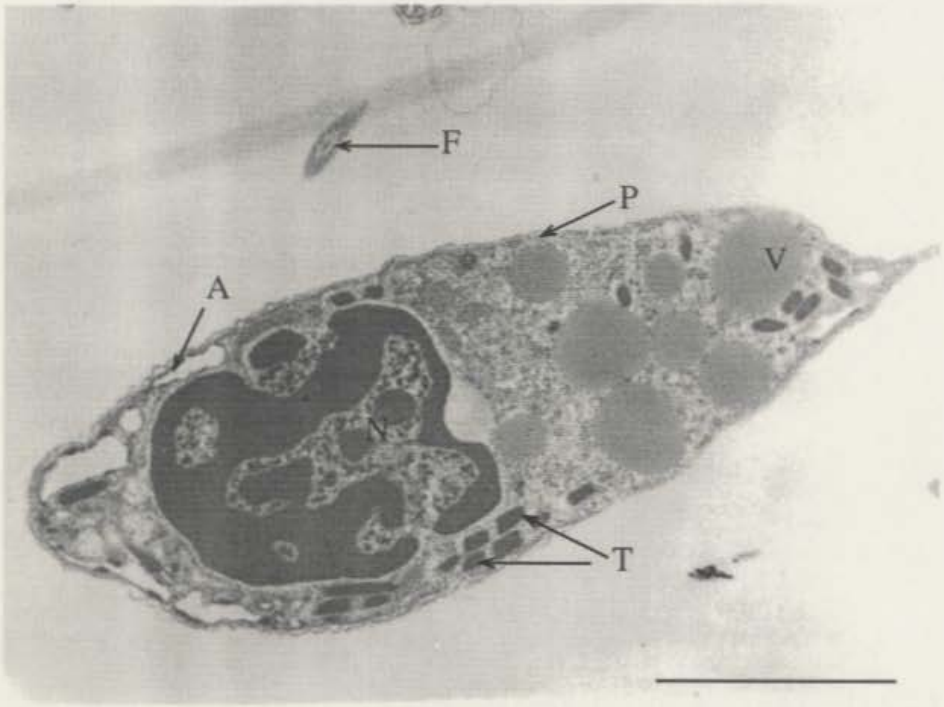

Figure 21. Transmission electron micrograph of the dinospore stage of *Hematodinium* sp. showing the fine structural details, flagella (F), lipid vacuole (V), trichocyst (T), amphesial alveolus (A) and pellicle (P). Bar = 1μm.

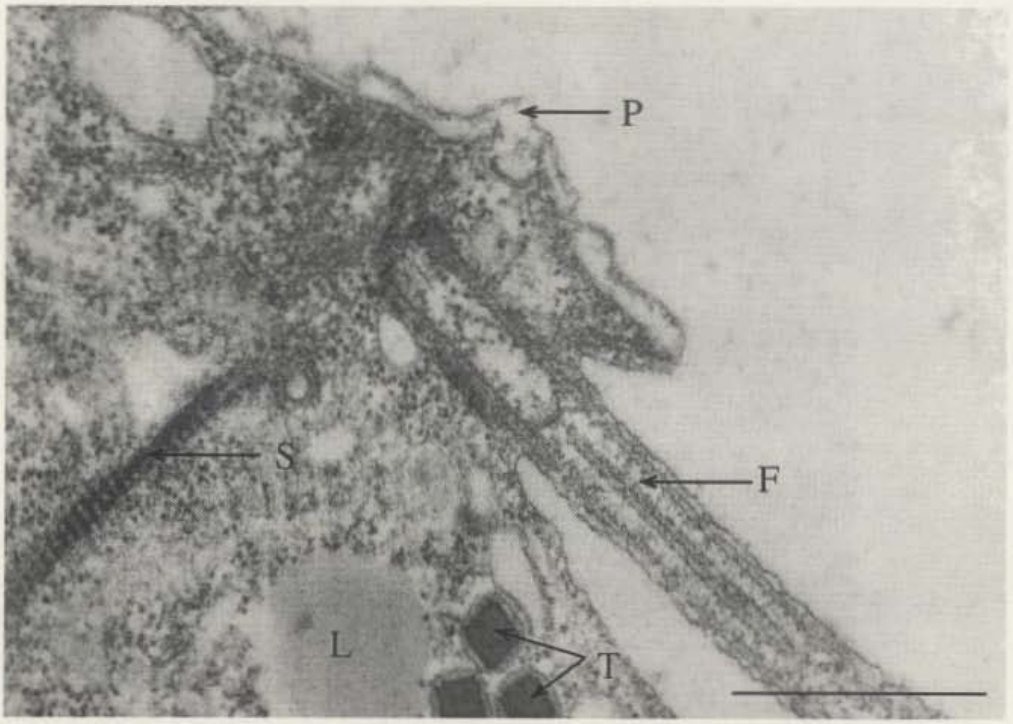

Figure 22. Transmission electron micrograph of flagella insertion in dinospore of *Hematodinium* sp. showing flagella (F), striated rootlet (S), trichocyst (T), lipid body (L) and pellicle (P). Bar = 1µm.

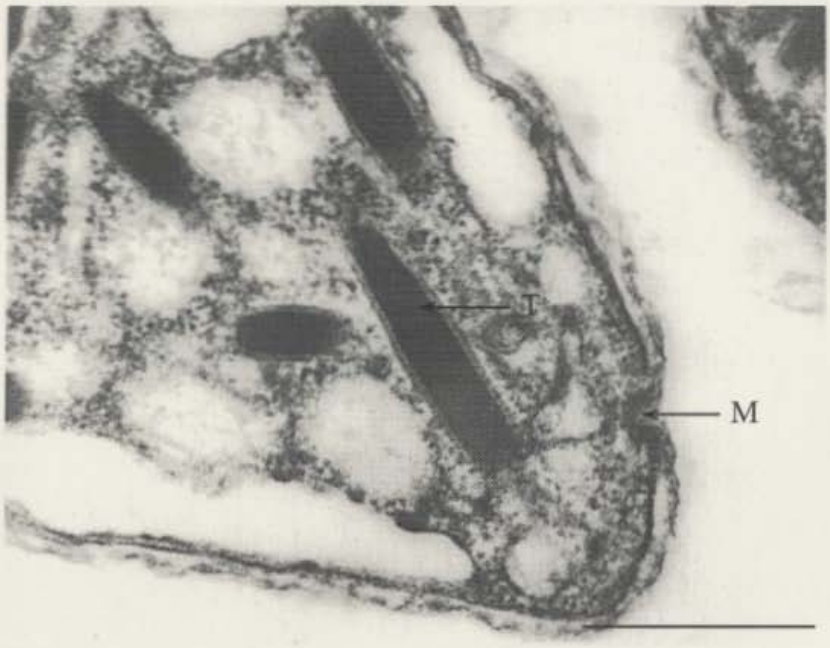

Figure 23. Transmission electron micrograph of the dinospore stage of *Hematodinium* sp. showing a micropore (M) and trichocyst (T). Bar = 1μm.

DISCUSSION

4.1 Historical

The present and a previous study (Taylor and Khan, 1995) document the presence of snow crab showing macroscopic signs of *Hematodinium* sp. infections in Newfoundland waters. The diseased snow crab were first noted by D.F.O. personnel during the annual stock assessment surveys conducted during 1990 in Bonavista Bay (Taylor and Khan, 1995). Subsequent to these findings diseased crab have been caught at a number of locations in Newfoundland waters by fishermen, D.F.O. personnel and by researchers participating in the present study.

In Alaska a disease condition known as Bitter Crab Disease "BCD", (or Syndrome "BCS") affected the commercially fished crab populations dramatically during the period 1985-1991 (Meyers et al., 1996). The diseased crab were first noted by the commercial fishermen and in follow - up studies by Alaska Fish and Game Department personnel the causative agent was identified as *Hematodinium* sp. (Meyers et al., 1987). In some of the Alaskan commercial crab stocks prevalence of infection reached 95% (Meyers et al., 1996). In contrast, prevalence in commercial stock observed during the present study was low and did not exceed 4%.

While the prevalence found in the present study of *Hematodinium* sp. in snow crab is low, it may be an artifact of the sampling technique. Samples of snow crab examined in this study were obtained from Conception Bay, Bonavista Bay and east of St. John's, where crab were captured using the commercial Japanese conical trap. This sampling technique requires the active participation of the crab and very few crabs <95 mm carapace width are caught because of the mesh size of the trap. All of the snow crabs caught during the present study and subsequently confirmed to harbor *Hematodinium* sp. displayed outward signs of lethargy, drooping mouth parts in addition to a pink (cooked) coloration. A sample from White Bay was obtained using mobile gear (modified shrimp

trawl). The trawl used in this study was towed and had the potential to capture snow crab of various sizes. The White Bay sample contained an observed infection rate of 1.8% and the only positively identified infected female snow crabs in this study. It is possible therefore that the Japanese conical pot capture method underestimates the prevalence of infection by excluding female and smaller size classes from the sample. A similar trawl sample was conducted off the north east Avalon in June 1996 to substantiate this prediction, but it did not capture a wide size range of crabs, or infected individuals.

There are large variations in the prevalence of infection in crustacean populations elsewhere. Eaton et al. (1991) report infection rates up to 95% in *C. bairdi* in Alaska. Off the east coast of Scotland investigators report prevalence in *Nephrops norvegicus* to reach 70% (Field et al., 1992). In other areas the level of infection reported was lower. In Florida the prevalence of infection by *Hematodinium* sp. in blue crab (*Callinectes sapidus*) reached a high in one catch of 30% (Newman and Johnson, 1975). The higher levels of infection in Alaska and Scotland were attributed to poor water circulation in the fjords and firths (Hudson and Shields, 1994). Salinity is an oceanographic factor reported to affect the distribution of *Hematodinium* sp. as infections did not occur below 11 ppt when this relationship was investigated (Newman and Johnson, 1975). The salinity of water in the northwest Atlantic is between 32-35 ppt, well above the lower threshold suggested for infection, thus salinity is unlikely to be a factor contributing to the low prevalence observed in this study.

All the crab examined in this study were "post molt shell condition 2" (Miller and O'Keefe, 1981). Examination of snow crab hemolymph in this study was restricted to these recently molted individuals because other studies report a correlation between molt cycle and presence of infection. In Alaska, 81% of new shelled Tanner crab (*C. bairdi*) showed signs of *Hematodinium* sp. infection compared to 24% of crab with older and

harder shells (Eaton et al. 1991). Messick (1994) also reports that in the coastal bays of Maryland and Virginia that there was a higher prevalence of *Hematodinium perezi* infection in post–molt blue crab (*Callinectes sapidus*) when compared to premolt.

4.2 Methods of identification

The snow crab in the present study were examined macroscopically for evidence of infection. A number of apparently healthy crab were bled for hemolymph examination to search for individuals with low levels of infection that did not exhibit the outward characteristics of infection. None of the hemolymph samples from these crab showed presence of *Hematodinium* sp. (n = 525).

In Scotland *Hematodinium* sp. infection in Norway lobster was identified and the level of infection was graded by pleopod examination (Field et al., 1992). The method developed by Field et al. (1992) was modified by Messick (1994) to detect infection in juvenile blue crab. The method employed by Field et al. (1992) and Messick (1994) was not applicable during the present study because the exoskeleton of adult snow crab is too thick to allow observation of the hemolymph by back illumination.

A number of methods have been used as alternatives to macroscopic and microscopic examination to identify sub-clinical infections. These include an indirect antibody technique (Field and Appleton, 1996). This method identified infections in Norway lobster that previously were not detected through hemolymph examination. The indirect antibody method can also be applied to tissue samples. When applied it confirmed previously undiagnosed tissue infections (Field and Appleton, 1996).

Field and Appleton (1996) also identified parasitic syncytia within the hepatopancreas of the Norway lobster using antibody methods, previously these syncytia were only observed within the heart and abdominal muscle under light microscopy (Field

and Appleton, 1995). Parasite syncytia were not observed in the hepatopancreas of snow crab during the present study, but heart and abdominal muscle were not examined. Polymerase chain reaction techniques (PCR) have been developed in Australia to identify *Hematodinium* sp. infections in decapod crustaceans (Hudson and Adlard 1994). Researchers used samples of *Hematodinium* sp. collected from five species of decapod Crustacea from various geographic locations including Alaska, Scotland, Virginia and Australia. Hudson and Adlard (1994) conclude that a positive PCR indicates presence of non – host DNA and recommend that *Hematodinium* sp. infection should be confirmed by examination of the hemolymph.

4.3. Pathology associated with *Hematodinium* sp. infections

Microscopic examination of the snow crab tissues in the present study revealed that the parasite infection is restricted to the hemal spaces. This observation is in accord with reports by other researchers who found that the parasite was restricted to the hemolymph (Hudson and Shields, 1994; Messick, 1994; Meyers et al., 1987). Pathology associated with infections observed in this study include the displacement of normal hemocytes, degeneration of muscle tissue, vacuolization and lysis of hepatopancreas epithelia and disruption of connective tissue within the testis and gills. The large number of *Hematodinium* sp occupying the hemal spaces likely cause anoxia. This is due to blockage of the hemal channels and competition between the host and parasite for oxygen within the hemolymph (Taylor, 1996) The ultimate consequence of the infection is death of the host. The pathologies found in infected snow crab were similar to those described in *C. bairdi* (Meyers et al., 1987) and *Callinectes sapidus* (Messick, 1994).

The present study documents the infiltration of the hemal spaces and disruption of connective tissue in the testis by the parasite with no infiltration of the testis. Meyers et al. (1996) found the parasite within the seminal fluid surrounding the spermatophores. Meyers

et al. (1996) suggested that although this is a passive infiltration of the testis caused by the large numbers of *Hematodinium* sp. present, there is potential for sexual transmission of the parasite between individual crabs. In crabs where extremely high numbers of *Hematodinium* sp. resulting in passive infiltration of seminal fluid, the muscle and other tissues likely have been affected. A host with this level of infection would be moribund and would be unable to copulate.

4.4 A comparison of the microscopic characteristics of *Hematodinium* sp.

Hematodinium sp. in the present study were compared to the type description (Chatton and Poisson, 1930) and to other descriptions in the published literature. These comparisons are summarized in Table 3. The type species description of *H. perezi* by Chatton and Poisson (1930) was based on light microscopic studies of the vegetative and plasmodial stages without a record of the dinospore stage.

The characteristic features of *Hematodinium perezi* include presence of trichocysts within the cytoplasm, dinomitosis, a continual state of mitotic activity within the nucleus and apparent absence of a nuclear membrane (Chatton and Poisson 1930). In the present study trichocysts were a feature of all life history stages. The size of the vegetative stage in the present study is 8.0-10 µm in diameter similar to *H. perezi* (8.0-9.0 µm) (Chatton and Poisson, 1930). However the shape of the plasmodia differ. In the present study plasmodia appeared round whereas Chatton and Poisson (1930) described the plasmodia as vermiform. This difference could be attributed to a host influence. *H. perezi* plasmodia isolated from *Ovalipes ocellatus* were round and non–motile (MacLean and Ruddell ,1978) whereas the *H. perezi* plasmodia from *Carcinus maenas* were vermiform and highly motile (Newman and Johnson 1975). Plasmodia observed during the present study were non–motile.

Comparing the *Hematodinium* sp. isolated from *C. opilio* in the present study with the *Hematodinium* – like dinoflagellate isolated from *C. bairdi* and *C. opilio* in Alaska, two main differences are evident. In *Hematodinium* sp. isolated from *C. opilio* , (present study), trichocysts are present in the vegetative stage. In *Hematodinium* sp. isolated from *C. bairdi* the vegetative stage lacked trichocysts.Two types of dinospores (15.2 µm x 11.4 µm. and 12.0 µm x 4.4 µm) were observed in the *Hematodinium* sp. isolated from *C.bairdi* . Only one size dinospore was isolated from *C. opilio* in the present study (10.8 x 3.6 µm) comparable to the smaller size of dinospore (12.0 x 4.0 µm) observed in *C. bairdi* .

Table 3.Comparison of the characteristics of *Hematodinium* spp. in decapods from various geographic locations. na = not reported by authors.

Host	Parasite I.D.	Vegetative size µm	Presence of trichocysts	Shape of plasmodia	motile plasmoidia	Dinospore size in µm	Source
Carcinus maenas, Liocarcinus depurator	*Hematodinium perezi*	8.0-9.0	present	vermiform	na	na	Chatton and Poisson, 1930
Callinectes sapidus	*Hematodinium perezi*	6.4-10.4	present	vermiform	highly motile	na	Newman and Johnson, 1975
Ovalipes ocellatus, Cancer irroratus, C. borealis	*Hematodinium perezi*	9.0-14.0	na	round	na	na	MacLean and Rudell, 1978
Chionoecetes biardi	*Hematodinium sp*	15.6 x 12.8	absent	round	na	15.2 x 11.4 and 12.0 x 4.4	Meyers et al., 1987
Cancer pagarus	*Hematodinium-like*	10.0-15.0	present	round	na	no	Latrouite et al., 1988
Chionoecetes biardi	*Hematodinium-like*	6.0-11.0	na	round	na	two types not meas.	Eaton et al., 1991
Nephrops norvegicus	*Hematodinium like*	6.0-10.0	present	vermiform and round	na	na	Field et al., 1992
Trapzia areolata	*Hematodinium sp.*	6.9-12.8	present	round	na	na	Hudson et al., 1994
Portunus pelagicus	*Hematodinium australis*	9.9-11.9	present	round	na	na	Hudson and Sheilds, 1994
Callinectes sapidus	*Hematodinium perezi*	10.5	na	elongate	amoeboid movement	na	Messick,1994
Necora puber	*Hematodinium sp.*	12.0-25.0	no	na	na	no	Wilhelm and Mialhe,1996
Chionoecetes opilio	*Hematodinium sp.*	8.0-10.0	present	round	no	10.8 x 3.6	present study

Ultrastructural examination of *Hematodinium* sp. isolated from *C. opilio* in the present study revealed the presence of micropores in the dinospore stage. The micropore is a structure characteristic of Apicomplexa (Levine et al., 1980). Appeton and Vickerman (1996) also noted the presence of these micropores in the biflagellate dinospore stage of *Hematodinium* sp. isolated from *in vivo* and *in vitro* culture. Appleton and Vickerman (1996) demonstrated a cytosomal function of the micropore by labeling culture media with colloidal gold and ferritin and demonstrated its uptake at a micropore sites using electron microscopy. Appleton and Vickerman (1996) suggest relationships between apicomplexans, dinoflagellates and ciliates based on molecular systematics to account for the presence of a apicomplexan type micropore in a dinoflagellate. Although vegetative, plasmodial and dinospore stages were examined in the present study, micropores were only observed in the flagellated dinospore stage.

Vickerman (1994) has been able to culture *in vitro Hematodinium* sp. isolated from *Nephrops norvegicus* and to examine uninucleate cells, multinucleate (plasmodial) cells, a filamentous network syncytia and two sizes of dinospore. The two sizes of biflagellate dinospores identified *in vitro* culture have not been described from *Nephrops norvegicus* .

The vegetative stage of *Hematodinium* sp. from *C. opilio* in the present study is similar in size to the vegetative stage of *H. australis* (Hudson and Shields, 1994). Trichocysts are present in the late vegetative stage of *H. australis* but not in the earlier stage. In this study trichocysts were observed in all stages. However the presence or absence of trichocysts may be dependent on the host. Trichocysts were absent in the *H. australis* vegetative stage isolated from *Scylla serrata* but were present in the *H. australis* vegetative stage isolated from *Portunus pelagicus* (Hudson and Shields, 1994). Flagellated dinospores of *H. australis* were not described.

Morphological studies of an organism can only reveal the phenotypic expression and these physical characteristics might be influenced by the host environment. Recent nucleotide sequencing by Hudson and Adlard (1996) on *Hematodinium* spp. isolated from several Crustacea hosts may help in identifying different species within the genus. Nucleotide sequencing of *Hematodinium perezi* isolated from *Callinectes sapidus* and of *Hematodinium* -like dinoflagellates isolated from *Nephrops norvegicus*, *Chionoecetes bairdi* and *C. opilio* (from Alaska) reveal that the parasites all belong to the same genus (Hudson and Adlard 1996). Differences in the nucleotide sequencing suggested that two new species be named; one isolated from *Nephrops norvegicus* and a separate *Hematodinium* sp. isolated from both *Chionoecetes bairdi* and *C. opilio* in Alaska (Hudson and Adlard, 1996). The authors do not suggest names for these two new forms.

4.5. Life cycle

The life cycle of very few marine parasitic dinoflagellates have been illustrated and our understanding of the parasite's survival strategy during seasonal host absences is incomplete (Drebes, 1984). Field and laboratory studies by Meyers et al. (1987) suggest that the incidence of Bitter Crab Disease follows a yearly cycle reflecting pattern in the life history of *Hematodinium* sp. They propose that the *Hematodinium* sp. infective agent is either the dinospore, or a zygote produced by fusion of the two sizes of dinospore. The dinospores isolated by Meyers et al. (1987) were viable in sea water for up to three months. The higher prevalence of infection in newly molted crabs suggests a route of infection by the dinospore or zygote through the carapace during this molt period.

Meyers et al. (1996) proposed an alternate route of transmission through a vegetative form released from infected and dying crabs, or excreted from the urine and feces thus infecting other crabs that have aggregated for molting. *Hematodinium* sp. may infect host crustacea passively through breaks in the newly formed cuticle or by

cannibalism of infected crabs. Meyers et al. (1987) and Love et al. (1991) found that there was significantly greater prevalence of infection in newly molted crab. The snow crab examined in the present study were all "stage two" (recently molted), selected to increase the chances of encountering crab infected with *Hematodinium* sp. Crab with a range of shell stages were not examined. The molt period of Tanner crab in Alaskan waters occurs during the spring yet the peak of spore production was detected in August and September (Eaton et al., 1991), thus the role of spores is not clear.

There is no evidence of cyst formation in *Hematodinium* sp. as a survival strategy during host absence. In the present study, water and sediment from a tank used to contain infected crab was examined microscopically for evidence of dinospore survival and cyst formation following the death of infected *C. opilio*. There was no evidence of dinospore survival within tank contents or of cyst production from the two crab remains examined (Williams-Ryan, unpublished data). The portion of the life cycle of *Hematodinium* sp. which occurs outside of the crab host and the mode of transmission between hosts remain unknown.

4.6 Recommendations for future studies

The present study documents the occurrence of *Hematodinium* sp. infections of snow crab (*C. opilio*) within the boreal and subarctic waters of the northwest Atlantic. There are aspects of the *Hematodinium* sp. infection and the relationship with the snow crab that need further investigation. The focus of future studies should establish the prevalence of infection and mode of transmission within the snow crab population of the northwest Atlantic. This could be accomplished by a two fold approach: a) inspection of commercial snow crab catches (crab>95mm carapace width and usually male) and

b) examination of smaller snow crab (including immature male and females) from specific locations. These measures would provide data on the prevalence of infection across a broad size range. It is expected that there would be a greater level of infection in the smaller size classes because infection is reported to be higher in newly molted individuals (Meyers et al., 1990) and smaller crab molt at more frequent intervals than larger crab (Miller and O'Keefe, 1981). High prevalence of infection and associated mortality of immatures and females would affect recruitment and impact the commercial fishery directly. Improved knowledge of infection rates would enable conservation practices to be implemented. Within stocks affected by this parasite, mortality from both commercial harvest and *Hematodinium* sp. infection could be taken into account by fisheries managers.

Education of commercial fishermen that would better enable them to identify infected snow crab, and emphasis on correct disposal of infected crab are two useful conservation measures. Information gathered by the commercial fishermen could provide data on infection prevalence within the commercial size (>95 mm.) population and supplement directed surveys. *Hematodinium* sp. infected crabs should be disposed of on land, not thrown back in the ocean. Land–based disposal should reduce the potential for widespread infection of crab populations.This may occur if harvesters discard infected crab onto uninfected areas of crab while steaming back to port. Previously uninfected populations of snow crab and possibly other Crustacea could become infected in this way. Reports to D.F.O. of "sick crab" have increased, indicative that fishermen are noticing *Hematodinium* sp. infection in their snow crab catches (D. Taylor personal communication). Unused crab and by-products from snow crab processors should not be dumped in inshore waters without some testing for presence of *Hematodinium* spp. because there is the potential to introduce *Hematodinium* spp. to possible hosts such as the rock crab (*Cancer irroratus*) in nearshore waters.To date there have not been any reports of *Hematodinium* sp. infections of other Crustacea that inhabit the inshore waters of

Newfoundland however MacLean and Ruddell (1978) report infections in *Cancer irroratus*, *Cancer borealis* and *Ovalipes ocellatus* from the inshore and offshore waters of the mid Atlantic Bight. This suggests another area of future study, i.e., research to determine whether there are other species of crab either inshore or offshore that harbor *Hematodinium* sp. infections.

The complete life cycle of *Hematodinium* sp. and the natural route of transmission have to date not been documented. Investigation into both these aspects of the parasite's biology would help fisheries managment personnel to evaluate the implications and significance of *Hematodinium* sp. infections within commercially harvested species. Studies in Newfoundland snow crab could include prevalence by month and by shell condition to determine if there is a correlation between molt condition and infection. The developmental stages of *Hematodinium* sp. should be documented to ascertain if there are correlations between development stage, season and host condition. Sediment from areas known to contain molting crab or from laboratory experiments could be examined at the time of ecdysis for the presence of cystic stages. Methods used to examine the sediment would include direct microscopic examination of sediment and sucrose gradient analysis (Schwinghamer et al., 1991) of the sediment for cystic stages.

Laboratory studies could also help in elucidating the life cycle of *Hematodinium* sp. Snow crab infected with various life history stages of *Hematodinium* sp. (vegetative cells, plasmoidia and dinospores) could be held in tanks with snow crab induced to undergo ecdysis. The rate of infection of molting crab held with infected crab may provide positive evidence for mode of transmission.

Nucleotide sequencing modeled after Hudson and Adlard (1996) would provide a comparison of the *Hematodinium* sp. found in the present study to other *Hematodinium* spp. described in the literature. Standards for naming the different forms described to date

would clarify the literature and make comparison easier for future research. Investigators may then be able to determine whether morphological differences between species are a result of host influence versus intrinsic variation between species.

CONCLUSIONS

5.1 Conclusion

The present study has identified a species of *Hematodinium* isolated from the snow crab (*C. opilio*) which has the following characteristics. The vegetative stage is spherical 8.0 µm to 10.0 µm in diameter with no obvious thecal structures or microtubules. The ultrastructural features include: abundant trichocysts, tubular cristae, vacuoles which are empty or contain a lipid like material and a nucleus containing condensed chromatin. The plasmodial stage is nonmotile, round or irregular in shape with up to eight nuclei present with condensed chromatin. The plasmodial cytoplasm is similar to cytoplasm observed in the vegetative stage, however the vacuoles are smaller in size. Presumably the uninucleate vegetative stage is derived from the plasmodial form which subsequently develops into the dinospore. The dinospore is pyriform in shape 10.8 µm x 3.6 µm with two flagella. The dinospore cytoplasm contain trichocysts, lipid containing vacuoles, tubular cristae and the cell membrane has a micropore.

The *Hematodinium* sp. examined in the present study is not identified to a particular species as insufficient evidence exists within the published literature to be confident with a species name. Furthur investigations may provide evidence (eg. a second size of dinospore, chromosomal) that will enable a species name to be assigned.

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APPENDICES

Karnovsky Fixative

2 gm paraformaldehyde

50 ml water

10 ml gluteraldehyde 25%

2M sodium cacodylate buffer made up in filtered sea water pH 7.5

Heat the water with the paraformaldehyde to 65° C add 1-3 drops of 1N sodium hydroxide (solution should clear); cool to room temperature then add the gluteraldehyde. Make the solution up to 50 ml. using 2M. sodium cacodylate buffer. Check and adjust pH to 7.5.

Method for wax embedding

Processing was performed on a Fisher™ Tissuematon automatic processor using the following processing schedule:

70% Ethanol – one hour.

80% Ethanol – one hour.

100% Ethanol, two changes of thirty minutes and one hour.

Chloroform, two changes of thirty minutes and one hour.

Wax, two changes of one hour each.

Haematoxylin and Eosin Staining Method

Dewax slides in two changes of xylene 5 min. each

rehydrate with a descending alcohol series:

absolute ethanol two changes 5 min. each

95% ethanol 5 min.

70% ethanol 5 min.

water 10 min.

Erlichs Haematoxylin 7 min.

water rinse 5 min.

1% Lithium Carbonate 2 min. (to "blue")

water rinse 5 min.

70% ethanol 5 min.

1% eosin in 95% ethanol acidified with acetic acid 3 min.

Absolute ethanol two changes of 3 min. each

Xylene two changes of 3 min. each

Mount in permount with cover slip.