6.2.5 Infection with *Hematodinium*

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A. Name of Disease and Etiological Agent

Hematodiniasis occurs in a wide range of crustaceans from many genera and results from a systemic infection of hemolymph and hemal spaces by parasitic dinoflagellates of the genus *Hematodinium* (superphylum Alveolata, order Syndinida, family Syndiniceae). The type species is *H. perezi*, morphologically described from shore crab (*Carcinus maenas*) and harbor crab (*Portunas* (*Liocarcinus) depurator*) (Chatton and Poisson 1931) from the English Channel coastline of France. A second *Hematodinium* species was described as *H. australis* (based entirely on different morphological characteristics) from the Australian sand crab (*Portunus pelagicus*) (Hudson and Shields 1994). Without molecular sequence data to allow comparative determination of the number of species or strains that might be infecting various crustacean hosts there has been an emergence of numerous *Hematodinium* and *Hematodinium*-like descriptions in the literature. These have been based on dinoflagellate-specific morphological features that cannot discriminate among species or strains and may also be somewhat plastic depending on environmental or host factors. Later sequencing of the partial small subunit ribosomal DNA (SSU rDNA) gene and internal transcribed spacer (ITS1) region of *Hematodinium* and *Hematodinium*-like dinoflagellates suggested there are three species (Hudson and Adlard 1996). A more recent similar comparison of the same sequences from the re-discovered type species *H. perezi* with other publicly available sequences indicated there are three distinct *H. perezi* genotypes (I, II, III (Small et al. 2012); also known as Clade A (Jensen et al. 2010)) found infecting several portunid crab species from distant geographical locations (Europe, USA, Asia). This finding supports the delineation of *H. perezi* from the *Hematodinium* infecting many other crustacean host species (known as Clade B; Jensen et al. 2010) in the Northern Hemisphere including *Chionoecetes* sp. and *Nephrops norvegicus*. Additional studies using small 18S rDNA fragments of *Hematodinium* amplified from red king crab (*Paralithodes camtschaticus*) showed 100% sequence similarity to respective gene fragments of *Hematodinium* reported in *Chionoecetes* and *Lithodes* crab hosts. A similar infection was observed in blue king crabs (*P. platypus*) but molecular work was not done (Ryazanova et al. 2010). In this chapter, the term *Hematodinium* is used to recognize that further molecular work is needed to define species boundaries.

B. Known Geographic Range and Host Species of the Disease

Hematodinium and like parasites have been reported in 33 species of decapod crustaceans and 13 species of amphipods found over an extensive geographic range (Table 1). Infected hosts have been reported from Denmark, France, United Kingdom and Scotland, Ireland, Russia, China, Australia, Greenland, east/southeast Atlantic and Gulf coasts of North America and the coasts of Alaska and British Columbia in the Pacific Northwest.

Table 1. *Hematodinium* reported from marine decapod crustaceans[a] and benthic amphipods.

Host Species	Common Name	Location	Reference
Carcinus maenas	Shore crab	France, E.USA	Chatton and Poisson 1931; Messick and Shields 2000
Liocarcinus depurator	Harbor crab	France	Chatton and Poisson 1931
		Denmark	Eigemann et al. 2010
Portumnus latipis	Pennant's crab	France	Chatton 1952
Necora puber	Velvet crab	France, UK	Wilhelm and Boulo 1988; Stentiford et al. 2003
Callinectes sapidus	Blue crab	E. USA	Newman and Johnson 1975
Callinectes similis	Lesser blue crab	GA, USA	Messick and Shields 2000
Ovalipes oscellatus	Lady crab	E. USA	MacLean and Ruddell 1978
Cancer irroatus	Rock crab	E. USA	MacLean and Ruddell 1978
Cancer borealis	Jonah crab	E. USA	MacLean and Ruddell 1978
Cancer magister	Dungeness crab	AK, USA	Meyers and Burton 2009
Cancer pagurus	Edible crab	France, UK	Latrouite et al. 1988; Stentiford et al. 2002 Chualain et al. 2009
Amphipods 13 species	Amphipods	E. USA	Johnson 1986; Messick and Shields 2000
Chionoecetes bairdi	Tanner crab	AK, USA	Meyers et al. 1987, Meyers et al. 1990
Chionoecetes opilio	Snow crab	AK, USA	Meyers 1990; Meyers et al. 1996
		NF, Canada	Taylor and Khan 1995
		Greenland	Eigemann et al. 2010
Chionoecetes tanneri	Deep Tanner	BC, Canada	Bower et al. 2003
Chionoecetes angulatus	Triangle Tanner	BC, Canada	Jensen et al. 2010
Portunus pelagicus	Sand crab	Australia	Shields 1992; Hudson and Shields 1994
Portunus trituberculatus	Swimming crab	China	Xu et al. 2007
Scylla serrata	Mangrove crab	Australia, China	Hudson and Lester 1994; Li et al. 2008
Trapezia areolata	Coral crab	Australia	Hudson et al. 1993
Trapezia coerulea	Blue coral crab	Australia	Hudson et al. 1993
Nephrops norvegicus	Norway lobster	Scotland	Field et al. 1992, 1998
		Denmark	Eigemann et al. 2010
Libinia emarginata	Spider crab	GA, USA	Sheppard et al. 2003
Neopanope sayi	Mud crab	SE USA	Messick and Shields 2000; Sheppard et al. 2003
Hexapanopeus angustifrons	Smooth mud crab		
Panopeus herbstii	Atl. mud crab		
Menippe mercenaria	Stone crab	GA, USA	Sheppard et al. 2003
Exopalaemon carinicauda	Ridgetail prawn	China	Xu et al. 2010
Pagurus bernhardus	Hermit crab	UK	Small et al. 2007
		Denmark	Eigemann et al. 2010
Hyas araneus	Great spider crab	Greenland	Eigemann et al. 2010
Hyas coarctatus	Arctic lyre crab	AK, USA	Jensen et al. 2010
Lithodes couesi	Scarlet king crab	BC, Canada	Jensen et al. 2010
Paralithodes camtschaticus	Red king crab	Russia	Ryazanova et al. 2010
Paralithodes platypus	Blue king crab	Russia	Ryazanova et al. 2010

[a]Subsequent molecular testing of putative *Hematodinium* in *Pandalus platyceros* and *P. borealis* (Bower et al. 1993; Meyers et al. 1994) identified a Haplosporidian instead (Reece et al. 2000).

C. Epizootiology

Although *Hematodinium* was reported as early as 1931 in the green shore (*Carcinus maenas*) and harbor (*Portunus (Liocarcinus) depurator*) crabs (Chatton and Poisson 1931), more recently this parasite has emerged as a significant pathogen causing epizootic mortality in several other commercially important crustacean hosts from various oceans of the world including: Tanner (*Chionoecetes bairdi*; Meyers et al. 1987; Meyers 1990) and snow crabs (*C. opilio*; Meyers et al. 1996; Wheeler et al. 2007) from Alaska, USA and Newfoundland, Canada; Norway lobster (*Nephrops norvegicus*; Field et al. 1998) in Scotland; velvet swimming crab (*Necora puber*; Wilhelm and Boulo 1988; Wilhelm and Mialhe 1996) in France; edible crab (*Cancer pagurus*; Latrouite et al. 1988, Stentiford et al. 2002) in France and the UK; blue crab (*Callinectes sapidus*; Messick and Shields 2000) from the southeastern coast of the USA; and cultured mud crabs (*Scylla serrata*; Li et al. 2008) and ridgetail white prawns (Xu et al. 2010) in China. The parasite in *N. norvegicus* has been grown in culture resulting in various morphological stages not observed in naturally infected animals (Appleton and Vickerman 1998) which may or may not be representative of parasite development in the host. Interestingly, experimental infections of *N. norvegicus* using both cultured *Hematodinium* and parasites taken directly from infected lobsters have not been successful suggesting a more complex mode of transmission (Stentiford and Shields 2005). The life cycles of *Hematodinium* parasites, when naturally occurring within their various hosts, are only partially known in two host species where experimental infections have been successful; *C. sapidus* on the southeast coast of the US and *C. bairdi* from Alaska (where the disease is referred to as Bitter Crab Syndrome or BCS). The morphological terminology for BCS is used in the following paragraph to illustrate the life history features typical of *Hematodinium*. The *Hematodinium* species associated with BCS has some similarities with the dinoflagellate parasite in *C. sapidus* and probably other host infections by *Hematodinium* in other crustacean species regarding morphology and the epizootiology of the disease. For a complete review of *Hematodinium* with known life stages and variations in the pathogenesis in different hosts consult Stentiford and Shields (2005).

Although the natural route of transmission of *Hematodinium* has not been established, there are likely several avenues of horizontal transmission (as shown in Figure 1), including: 1) from stages of the parasite released into seawater from other infected crabs that passively enter breaks in the host cuticle during mating and molting, i.e., epizootics of *Hematodinium* in snow crabs have been linked to synchronized molting events (Shields et al. 2007); 2) cannibalism or feeding on detritus containing resting spores, i.e., there are conflicting reports of dietary infection in *C. sapidus* (Sheppard et al. 2003; Walker et al 2009; Li et al. 2011) and failure by ingestion reported for *P. pelagicus* (Hudson and Shields 1994) ; 3) potential sexual transmission via seminal fluids of parasitized male crabs (Meyers et al. 1996); and 4) possible transmission from a reservoir host such as bottom dwelling amphipods either from being eaten or from shedding of infectious stages. The parasite and disease in *C. bairdi* have been transmitted in the laboratory by injection of hemolymph containing vegetative trophonts and dinospore stages (Meyers et al. 1987; Eaton et al. 1991). Life history stages include trophonts (Figure 2) which are single cells of varied size that have slower division rates, larger plasmodia with multiple nuclei, and pre-spores or sporonts with multiple dense nuclei and rapid division followed by sporulation of biflagellated spores with a single nucleus. Spores are of two types, a large slow moving macrospore and a small fast moving microspore, generally only one spore type occurring per host. Spores may possibly have a disseminatory or resting stage function rather than transmission. Sporulation kills the crab host and spores exit through the gills. Laboratory studies suggest that the life cycle of the parasite in *C. bairdi* occurs over a 15 to 18 month period. Infection likely occurs from the trophont stage during the spring molting period from mid-March to May. Crabs dying in the summer and fall

following sporulation were likely parasitized in the spring of the previous year. After sporulation in the fall the prevalence of parasitism eclipses (becomes almost undetectable) until the new infections of the previous spring build to detectable levels that can be observed the following late winter and early spring.

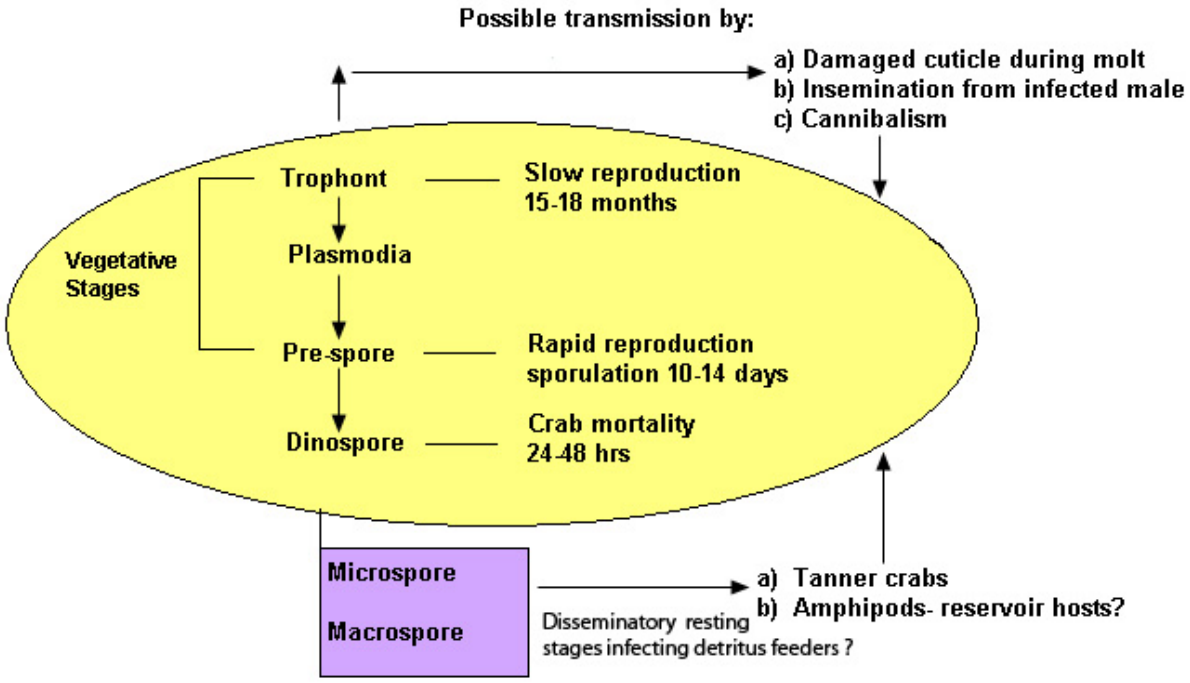

Figure 1. Possible life cycle of *Hematodinium* in *Chionoecetes bairdi* (from Meyers and Burton 2009).

D. Disease Signs

1. Gross signs

A universal gross clinical sign in crustaceans infected with *Hematodinium* is general lethargy and cloudy to very thick, milky appearing hemolymph in peripheral circulation and on the surface of the internal viscera. Additional gross signs include hyper-pigmentation of the carapace or reddish to pink coloration, especially visible in the arthrodial membranes, reported in *N. norvegicus*, *C. bairdi*, *C. opilio* and *C. pagurus* (Stentiford and Shields 2005). Meat texture is flaccid, chalky and has an astringent off-flavor when cooked as first reported for *C. opilio* and *C. bairdi*, hence the name Bitter Crab Disease as coined by Meyers et al. (1987). Severely infected crabs may die when handled or transported due to respiratory dysfunction, tissue hypoxia and other deleterious biochemical changes causing depletion in the protein and carbohydrate stores of the host (Stentiford and Shields 2005).

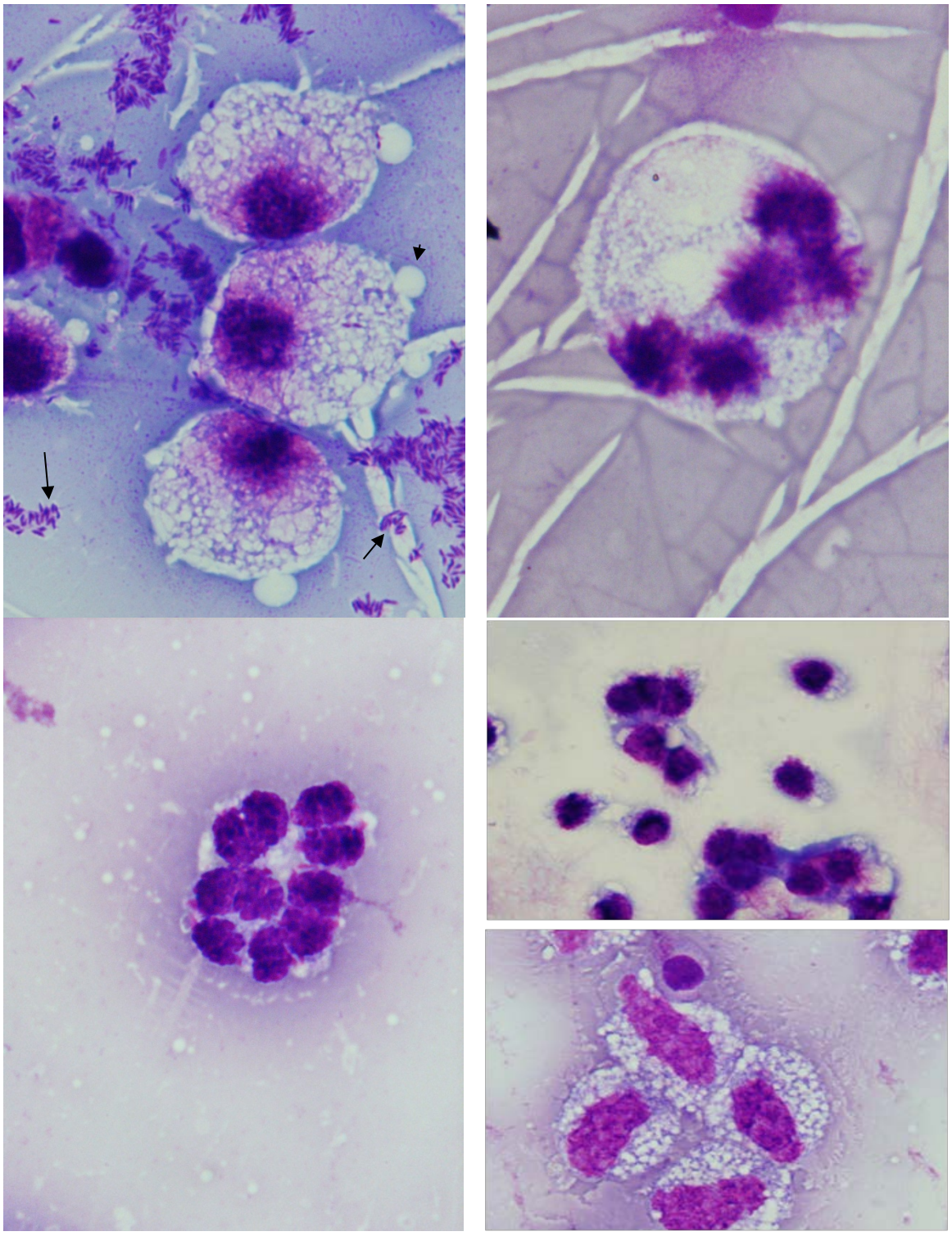

Figure 2. *Hematodinium* in *Chionoecetes bairdi* hemolymph smears: Clockwise from top left: trophonts with vacuolated cytoplasm, indistinct nuclear border and surface blebbing (arrowhead), secondary bacterial infection (arrows); multinucleated plasmodium; micro-dinospores with scant cytoplasm; macro-dinospores with vacuolated cytoplasm; multinucleated sporont (1000 X). Photo by Ted Meyers.

2. Microscopic and histologic signs

Wet mounts of peripheral hemolymph, stained hemolymph smears and histologic sections of nearly all tissues from infected hosts show myriad numbers of single celled dinoflagellate organisms (Figure 3). The parasites fill all hemal spaces, cause hemocytopenia, coagulopathy and displace normal visceral organs which degenerate in appearance and function with no apparent host cellular response. Secondary systemic infections by opportunistic bacteria, yeasts and other protozoa often occur (Figure 2).

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Clinical gross signs are pathognomonic for parasitic dinoflagellate infections in many host species but only presumptive for others. Wet mounts from fresh hemolymph provide further presumptive evidence that a protozoan is present. Wet mounts also allow observation of the behavioral differences in the motile biflagellated micro and macro-dinospores if present.

Removal of a pleopod in *N. norvegicus* (pleopod staging) has been used for diagnosing dinoflagellate (*Hematodinium*) infection and severity. A pleopod is examined under about 40 X magnification to observe the density of the layer of aggregation of hemocytes and parasites beneath the cuticle and rated from zero (0) to I-IV. The pleopod of an uninfected lobster (0) appears relatively transparent while parasitized animals have pleopods of varying degrees of opacity depending upon severity caused by the presence of parasite cells (Field et al. 1992).

2. Confirmatory Diagnosis to Dinoflagellate and Genus

In the case of *C. sapidus* and *C. maenus*, wet mounts stained with neutral red demonstrate the lysosomes of *Hematodinium* trophonts (Chatton and Poisson 1931; Stentiford and Shields 2005) thus providing a confirmatory diagnosis to genus in these two host species. Neutral red is not taken up by *Hematodinium* trophonts found in *N. norvegicus* (Stentiford and Shields 2005).

Hemolymph smears containing the parasite cells are fixed with methanol on glass slides and stained with commercially available two part stains consisting of eosin and Giemsa or methylene blue. This method provides consistent results using manufacturer instructions (Meyers et al 1987). A second method utilizes a hemolymph smear prepared on a poly-L-Lysine coated slide that is rapidly fixed in Bouin's solution or 10% neutral buffered formalin and then stained using standard procedures with Giemsa or hematoxylin and eosin (Messick 1994; Pestal et al. 2003). Microscopic examination of hemolymph smears is non-destructive and has been useful for determining the marketability and palatability of *C. bairdi* infected by *Hematodinium*. Parallel organoleptic testing correlated very well with results from stained hemolymph smears where market grading rejected all but the most lightly infected crabs (Imamura and Woodby 1994).

Staining allows observation of typical dinoflagellate parasite life stages described for the various host species (trophonts - often dividing and binucleated, multinucleated plasmodia, pre-spores or sporonts, two morphologically dissimilar micro and macro biflagellate dinospores) and cell details including cytoplasmic vacuolation, surface blebbing (Figure 2) of waste materials and dinokaryon nuclei with occasional V-shaped chromosomes (Figure 3).

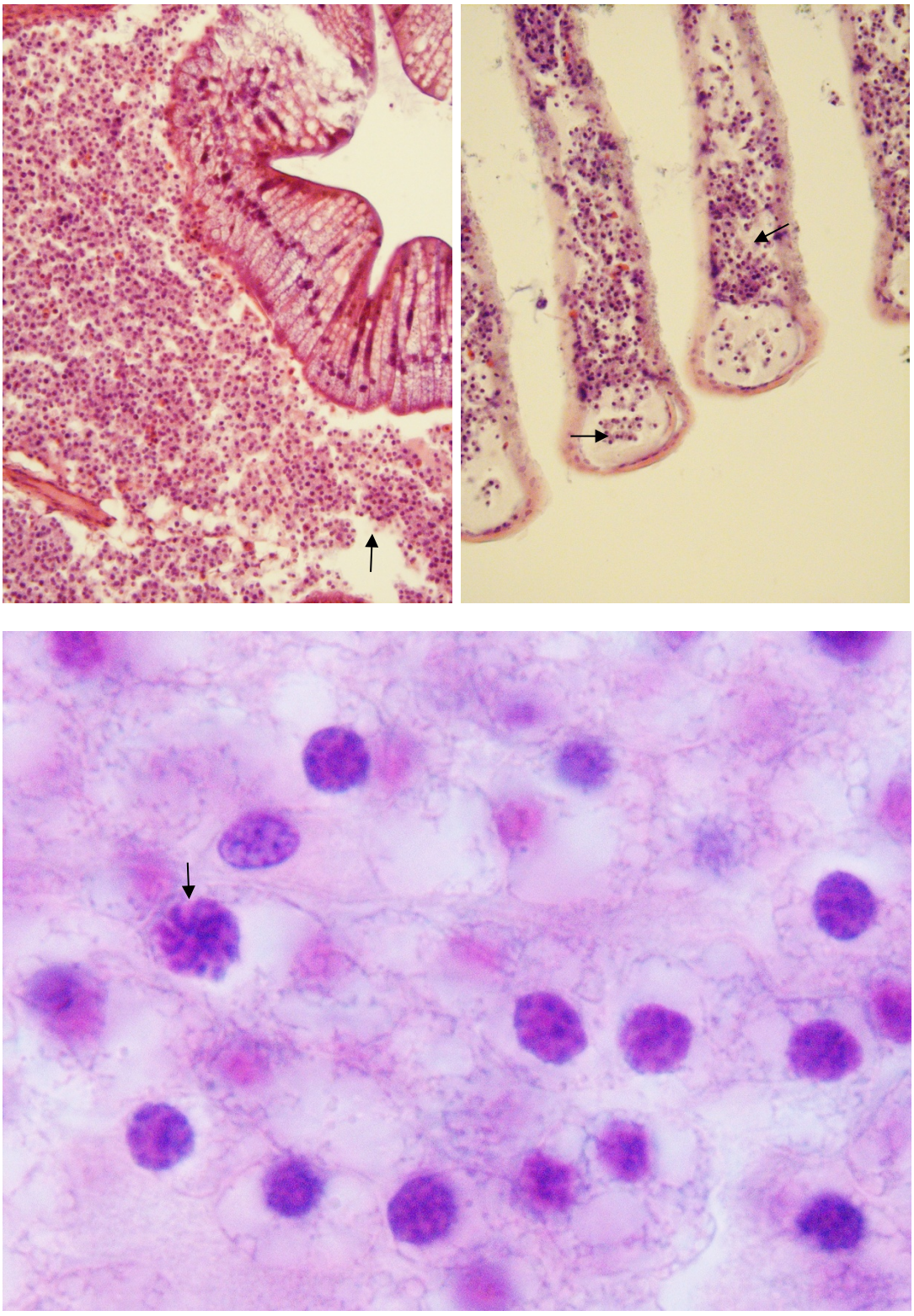

Figure 3. Histological sections (H&E) of *Hematodinium* in *Chionoecetes bairdi*: Top left and right- Myriad numbers of trophonts (arrows) filling tissue spaces of digestive gland and gills X 100; Bottom – Trophonts with dinokaryon nuclei and occasional V-shaped chromosomes (arrow) X 1000. Photo by Ted Meyers.

Chromosomes in a dinokaryon nucleus are attached to the nuclear membrane and lack histones, thus remaining condensed throughout interphase rather than just during mitosis. These morphological features establish that the parasite is a dinoflagellate and typical of the *Hematodinium* genus which infects decapod crustaceans.

Histological examinations allow for further visualization of parasite cell morphology and the typical pathogenesis of the systemic disease involving the hemal spaces of the crustacean host. Dinokaryon nuclei are better observed in stained histological sections.

Standard methods of transmission and scanning electron microscopy allow further confirmation of dinoflagellate and *Hematodinium* morphology such as a trilaminar alveolate pellicle, beaded chromatin in the nuclei of some life stages, large lipid containing vacuoles compressing the cytoplasm (some causing outward bulging of the cytoplasm), micropores associated with the pellicle, trichocysts in certain life stages and flagella present on the dinospores. See the review by Stentiford and Shields (2005) for morphological measurements and figures for the *Hematodinium* parasites of different crustacean host species.

3. Confirmatory diagnosis to genotype and species

Several experimental PCR assays have been published for detection of *Hematodinium* in respective host crabs including *C. sapidus* (Gruebl et al. 2002; Nagle et al. 2009), several species of marine decapods from waters of Denmark and Greenland (Eigemann et al. 2010), *N. norvegicus* (Small et al. 2006) and *C. bairdi* and *C. opilio* (Jensen et al. 2010). Most of these assays have been designed specifically for the *Hematodinium* parasites in a particular host species while the assay of Jensen et al. (2010) may be useful as a general detection method for identifying genotypes or clades in other decapod species. However, none of these assays have been validated and standardized to allow for recommendation as a test method for confirmatory diagnosis of all *Hematodinium* or like parasites in all reported host species. Furthermore, the assignment of *Hematodinium* species has not been established and is still being investigated through genetic sequencing.

Table 2. Methods used for diagnosing clinically diseased hosts[a].

Methods	Screening	Presumptive	Confirmatory	
			Dinoflagellate	Genus
Gross signs[b]	++	++	+ +	+
Hemolymph wet mount	+	+	+	+
Pleopod staging[c]	+	+	+	+
Stained hemolymph smear	+++	+++	+++	++
Histopathology	-	++	+++	++
Electron microscopy	-	-	+++	++

[a]Key: (-) the method is presently unavailable or unsuitable, (+) the method has good application in some situations, but is not practical for field use, or is difficult to evaluate or has been used for only a particular *Hematodinium* infection in one host species which severely limits its usefulness on other *Hematodinium* infections until validated, (++) the standard method, (+++) the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

[b]The specificity is high and pathognomonic for several species of clinically diseased crustaceans but the sensitivity is moderate since a large percentage of sub-patent infections may be missed. Less suitable for use with *C. sapidus*.

[c]Used only on *N. norvegicus* for evaluating the severity of infection.

F. Procedures for Detecting Subclinical Infections

1. IFAT

An indirect fluorescent antibody test (IFAT) was developed by Field and Appleton (1996) for detection of *Hematodinium* in *N. norvegicus* in Scotland. The tested samples consisted of hemolymph smears fixed in 70% ethanol or tissue smears fixed in a saline phosphate buffered mixture of 0.1% glutaraldehyde and 4% sucrose with a pH of 7.4. The primary antibody was rabbit anti-*Hematodinium* antiserum followed by a donkey anti-rabbit fluorescein conjugate with a DAPI (4',-6-diamino-2-phenylindole) counter stain. Rinses between antibody stains used a PBS/0.1% BSA. The IFAT detected 1.8% more infected lobsters than did blood smears stained with Leishman's stain (Field and Appleton 1996).

2. Western Blot

A western blot was developed (Stentiford et al. 2001) to examine prevalence of *Hematodinium* in *N. norvegicus* from the Clyde Sea area of Scotland using denatured hepatopancreatic tissue samples, a rabbit anti- *Hematodinium* antiserum developed by Field and Appleton (1996), a goat anti-rabbit horseradish peroxidase conjugate and chemiluminescence reagents as developer. By comparing the assay results with known parasite numbers in the hemolymph of infected lobsters, the immunoassay minimum sensitivity was determined to be 204 parasites per cubic mm of hemolymph which was 10 times more sensitive than the pleopod method (Stentiford et al. 2001). The assay worked well for both diluted hemolymph and tissue samples. Latent infections in *N. norvegicus* may be detectable in tissues such as the hepatopancreas and muscle before parasites become numerous enough to detect in the hemolymph (Field and Appleton 1995).

3. ELISA

An enzyme-linked immunosorbent assay (ELISA) was developed for surveillance of *Hematodinium* in *N. norvegicus* in the coastal waters of Scotland (Small et al. 2002). This ELISA used hemolymph samples, a polyclonal rabbit anti-*Hematodinium* antisera (Field and Appleton 1996), a goat anti-rabbit horseradish peroxidase conjugated antibody and tetramethyl benzidine reagent for color development. The assay had a detection limit of 5×10^4 parasites per ml of hemolymph and was significantly more sensitive than the pleopod method, more sensitive by a factor of 4 than the western blot and equally sensitive as the IFAT, although much easier to perform on larger numbers of samples (Small et al. 2002). The assay was also reported able to detect *Hematodinium* in *C. pagurus*.

4. **PCR**

PCR tests for *Hematodinium* infections in specific host crustaceans were discussed above.

G. Procedures for Determining Prior Exposure to the Etiological Agent

There have been no procedures published for determination of prior crustacean exposure to *Hematodinium.*

H. Procedures for Transportation and Storage of Samples

Diagnostic methods are best performed on live crustaceans. If live crustaceans are to be transported for testing then conditions should include adequately insulated containers for shipping, refrigeration, reduced time out of sea water and consideration of animal health and condition when collected regarding survivability in transit. Hemolymph smears collected from

live crustaceans in the field should not be contaminated with sea water, should be completely air dried and placed into slide boxes and shipping containers with adequate packing to avoid breakage. Tissues for histological or ultrastructural examinations should be from live animals and placed into the appropriate standard fixatives for transport to the laboratory. Hazardous material requirements for transport may be avoided by rinsing fixed tissues in water or buffer and shipping in water or buffer. Transport and storage of tissues or hemolymph for immunoassays or molecular methods have their own specific protocols but generally include one or a combination of the following: placing into a tissue stabilizer, freezing to at least -20^{o}C or fixation in 100% ethanol.

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