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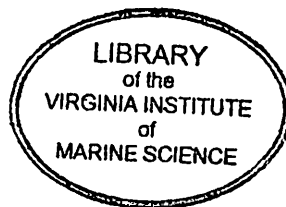
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MORTALITY AND PATHOPHYSIOLOGY STUDIES OF BLUE CRABS INFECTED  
WITH THE PARASITIC DINOFLAGELLATE *HEMATODINIUM PEREZI*

by

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## Saltonstall-Kennedy Program Grant

### FINAL REPORT

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**D. Grantee:** Virginia Institute of Marine Science

Jeffrey D. Shields, Virginia Institute of Marine Science, The College of William & Mary, Gloucester Point, Virginia 23062

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**Abstract:** On the eastern seaboard of the USA, populations of the blue crab, *Callinectes sapidus*, experience recurring epizootics of a parasitic dinoflagellate. The parasite, *Hematodinium perezii*, fulminates in the summer and autumn causing mortalities in high salinity embayments and estuaries. In laboratory studies, we experimentally investigated host mortality due to the disease, assessed differential hematological changes in infected crabs, and examined proliferation of the parasite. Mature, overwintering, non-ovigerous, female crabs were injected with  $10^3$  or  $10^5$  cells of *H. perezii*. Mortalities began 14 d after infection, with a median time to death of  $30.3 \pm 1.5$  d (se). Subsequent mortality rates were greater than 86% in infected crabs. A relative risk model indicated that infected crabs were 7 to 8 times more likely to die than controls, with decreases in total hemocyte densities covarying significantly with mortality. Hemocyte densities declined precipitously (mean = 48 %) within 3 d of infection and exhibited differential changes in subpopulations of granulocytes and hyalinocytes that lasted throughout the course of the infection. Crabs that did not present infections after injection (i.e., "immune") did not show hemocytopenia, and exhibited significant long-term (21-27 d) granulocytemia. Detection of the parasite in the hemolymph of infected crabs increased from approximately 30% after 14 d to 60% after 21 d to 100% after 35 d. Plasmodial stages were, however, detectable in histological preparations of the heart within 3 d of infection and increased in number over 5 and 7 d. Sporulation of the parasite occurred over a short time (at least 4 d, after 43 d infection) and did not culminate in the immediate death of the host. The mortality studies indicate that *H. perezii* represents a significant threat to the blue crab fisheries in high salinity estuaries, and may have a greater effect on mature females that move to higher salinities to breed.

## Executive Summary

With the decline of the oyster and the moratorium on scallops, blue crabs now support the largest commercial fishery within Chesapeake Bay, and the second largest fishery in Virginia (Kirkeley, 1997). The crab industry harvests from 80-120 million pounds of crabs from Chesapeake Bay annually. Significant declines in crab catches in 1998 and 1999 indicate that mortality processes in blue crabs are not well understood. Fishing pressure is often cited for declining stocks, but diseases and environmental processes have not been well examined. Both processes have contributed to declines and population cycles in other crab fisheries (e.g., *Cancer magister*, *Paralithodes camtschaticus*).

Recent outbreaks of the lethal parasite, *Hematodinium perezii*, have negatively affected the coastal blue crab fishery of the Delmarva peninsula. Species of *Hematodinium* or *Hematodinium*-like parasites have seriously affected several other crustacean fisheries; hence, it is vital that we investigate the biology and ecology of *H. perezii* in the blue crab, *Callinectes sapidus*. Unfortunately, little is known of *Hematodinium* infections because they can be difficult to diagnose. The present study undertook to examine aspects of the mortality and pathophysiology of infected crabs.

Mortality studies uncovered several aspects of the biology of *H. perezii*. The parasite proliferates rapidly in the blue crab, and can kill its host within 3 to 6 weeks. Early occult infections take approximately 1 to 4 weeks to become detectable via standard methods. Acute infections show significant mortality with low levels of the parasite, while chronic, longer term infections become virtual monocultures that can reach densities of 100 million parasites ml<sup>-1</sup> of hemolymph. Sporulation may occur sporadically in acute infections or it may become more

synchronous in chronic infections. Sporulation was thought to culminate in host death, but we have observed several bouts of sporulation in chronically infected crabs.

The cellular immune response of infected crabs is affected by the parasite. In early infections, circulating hemocytes show significant declines, that are associated with the mortalities of individual crabs. Declines of as much as 80% of circulating hemocytes are associated with host death. In infection studies, a few crabs survived the infection. These crabs showed consistently high numbers of granulocytes compared to controls, and infected animals. The granulocytopenia was apparent over a time course of 21 to 27 days and indicates an aggressive cellular response to the disease.

Infected crabs frequently show signs of weakness and lethargy, and often die to stress-related handling or fishing. Radical changes in the chemistry of the hemolymph are obvious by the lack of clotting ability, and by its frequently observed discoloration. Serum proteins, serum acid phosphatase, hemolymph enzyme activity, and tissue glycogen levels all showed changes with infection, and indicated a gradual decline in the metabolic resources of the host. Hemagglutination, an indicator of innate, humoral defense activity was not affected by the parasite, but the cellular response was clearly compromised by infection.

Continued studies of the disease are justified given the value of the region's blue crab fishery, and the impact of other species of *Hematodinium* on several commercially important crab and lobster fisheries.

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Signature of Principal Investigator

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Date

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**Eight Tables and Fourteen Figures are presented at the end of the document.**

**Appendix A: Manuscript on culture activities partially supported by this grant.**

## INTRODUCTION

### Identification of priorities/problem(s)

*Hematodinium perezii* is a parasitic dinoflagellate that proliferates in the hemolymph of the blue crab, *Callinectes sapidus*. The disease occurs in blue crabs in high salinity (> 11 ‰) waters from Delaware to Florida, and the Gulf of Mexico (Newman & Johnson, 1975; Messick & Sinderman, 1992). In 1975, a 30% prevalence was reported in blue crabs from Florida, where its impact on the population was thought to be high (Newman & Johnson, 1975). In 1991 and 1992 an epizootic<sup>1</sup> of the parasite affected 70 % to 100 % of the blue crabs in the seaside bays of Maryland and Virginia (Messick, 1994). Commercial watermen reported reduced catches, lethargic, moribund and dead crabs in pots and shedding facilities. The epizootic virtually shut down the blue crab fishery in seaside bays of the Delmarva Peninsula. In 1996 and 1997, prevalences ranged from 10 to 40 % in the eastern portions of lower Chesapeake Bay (Shields, 1997). Enzootic outbreaks occurred in Chincoteague Bay in 1998 (Shields, unpubl. data).

In 1997, blue crabs supported the largest commercial fishery within Chesapeake Bay, and the second largest fishery in Virginia (Kirkeley, 1997). The crab industry harvests from 80-120 million pounds from Chesapeake Bay annually; of that, approximately 10-14 million pounds are soft-shell crabs (Casey et al., 1991; Rugolo et al., 1998). Declines in crab catches in 1998 and projected declines in 1999 indicate that mortality processes in blue crabs are not well understood. While fishing pressure continues to be cited for the recent declines, disease and environmental processes have not been well examined. Both processes have contributed to declines and population cycles in other crustacean fisheries (see Kuris & Lafferty, 1992 for review.).

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<sup>1</sup> An epizootic is an epidemic or outbreak in an animal population.

*Hematodinium perezii* occurs in high salinity waters (Newman & Jonson, 1975). Since larval and juvenile crabs must pass through high salinity waters, they may risk exposure to infectious stages of *H. perezii*. The occurrence of the parasite on both sides of the Delmarva Peninsula indicates that it may threaten several important subpopulations of the blue crab (Figure 1). Indeed, it occurs at low to moderate (1-15%) levels in female crabs in the prime breeding grounds of the lower bay (Shields, 1997; Messick & Shields, in prep.). Unfortunately, background mortalities due to *Hematodinium* are difficult to assess because dead crabs sink, and quickly become undiagnosable. With the decline of blue crab stocks in Chesapeake Bay, it is imperative that we understand the life cycle, transmission patterns, and fulmination of epizootics of this important disease agent.

The Delmarva Peninsula possesses several characters that may facilitate epizootics of *H. perezii*, including relatively closed crab populations (i.e., those with little immigration and emigration of juveniles and adults), relatively high salinity with little water exchange between the open ocean and backwaters, and stressful conditions such as heat stress, seasonal hypoxia, seasonal fishing and predation pressure (Shields, 1994). Similar conditions exist in many small estuaries along the mid-Atlantic and southeastern USA. The effects of *Hematodinium* spp. on the Tanner crab (Meyers et al., 1987, 1990; Taylor & Khan, 1995), Norway lobster (Field et al., 1992), and velvet crab fisheries (Wilhelm & Miahle, 1996), and our current mortality data indicate that *H. perezii* has a significant impact on the coastal blue crab fisheries along the Atlantic seaboard of the USA.



Infections of *Hematodinium* spp. or *Hematodinium*-like species have been reported from a variety of different hosts, including amphipods, crabs and lobsters (see Shields, 1994 for review). There are, however, only two described species of *Hematodinium*: *H. perezii* Chatton & Poisson, 1931, and *H. australis* Hudson & Shields, 1994. While there is some question as to the identity of the parasite, by convention (Newman & Johnson 1975; MacLean & Ruddell 1978) and morphological characters, I refer to *Hematodinium perezii* as the infectious species in the American blue crab.

### **PROJECT GOALS AND OBJECTIVES**

**Goal:** . We want to clarify the potential threat of this parasite to the blue crab fishery by better understanding its epizootiology and pathology. In this study we examined the ecological and physiological factors associated with host mortality.

Our primary objective was to (1) examine host mortality from laboratory studies to estimate the potential loss of crabs to the fishery. Estimates of mortality can then be incorporated into current models for management of the fishery. Our secondary objective was to (2) determine the pathophysiological mechanisms underlying the cause of death in crabs infected with *Hematodinium perezii*. This consisted of investigations into changes in blood and tissue chemistry in infected versus uninfected crabs. We included host sex as a factor on various physiological constituents between infected and uninfected crabs. (3) In addition, in Fall, 1997, we undertook to continue the broad-scale sampling (as described in the previous Saltonstall-Kennedy grant; Shields, 1997) to further examine the epizootiology of the disease.

## APPROACH

### METHODS

#### Collection sites

Blue crabs were collected from the coastal bays and creeks on the "seaside" (e.g., Red Bank Creek, Wachapreague), and "bayside" (e.g., Nassawadox Creek, Hungars Creek, Cape Charles) of the Delmarva Peninsula, and from many locations within the mainstem of the lower Chesapeake Bay. Additional collections were made in the York, James, and Rappahannock Rivers. Regular seasonal sampling was done via pot and trap fishing (Mr. Seth Rux) at two reference locations: bayside at Hungars Creek, and seaside at Red Bank Creek (Figure 1). Since 1996 was one of the coldest and wettest years on record (i.e., an anomalous year for studying temperature and salinity relationships), the broad scale sampling was continued in 1997 from the lower Chesapeake Bay and the two reference locations.

#### Collection method

Crabs were collected by a number of methods. The broad-scale sampling was done in conjunction with the VIMS Trawl Survey (April through December), and the VIMS Blue Crab Dredge Survey (November through March). Additional samples were collected via trawl. In most cases, crabs were chilled on ice for transportation to the laboratory. For the 1997 sampling, up to 60 crabs from each trawl or dredge were examined for *Hematodinium perezii*. We sampled all of the crabs over 28-30 mm CW from most of the sites in the VIMS Dredge Survey. Low salinity locations (e.g., York River near West Point) were not sampled for the disease, but subsamples (n=60-75) of several hundred crabs were sampled from the York River. Temperature, salinity and dissolved oxygen were recorded for each station in the Trawl and Dredge Surveys.

### Hemolymph analyses

Crab hemolymph was removed from the axillae of the 5th walking leg using a 27 ga. needle with a tuberculin syringe. The hemolymph was examined as a wet smear, with an additional smear being processed and stained as described in Messick (1994). Briefly, acid-cleaned, poly-l-lysine-coated microslides were smeared with 2-3 drops of fresh blood, allowed to sit for 2-3 min, then fixed in Bouin's fixative. The smears were then processed through a routine hematoxylin and eosin procedure. Wet smears were read at 400x, prepared smears with oil immersion at 1000x. Host and parasite densities were estimated with a hemacytometer (Neubauer Bright-Line). Note that intensity (number of parasites per 100 host cells) could easily be obtained in this manner.

### **Methods 1: Mortality studies**

Uninfected crabs were housed together for three to seven days prior to treatment to insure acclimation and absence of overt bacterial or protozoal diseases (as assayed below). During the experiments, crabs were fed fish and squid semiweekly and held individually in aquaria (5 gal., 19 l) at 20° to 21° C, and 24 ppt salinity. Only mature, non-ovigerous female crabs (healthy, orange maturing gonads, little to no shell damage) were used in the experiments. Females were used to avoid potential confounds between sexes, and to improve sample sizes in experiments using limited numbers of aquaria and replicates.

*Hematodinium perezii* was maintained in the laboratory by serial passage of infected hemolymph. Initially, hemolymph from naturally infected crabs was injected directly into naïve crabs. Naïve crabs and crabs used for inoculation experiments were obtained from low salinity non-enzootic

locations. Infected and inoculated crabs were housed separately and used as hemolymph donors to inject ( $10^5$  -  $10^6$  parasites per host) naïve hosts. We have maintained *H. perezi* for over seven months using this method with no apparent loss of pathogenicity.

Two mortality, and one early life history experiment are presented. A variety of other experiments were also undertaken to test the use various support buffers, etc. These other experiments will not be reported here. Experiment I was a mortality study that used raw, infected hemolymph as the inoculant. While appropriate for maintaining infections in the laboratory, raw hemolymph cannot be adjusted to manipulate parasite densities without the use of physiological buffers, nor can it be guaranteed as sterile without appropriate assessment (see Welsh & Sizemore, 1985). Preliminary experiments with sterile sea water, physiological buffers, or infected hemolymph indicated that buffer-washed parasites remained infectious, and could, therefore, be adjusted to consistent densities appropriate to controlled experiments. Experiment II was a mortality study that used buffer-washed parasites adjusted to a similar density as in Experiment I. Experiment II closely resembles a replicate of Experiment I except for (1) centrifugation, (2) the use of the physiological buffer in handling the parasites, and (3) use of plasmodial vs. uninucleate stages of the parasite. Controls in both experiments were used to establish background densities of hemocytes. Experiment III was designed to examine the effects of early infections on the hematology of the host and the early life history of the parasite. Experimental densities in Experiment III were four times higher than those in the previous experiments, and were arbitrarily higher to insure observation of parasites prior to their proliferation.

Experiments I and II were mortality studies using trophonts and plasmodia (for definition see below), respectively. Experiment I consisted of a control group of uninfected crabs ( $n = 22$ ) injected individually with 100  $\mu$ l of hemolymph from an uninfected donor crab and an experimental group ( $n = 20$ ) injected individually with 100  $\mu$ l of infected hemolymph from a donor crab containing an estimated  $1.3 \times 10^6$  trophonts  $\text{ml}^{-1}$  ( $1.3 \times 10^5$  trophonts per crab).

Experiment II consisted of a control group ( $n = 8$ ) injected individually with 100  $\mu$ l of physiological saline buffer (modified from Appleton & Vickerman, 1998; NaCl, 19.31 g/l; KCl 0.65 g/l;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  1.38 g/l;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1.73 g/l;  $\text{Na}_2\text{SO}_4$  0.38 g/l; HEPES 0.82 g/l;) adjusted to pH 7.8, with added glucose (1.0 mg/ml) and two groups of experimental crabs injected with 100  $\mu$ l of buffer containing an estimated  $1.0 \times 10^5$  parasites per crab ( $n = 10$ ) and  $1.0 \times 10^3$  parasites per crab ( $n = 10$ ).

For Experiment II, 2.0 ml of infected hemolymph were drawn from a donor crab infected with  $6.15 \times 10^7$  parasites  $\text{ml}^{-1}$  (comprised of 97% plasmodia; 3% trophonts). The infected hemolymph was diluted 1:1 with buffer, centrifuged at 4000 rpm for 10 minutes, the supernatant decanted, and the cells resuspended in buffer. The cells were then adjusted to  $1.0 \times 10^7$  parasites  $\text{ml}^{-1}$ , centrifuged and washed twice more, and serially diluted to attain densities of  $1.0 \times 10^6$  parasites  $\text{ml}^{-1}$  and  $1.0 \times 10^4$  parasites  $\text{ml}^{-1}$ . Aliquots of 100  $\mu$ l were then injected into each crab as described above.

In both experiments, crabs were monitored daily for mortalities. Deaths within the first 9 days of each experiment were excluded due to handling stress arising from infrequent, bacterial infections

(e.g., Johnson, 1976). None of the crabs in the experiments presented with amoebae, microsporans, or overt bacterial infections (but see Welsh & Sizemore, 1985 for background levels of *Vibrio* spp. in hemolymph of *C. sapidus*). Ten crabs from each treatment in Experiment I, and all of the crabs in Experiment II were bled weekly to assess infection status. In Experiment I, the same crabs were bled weekly until they died, other crabs within the experiment were then added as replacements.

Crab hemolymph was taken using a tuberculin syringe (1 ml) with a 25½ ga. needle from the arthrodistal membrane at the juncture of the basis and the ischium of the 5th pereopod (swimming leg). Ethanol (70%) was used to sterilize the site of inoculation and blood letting. Total and differential counts of host hemocytes and estimates of parasite density were obtained with a hemocytometer (Neubauer improved, Bright Line) using phase contrast microscopy at 400x. Host hemocytes were identified as granulocytes, semi-granulocytes (intermediate cells with relatively few granules, Bodammer, 1978; Johnson, 1980) and hyalinocytes (cell types defined in Söderhäll & Cerenius, 1992). Hemocyte and parasite densities higher than  $1.0 \times 10^7$  cells ml<sup>-1</sup> were diluted 1:5 with the buffer and recounted to provide better estimates of cell density. Parasites were easily distinguished from host cells using phase contrast microscopy: uninucleate trophonts (9-15 µm) possessed few small, refractile vacuoles, and were rounded or amoeboid, without filopodia; multinucleate plasmodia (20-100 µm) were slender, vermiform and motile. The density of infection refers to the number of parasites per ml of hemolymph. Total hemocyte density refers to the number of hemocytes per ml of hemolymph. Mean intensity refers to the mean number of parasites per quantity of *infected* host tissue (Margolis et al., 1982).

Permanent preparations of hemolymph were processed and stained as described in Messick (1994). Briefly, acid-cleaned, poly-l-lysine-coated microslides were smeared with fresh hemolymph, let stand for 2-3 minutes, and fixed in Bouin's fixative. The smears were processed through a routine Harris hematoxylin and eosin-Y procedure (Humason, 1979, p. 123 without acid destain).

Experiment III, the Early Infection Experiment, consisted of a control group ( $n = 5$  crabs) injected individually with 100  $\mu$ l of hemolymph from an uninfected donor crab and an experimental group ( $n = 20$ ) injected with 100  $\mu$ l of hemolymph from a donor crab containing an estimated  $4.1 \times 10^6$  parasites  $\text{ml}^{-1}$  ( $4.1 \times 10^5$  parasites per crab; comprised of 79% plasmodia, 21% trophonts). Three days prior to infection, cell counts were conducted on all crabs to serve as a benchmark (presample) for before-after comparisons. On Days 3, 5, and 7, post-inoculation, five infected crabs were bled and dissected. Differential cell counts were conducted and tissue samples taken for histological analysis. Tissue samples were processed through a routine hematoxylin and eosin procedure and included muscle, hepatopancreas, heart, and, in some cases, foregut. A Day 10 sample was terminated as three inoculated crabs died during the experiment; the remaining two crabs would not provide an adequate sample size for analysis. The control crabs were bled and tissue samples taken 10 days post injection.

For statistical analyses, the proportional hazards model with the Weibull distribution was used to examine survival data and associated variables (Cox & Oakes, 1984). The Tarone-Ware log-rank test was used to examine differences between survivorship curves (Wilkinson, 1997). ANOVA was used to analyze relationships in hemocyte densities and proportion of cell type (cell type

density divided by total hemocyte density) between inoculated and uninfected crabs. Similar trends were noted in hematology and survival between Experiments I and II; hence, data were combined for the analyses. Where similar trends were noted between statistics for injection dosage ( $10^3$  vs.  $10^5$ ), data were also combined for the analysis (i.e., survivorship, hematology). SYSTAT (Wilkinson, 1997) and SAS were used to perform the statistics. A significance level of  $p < 0.05$  was accepted as significant.

## **Methods 2: Pathophysiological Indicators**

### **Hemolymph and tissue constituents**

Hemolymph samples were drawn with a sterile syringe, and placed in a microcentrifuge tube. For serum samples, fresh hemolymph was clotted on ice for 30-60 min, macerated with a tissue grinder, centrifuged for 10 min at 4000 rpm at room temperature, and the supernatant frozen at  $-80^{\circ}\text{C}$ . For plasma samples, 500  $\mu\text{l}$  fresh hemolymph was drawn into a syringe containing 500  $\mu\text{l}$  ice-cold anticlotting medium (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6 – Soderhall & Smith, 1983), centrifuged for 10 min at 4000 rpm at room temperature, and the supernatant frozen at  $-80^{\circ}\text{C}$ . Other tissues (muscle and hepatopancreas) were dissected, wrapped in foil or in tubes, frozen and stored in an ultracold freezer for later analyses.

For hemagglutination tests, red blood cells were prepared by rehydrating sheep, chicken or horse cells in a Tris-NaCl buffer (0.15 mM NaCl, 50 mM Tris), centrifuging 3-4 times to wash the cells, and adjusting cell density to a 2% (v:v) concentration. Serial dilutions of hemolymph serum, and in some cases plasma, were done in 12 steps (full strength to 1/1028), with suspensions of RBCs.



The plates were incubated for 2 hrs, read with a dissecting microscope, and read again after 24 hrs. Controls consisted of wells containing only RBC preparations.

For measurement of total proteins and acid phosphatases, hemolymph, samples collected, iced, then frozen at  $-20^{\circ}\text{C}$  until processed. Total proteins were analyzed with the Biuret method using a standard kit (Sigma #541), and read with an absorbance maximum at 540 nm. Acid phosphatases were measured using the method of (Andersch & Sczypinski, 1947) using a standard kit (Sigma #104), and read with an absorbance maximum at 420 nm. For statistical analyses, samples that were below the minimum detectable level were assigned a value of half the minimum detectable level. Controls consisted of standards, blanks, and spiked samples. [NB: This is a conservative approach for the statistical analysis.]

Enzyme constituents were measured in fresh hemolymph samples. The following enzymes were tested with hemolymph sera using the apiZYM enzyme kit (bioMerieux, France): alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, beta galactosidase, beta galactosidase, beta glucuronidase, beta glucosidase, beta glucosidase, N-acetyl-beta glucosaminidase, beta mannosidase, beta fucosidase.

Hemocyanin and lactic acid: Hemocyanin measurements were carried out according to Engel et al. 1993. Briefly, hemolymph serum samples were diluted with 50 mM Tris / 10 mM  $\text{CaCl}_2$ , pH 8.0, and absorbancies measured spectrophotometrically at 334 nm. The concentration of hemocyanin were calculated as  $E_{334\text{nm}} = 2.33$  for intact undissociated hemocyanin (Nickerson &

Van Holde, 1971). Therefore, Hc concentration is (dilution\*absorbance/2.33) in g/100 ml. While hemolymph samples were collected, they have not completely processed. Lactic acid analyses was conducted using a lactate detection kit (Sigma # 735-10) with samples read at 540 nm. While some hemolymph samples were tested for lactate, at the time of the assays there were not enough infected crabs to reach a conclusion.

#### Tissue glycogen:

Inconsistencies in the published literature on glycogen required some unplanned comparisons and refinements in the methods. We finally settled on the anthrone oxidation method (modified after van Handel, 1965; Baturo et al., 1995). Enzymatic oxidation (glucose HK) was too expensive, and acid digestion (Dubois method) converted extraneous sugars, hence, gave unreliable results. While we ran glycogen tests on muscle, there was so little present that it quickly became unfeasible. We therefore focused on glycogen in the hepatopancreas.

Aliquots of hepatopancreas (0.5 g, all samples done in triplicate) were individually placed in 4.0 ml deionized water mixed with 1.0 ml 30% potassium hydroxide solution (30g KOH in 100ml deionized water) and boiled for approximately 20 minutes. The samples were cooled on ice for 2-3 min, then homogenized. The homogenates were then mixed with 6.0 ml 100% ethanol and 100µl saturated sodium sulfate solution (1g Na<sub>2</sub>SO<sub>4</sub> in 3.6ml deionized water), and boiled for 1 min. Upon removal, the samples were centrifuged at 2000xg for 20 minutes. The supernatant was removed from each tube, and the precipitates were dried in an oven until dry (or overnight) at 60° C. After drying, the precipitates were resuspended in 500 µl deionized water, mixed with 3.0 ml 0.15% anthrone reagent (in 70% reagent grade H<sub>2</sub>SO<sub>4</sub>, made fresh daily) and heated for 10-12

minutes in a 90°C water bath. The samples were cooled to room temperature and read on a spectrophotometer at 620 nm. The amount of glycogen in each sample was calculated based upon the absorbances of the standards, and dilutions and tissue weights of the samples were accounted for when performing these calculations. For each daily series, a stock solution of oyster glycogen (1 mg/ml) was prepared, and standards (1000, 500, 250, 125, 62.5, 31.25, and 0 µg/ml solutions) treated exactly as described above.

## RESULTS

### Results: Primary Objective - Host mortality studies

Inoculated crabs that became infected with *Hematodinium perezii* began dying two weeks after inoculation (Figure 2). Mortalities peaked at three weeks post-injection, with a continuous decline in survivors from Weeks 3 through 5. The mortality rate of the infected crabs was 86%, while only 20% of the controls died. Crab mortalities were similar over the time course of infection between Experiment I (infected hemolymph, uninucleate trophonts) and Experiment II (buffer-washed parasites, vermiform plasmodia) (Tarone-Ware, Chi-square = 1.212 with 1 *df*, *P* = 0.271), even between different initial doses of the parasite (Figure 2; Tarone-Ware, Chi-square = 0.738 with 1 *df*, *P* = 0.390). Uninfected crabs (controls) experienced significantly fewer mortalities than infected hosts (Figure 2; Tarone-Ware, Chi-square =, 19.267 with 1 *df*, *P* < 0.001). The controls for Experiment II did, however, exhibit background mortalities (Figure 2); but the mortality rate was not significantly different from controls in Experiment 1 (Tarone-Ware, Chi-square = 0.652 with 1 *df*, *P* = 0.419). None of the control crabs developed infections *with H. perezii*. Since mortalities were similar between experiments, data were grouped for further analysis.

The median time to death for infected crabs was  $30.3 \pm 1.5$  (se) days. Since the controls exhibited few mortalities, the median time to death for the uninfected controls could not be calculated. Infected crabs had a significantly higher mortality rate, 7 to 8 times greater than that of the uninfected controls (Figure 2; proportional hazards, Chi-square = 13.503,  $P < 0.001$ ; relative risk =  $e^{1.055/0.5174}$ ). Hemocyte and parasite density were jointly analyzed as covariates in the proportional hazards model. For injected crabs, the decline in  $\ln(\text{total hemocyte density})$  was significantly associated with mortality ( $\ln \text{ Day of death} = 0.875 + 0.1445 \ln \text{ Total hemocyte density} - 0.0166 \ln \text{ Parasite density} + 0.409 W$ ; Chi-square = 4.467 with 1 *df*,  $P < 0.05$ ). Hemocyte density (untransformed), and parasite density ( $\ln$ , and untransformed) were not associated with mortality (Chi-square,  $P = 0.07, 0.61, \text{ and } 0.47$ , respectively); thus, decreases in hemocyte density ( $\ln$ ), not parasite density, were associated with *imminent* death.

Direct observations from crabs used to maintain infections and experimental results indicated that the parasite was detectable in the hemolymph approximately two weeks after injection (Figure 3). While the parasite could be detected as early as one week post inoculation, detectability (the percentage of *infected* crabs exhibiting detectable parasites in the hemolymph) was relatively low (30-35%) after 14 to 18 days, reaching 80 to 85% of infected crabs after 26 to 32 days, with 100% prevalence after 35 days. (Detectability was based solely on inoculated animals that developed infections. The four crabs from Experiment II that did not present infections, hereafter referred to as "immune" crabs, were excluded from the analysis of detectability.) Proliferation and growth of the parasite followed a similar pattern as the prevalence of detection, and the two variables were clearly related (Table 1).

Weekly growth of the parasite showed a marked increase in the mean density of vermiform plasmodia over Days 18 to 26 (Table 1). The mean density of trophonts increased markedly over Days 32 to 35. Note, however, that to avoid mortalities from other causes (e.g., secondary infections) sampling could not be done on a daily basis.

Plasmodia were found within the hearts of 93% (n=14/15) of the injected crabs in the Early Infection Experiment. Plasmodia were found in 4 of 5 crabs as early as Day 3 (Table 2). Uninucleate trophonts were only observed in the heart on and after Day 7. Relatively more parasites were observed in the heart tissue over time (Table 2; Days 3 and 5d vs. Day 7); but no effort was made to standardize area in the histological preparations. Growth of the parasite was rapid in the heart. The dosage in the Early Infection Experiment was, however, four times higher than that in Experiments I and II; thus, results between experiments were not directly comparable.

Sporulation from the trophont stage to the dinospore stage was only observed in crabs that were used to maintain infections. Parasites in one crab sporulated at least twice with each event lasting less than 4 d (less than 2 d?). Parasite density was extraordinarily high ( $1.6 \times 10^8$  dinospores  $\text{ml}^{-1}$ ) during sporulation, and dropped to moderate levels ( $3.3 \times 10^6$  trophonts  $\text{ml}^{-1}$ ) thereafter. Dinospores were observed five times over the course of 26 d, beginning 43 d after injection. Additionally, some crabs injected with only the trophont (vegetative) stage were observed with plasmodia after 3 to 4 weeks of infection.

Hemograms of infected crabs were significantly different from those of uninfected controls (Table 3, Figure 4). Total hemocyte density was significantly depressed in infected crabs (Figure 4A; 2-way ANOVA by Group and Day,  $F = 5.033$ ,  $P < 0.001$ ). Total hemocyte density was not significantly different between crabs inoculated with different initial doses (2-way ANOVA,  $10^3$  vs.  $10^5$  parasites per crab and Day,  $F = 3.187$ ,  $df = 1,64$ ,  $P = 0.079$ ). Crabs that were injected and did not acquire the infection ("immune" hosts) did not have significant decreases in hemocyte densities (Table 3, Figure 4; 2-way ANOVA,  $F = 1.460$ ,  $df = 13,105$ ,  $P = 0.145$ ). While the experimental parameters (infectious dose, and sample size) were different in the Early Infection Experiment compared to I and II, the decrease in total hemocyte densities occurred within three days of injection (Table 4).

In addition to a decrease in cell density, the proportions of different host cell types (density of cell type divided by total hemocyte density) in infected crabs shifted to proportionally more granulocytes than hyalinocytes (Table 5, Figure 4B and 4D) (2-way ANOVA,  $F = 1.830$ ;  $df = 20, 149$ ,  $P < 0.05$ ). Significant shifts in the population of semigranulocytes were also noted ( $F = 2.506$ ,  $df = 20, 149$ ,  $P < 0.001$ ). "Immune" crabs exhibited a fluctuation in cell types with significantly higher proportions of granulocytes to semigranulocytes from Weeks One through Five ( $F = 4.353$ ,  $df = 5,18$ ,  $P < 0.01$ ). By Day 40, the hemograms of "immune" hosts were virtually identical to those of the uninfected controls (Table 5, Figure 4C and 4D).

In the Early Infection Experiment, hemocyte populations shifted within the first three days of infection (Tables 4 and 6; ANOVA, log hemocytes,  $F = 9.158$ ;  $df = 3,31$ ,  $P < 0.01$ ), with the proportion of granulocytes in infected crabs increasing significantly compared to the

semigranulocytes (ANOVA,  $F = 4.385$ ,  $P < 0.05$ ). Uninfected crabs in the Early Infection Experiment exhibited minor fluctuations in the proportion of granulocytes to that of hyalinocytes but the proportions were similar to those observed in Experiments I and II (Tables 5 and 6).

### **Results: Secondary Objective - Pathophysiological indicators**

#### Basic hemolymph chemistry

Hemolymph levels of total protein levels and acid phosphatase activity were examined as an initial characterization of pathological changes in infected crabs. Total protein levels were significantly different between infected and uninfected male crabs, but not between similar female crabs (Figure 5). Lipoprotein levels, an important confound in females, were not considered in the analysis. Lipoproteins are associated with oogenesis and vitellogenesis, and are mobilized in the hemolymph during these processes. In addition, gonadal indices (a crude but efficient marker of the reproductive state of the host) were not measured. Hence, total protein levels may change in infected females when lipoproteins are factored out. For male crabs, the data are consistent with that observed for hemocyanin concentrations in Norway lobster (Field et al., 1992).

Acid phosphatase activity in the hemolymph varied significantly with infection of the host (Figure 6). Diseased crabs had much higher levels of acid phosphatase activity in their hemolymph, and significantly more infected crabs had detectable levels of the enzymes (Table 7). The influence of host sex was not examined in detail, but given the low levels of enzyme activity in uninfected animals, it is doubtful that differences would be observed.

The acid phosphatase data showed that the parasite was responsible for the increase in the enzymes in the hemolymph. Hemolymph sera (i.e., no cells present) from infected and uninfected hosts showed no differences in acid phosphatase activity (cf. uninfected hosts in Figure 6, to sera only in Figure 7). Yet, there were significant differences with enzyme activity in whole hemolymph (i.e., cells present). The data indicate that acid phosphatase activity is intracellular (as opposed to lysozymes that can be found extracellularly), and that the enzymes are primarily located in the trophonts of *Hematodinium perezii*.

Enzyme tests (apiZym, bioMerieux, France) showed some differences between uninfected and infected sera. Infected sera showed significantly higher levels of acid phosphatase (see above), naphthol AS-BI phosphohydrolase, beta glucuronidase, and beta galactosidase. Uninfected sera showed significantly higher levels of alpha fucosidase. Beta glucuronidase showed reactions in 2/9 uninfected crabs vs. 7/8 infected crabs (chi-square would show this as significant but N is too low, therefore the data need augmenting), but the means were not significant (t-test). Sample sizes were small (9 uninfected vs 8 heavily infected) because the test kits were expensive, and the tests were not planned in the original study.

A large, replicated series of hemagglutination tests were performed using rabbit, chicken and sheep red blood cells. Initially, the hemolymph of infected crabs showed significantly fewer agglutinin reactions to rabbit red blood cells than did the hemolymph of uninfected crabs (Figure 8). The intensity of the reaction was not, however, different between infected and uninfected crabs (Figure 9). Upon further testing there were no differences in agglutination for sera exposed



to any of the rabbit, and sheep red blood cells (Table 8). Both infected and uninfected sera reacted poorly to sheep RBCs. Thus, agglutination does not appear to be affected by the parasite.

Glycogen data were conclusive. Infected crabs had significantly less glycogen in the hepatopancreas than did uninfected crabs (Figure 10). Infected males exhibited a larger decrease in glycogen levels than did infected females (70% vs. 48% decline). Muscle samples (1 gm tissue) showed little glycogen concentration between infected and uninfected animals and were not further examined. The glycogen data support an observation from the mortality studies. Infected crabs slow or cease feeding after about 3 weeks of infection. The cessation is lengthy and appears related to a decline in the activity of the infected animal.

#### **Results: Additional sampling – 1997 epizootiological study**

Due to the higher autumnal prevalence of the disease, we undertook additional broad-scale sampling from locations in the mainstem of the bay during September-December, 1997. Since 1996 was a "wet" year, and 1997 a "dry" year, we felt it was necessary to obtain additional data on the epizootiology of the parasite (as in the previous Saltonstall-Kennedy award). Partial results from the Fall 1997 sampling period have been reported (Shields, 1997; Messick & Shields, in prep.).

In 1997, a total of 870 crabs were sampled. Of these, 6 were sampled in the Winter, 148 in the Spring, 358 in the Summer, and 358 in the Fall. At least 47 crabs were infected, with the infected crabs evenly spread through the three seasons. (We still have to process some of these samples,

so prevalences will increase). In 1997, the prevalence in the Spring was already at 14%, and remained at 20-25% through the Summer months.

In 1997, *H. perezii* festered at moderate prevalences (10-20%) at both the seaside and bayside reference stations. In the Summer and Fall, 1997, the prevalence and distribution of the disease were higher than that observed in 1996. The disease was present at both reference locations during the Spring and Summer of 1997. The high prevalence of the parasite in Spring, 1997, and its increase at Hungars Creek (bayside), may have been related to the low rainfall and warm temperatures experienced during 1997. Hungars Creek had a surprisingly high prevalence of 20% during September, 1997. This creek is located over 30 miles from the mouth of the bay on the "bayside" of Delmarva Peninsula.

## FINDINGS

We have fulfilled a portion of Koch's Postulates through the introduction of *Hematodinium perezii* into naive hosts with the subsequent acquisition of disease. We have also achieved short-term primary culture of *H. perezii* (Attachment A, Shields et al., in review). Such primary cultures remained infectious for at least 14 days, further substantiating Koch's Postulates. Our inoculation studies involved serial passage of *Hematodinium* through two different 7 month periods. Infected crabs showed signs of weakness and lethargy, but only when the disease had progressed to moderate and heavy infections, i.e., after 14-35 days (at 20 ° C).

### DISCUSSION – Part 1: Mortality Studies

Estimates of host mortality are important in predicting the scale or impact of parasites and diseases on a fishery. In laboratory experiments, *Hematodinium perezii* caused significant mortality to infected mature, non-ovigerous blue crabs. Infections were not always fatal (4 crabs survived inoculation without developing infections), but the overall mortality to lab-injected crabs was high at 86% over 40 days. The proportional hazards model indicated that infected crabs were 7 to 8 times more likely to die than uninfected crabs. Infections in Tanner crab, *Chionoecetes bairdi*, and Norway lobster, *Nephrops norvegicus*, are frequently fatal to the host (Meyers et al., 1987; Field et al., 1992). The mortality of naturally infected Tanner crabs held in aquaria for 97 days was 67% ( $n = 11$ ) with hosts surviving from 20 to 158 days in the laboratory. Uninfected Tanner crabs experienced no mortality during the course of the experiment (Meyers et al., 1987). Naturally infected Norway lobsters suffered mortality rates of 86% to 100% over 27 d and 75 d respectively, and had mortality rates 2 - 4 times higher than uninfected lobsters, with most of the deaths occurring early in the course of the experiment (Field et al., 1992). Boreal hosts are large

and experience long courses of infection presumably because of inherent metabolic adaptations of cold water parasites. It may, therefore, be more difficult to estimate survival and recovery in these hosts. Infections in blue crabs kill the host very quickly; infected crabs apparently die before acquiring the bitter flavor found in infected Tanner crabs.

Survival analysis indicated that parasite density was not associated with mortality. Similarly, survival time of Norway lobsters did not show a significant relationship with severity of infection, but host mortality did increase with the progression of the disease (Field et al., 1992). In blue crabs, absolute declines in  $\ln(\text{total hemocyte density})$  were associated with host mortality. Hence, the cellular defensive response of the host appeared seriously compromised by infection. Observations on naturally infected, and experimental infections suggest that blue crabs experience three possible outcomes to the disease. (1) Crabs with acute infections, such as those reported here, show rapid mortalities typically dying within 40 d. Acute infections rarely lead to heavy infections ( $10^{7+}$  parasites  $\text{ml}^{-1}$ ), and may not lead to the development of dinospores. (2) Crabs with chronic infections (observed in very few naturally infected hosts,  $n = 4$ ) endure the acute stage, survive for longer periods (up to 90 days), and develop infections that lead to massive numbers of dinospores (Figure 11). (3) Some crabs successfully resist the parasite or are refractory to the infection. Preliminary experiments (not shown) suggest that resistant crabs ( $n = 10$ ) may become refractory to further inocula of *H. perezii*.

Blue crab catches fluctuate yearly in Chesapeake Bay but causes for these fluctuations are not well understood. Since salinity appears to limit the distribution of *Hematodinium perezii* (Newman & Johnson, 1975), the dinoflagellate could feasibly infect and cause significant

mortalities to juvenile and adult crabs in Chesapeake Bay where salinities are greater than 11 ‰; i.e., much of the mainstem of the bay. Current models for blue crab populations in Chesapeake Bay are based on population assessments from various surveys (Lipcius & Van Engel, 1990; Abbe & Stagg, 1996; Rugolo et al., 1998). These models project crab abundance for the fishery as a whole but do not separate the larger, low salinity "bayside" fishery from the smaller, high salinity "seaside" fishery where mortalities to *H. perezii* occur. Such projections include estimates of natural mortalities, but do not account for the potential epizootics and resulting mortalities caused by *H. perezii*. Differential models of exploitation by region may be warranted, especially during or immediately following epizootics. Disease estimates must, however, account for the variation in the prevalence of detection as the prevalence in field samples may be significantly underreported (see Fig. 3).

The life cycle of *H. perezii* from *C. sapidus* has not been fully documented. Culture attempts with *H. perezii* have been only partially successful (Shields et al., in review). Several morphological and life history differences, however, distinctly separate *Hematodinium* sp. ex *N. norvegicus* (Appleton & Vickerman, 1998) from *H. perezii* ex *C. sapidus*. For example, the syncytial and network forms of *Hematodinium* sp. ex *N. norvegicus* (Field & Appleton, 1995) have not been observed with *H. perezii* (Messick, 1994; present study); nor do the plasmodia (cf. filamentous trophonts of Appleton & Vickerman, 1998) of *H. perezii* develop as "gorgonlocks," rather they undergo budding to produce additional plasmodia, and schizogony (cf. segmentation in malaria life cycles) to produce uninucleate trophonts (Shields et al., in review). The trophonts then undergo further fission. Such differences may warrant generic separation between the two parasites.

*Hematodinium perezii* was successfully transmitted to blue crabs via injection. Transmission experiments with the parasite in Tanner crabs and Australian sand crabs (*Portunus pelagicus*) have been partially successful. Parasites from primary cell culture (using sterile hemolymph) were successful in establishing infections in Tanner crabs, but inoculation with vegetative stages did not produce infections (Meyers et al., 1987). Injections of trophonts (vegetative stages) were successful in producing infections in *P. pelagicus*, but other stages were not investigated (Hudson & Shields, 1994). Infection experiments with Norway lobster have not been reported. Transmission with cultured dinospores has yet to be achieved (Appleton & Vickerman, 1998). Sporulation is a rapid event with *H. perezii* presumably occurring over several hours instead of several days or weeks as that reported for *Hematodinium* sp. from Tanner crabs (Meyers et al., 1987, 1990). At lower temperatures and salinities, *H. perezii* apparently ceases to grow or slows its proliferation in naturally infected blue crabs.<sup>2</sup>

Densities of circulating hemocytes declined rapidly in infected crabs. The decline occurred within the first three days and progressed to an 48% decrease in total hemocyte densities within the first week of infection. After three weeks, absolute declines of up to 80% were noted for total hemocyte densities. The loss of cells was evident early in the infection even though the parasites were not detectable in hemolymph. Declines in hemocyte densities have been reported for starved lobsters, *Homarus americanus* (33-60% loss) (Stewart et al., 1967), *Aeromonas*-infected lobsters (80-84% loss) (Stewart et al., 1983), *Fusarium*-infected brown shrimp, *Penaeus californiensis* (approximately 88% loss) (Hose et al., 1984), and *Vibrio*-infected *Cancer irroratus* (95% loss)

(Newman & Feng, 1982). Reductions in hemocytes were noted for Norway lobster, *N. norvegicus*, infected with *Hematodinium* sp. (Field & Appleton, 1995), and for blue crabs, *C. sapidus*, infected with *Paramoeba pernicioso* (Sawyer et al., 1970), but the degree of loss, and differential counts were not quantified. Declines in hemocyte counts occur quickly in crayfish, *Pacifastacus leniusculus*, (10 min) and are associated with loss of resistance to *Aphanomyces* infections (Perrson et al., 1987). For crayfish, the declines are dependent upon the stimuli (yeast vs. zymosan vs. buffers), and are evident over the course of several days.

Crustacean cell types probably represent maturation of a single lineage (e.g., Bodammer, 1978, Bachau, 1981; Hose et al., 1990). Hyalinocytes represent younger cells that become semigranulocytes (intermediate hemocytes), then granulocytes. Infected crabs exhibited marked shifts in subpopulations of different hemocyte stages (cell types). Since there was an absolute decline in the total number of circulating hemocytes, and relative declines in hyalinocytes, and semigranulocytes, we suggest that cell death and differential sequestration occur in response to the disease. General declines in hemocyte density in *N. norvegicus* infected with *Hematodinium* sp. may occur from sequestration, other defense reactions, hydrostatic effects of heavy infections or clogging of hemal sinuses (Field & Appleton, 1995). In the present study, the rapid decline in total hemocyte density (within three to seven days) argues against hydrostatic effects and clogged sinuses. The shift towards proportionally more granulocytes than hyalinocytes may result from mobilization of tissue-dwelling reserves, differential cell death (Mix & Sparks, 1980), changes in mitotic stimuli of hemopoetic tissue (Hose et al., 1984), or sequestration of specific cell types in defense of the infection (e.g., nodule formation, Johnson, 1976, 1977). Hyalinocytes and

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<sup>2</sup> Messick, G., 1998, Oxford Cooperative Laboratory, 904 S. Morris St., Oxford, MD 21654, personal. comm.,

semigranulocytes are the major phagocytic hemocytes in crustaceans (Bachau, 1981; Hose et al., 1990). Such hemocytes form nodules in bacterial, amoebic, and *Hematodinium* infections in blue crabs, *Hematodinium* infections in *N. norvegicus*, and gaffkemia infections in *Homarus americanus* (Johnson, 1976, 1977; Johnson et al., 1984; Messick, 1994; Field & Appleton, 1995), are thus removed from circulation, and may account, in part, for the observed declines. In fungal infections of crayfish, *P. leniusculus*, hemocytic nodules do not dissociate in the presence of zymosan, a yeast derivative, and may last several days (Perrson et al., 1987). In *Aeromonas*-infected lobsters, the hyalinocytes increase in proportion to the other cell types, presumably via increased mitotic activity in hemopoietic centers, but there is a significant decline in hemocytes after five days of infection (Stewart et al., 1983).

Lastly, several blue crabs (n=4) successfully fought off the infection. These "immune" crabs exhibited significant sustained levels of granulocytes, did not suffer hemocytopenia, their hemolymph clotted normally, and they did not exhibit gross changes in morbidity. Histological preparations of heart, hepatopancreas, muscle, and hemopoietic tissues were negative for latent infections in the "immune" animals. The relative increase in semigranulocytes and sustained densities of hyalinocytes over time (Figure 4) suggests an increase in mitotic activity in hemopoietic tissue in response to the infection. This increase may not be sufficient to counter the parasite in susceptible hosts. In *N. norvegicus* infected with *Hematodinium* sp., the hemopoietic tissues show marked increases in activity. While changes in host cell densities were not quantified, apparent stem cells were present in the active nodes (Field & Appleton, their Tables 1

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unpubl. data.



and 2). The role of the granulocytes in the defense against *Hematodinium* infections and the underlying mechanisms leading to refractory hosts warrant further study.

## **DISCUSSION – Part 2: Pathophysiological indicators**

Blue crabs infected with *Hematodinium perezii* exhibit radical changes in the chemistry of the hemolymph. Gross changes include chalky or yellow discoloration of the hemolymph, lack of clotting ability, and a radical decline in the total hemocyte densities. Loss of clotting is a common endpoint for the hemolymph in many different decapod infections. The changes leading to the loss were not apparent in the present study. Declines in hemolymph proteins, and various enzyme systems suggest that an overall decrease in proteins associated with clotting may result from infection. Alternatively, the clotting mechanisms may be specifically suppressed or reduced. Pauley et al. (1975) suggest that the lack of clotting in *Paramoeba* infections may be due to alterations of the hemolymph via parasite modulated proteolytic activity, or from the loss of serum fibrinogen as a component of the total serum protein.

The total serum protein assays suggest that the disease may have a different time course in male crabs. Infected females may survive longer, and may show a less rapid depletion of resources than their male counterparts. Since female crabs have a larger lipid reserve than males, females may have more resources available to counter the infections, or may simply take longer to show signs of the disease. Note that the mortality experiments used female crabs that were in excellent physiological condition. The field prevalence data neither supports nor refutes the speculation. It needs further investigation.

Male blue crabs infected with *Hematodinium perezii* have lower total serum protein levels than uninfected crabs. Pauley et al. (1975) found that crabs heavily infected with *Paramoeba pernicioso* had significantly less total protein and glucose in the hemolymph than uninfected crabs. Uninfected male crabs had  $45.4 \pm 15.1$  (sd) vs.  $9.4 \pm 4.6$  mg ml<sup>-1</sup> total protein, a decrease of almost 81%. Uninfected females had  $14.6 \pm 5.6$  vs.  $7.4 \pm 3.9$  mg ml<sup>-1</sup> total protein in the hemolymph. Hemolymph glucose levels showed similar declines. A progressive loss of total protein was noted between uninfected, lightly, moderately and heavily infected hosts. Decreased levels of hemocyanin were reported by the declines were not quantified. Serum glucose may not be a good indicator of pathophysiology as it varies considerably with season and the physiological state of the organism (Lynch & Webb, 1973). With amoebic infections, the decline in total proteins probably accounts for the weakened condition and rapid decline of crabs (Pauley et al., 1975). Starvation from lethargy (as occurs with *Paramoeba* and *Hematodinium* infections) was not, however, considered. The loss of hemocyanin indicates that death is due to a combination of hypoxia, and loss of nutrients (starvation) (Pauley et al., 1975). Reduced hemocyanin levels and tissue anoxia may also explain the mortality of Norway lobster, *N. norvegicus*, due to *Hematodinium* (Field et al. 1992).

Blue crabs parasitized by the microsporidian parasite, *Ameson michaelis*, show lower levels of Cl<sup>-</sup> and Na<sup>+</sup> in the blood, and higher levels of free amino acids (Findley et al. 1981). In addition, infected crabs show increased levels of lactate and decreased levels of blood glucose in the hemolymph, thoracic muscle and hepatopancreas. Increased levels of amino acids in the hemolymph may have resulted from muscle proteolysis or leakage of amino acids from cell membranes. Increased lactic acid levels in hemolymph may result from the use of muscle tissue as

an energy source by the parasite or from parasite-induced stress (Findley et al. 1981). The changes in enzymes reported here (acid phosphatase, etc.), indicates that infected animals are experiencing significant changes to their hemolymph chemistry and metabolism.

Glycogen levels were significantly lower in infected blue crabs. Parasite-induced stress can result in altered physiological conditions such as decreased food consumption, digestion, assimilation, castration, and energy storage. (Andrews 1961, Newell 1985, Barber et al. 1988). Glycogen is the main storage substrate in many invertebrates, providing energy for several physiological processes (Bayne 1976, Gabbot 1976, 1983). Numerous studies have indicated that glycogen is stored in the body of the organism when food is abundant and later used for reproduction. The cessation of feeding in infected blue crabs may facilitate the depletion of glycogen. Notably, the decline in infected females was less dramatic than that in infected males.

Hemagglutinins were present, but showed no changes in infected crabs. Various host cellular and humoral factors contribute to extracellular and intracellular destruction of parasites in crustaceans (see Soderhall & Cerenius, 1992, for review). Serum lectins and agglutinins are components of hemolymph. They are known to agglutinate vertebrate erythrocytes and bacteria *in vitro*. Three lectins (serum-bound and cell-bound) have been discovered in the blue crab, *C. sapidus* (Cassels et al, 1986). In addition, a bacteriolytic factor has recently been found in the hemolymph of blue crabs (Noga et al. 1994). In crustaceans, agglutinins have been found against vertebrate erythrocytes (Chushin, 1967), bacteria (Huang et al. 1981), invertebrate sperm (Smith & Goldstein, 1971), protozoans (Bang, 1962), and other cells (Tyler & Metz, 1945). In most crustaceans agglutinins occur naturally in the plasma or serum. Foreign bodies cause an increase

in agglutination titers in the blue crab, *C. sapidus* (Pauley, 1973). The suggested defensive roles for humoral factors include: agglutination of bacteria by lectins and agglutinins leading to inactivation; lysis of inactivated bacteria by extracellular lysosomal enzymes; aiding hemocyte phagocytosis and encapsulation of agglutinated particles; and, recognition of non-self particles by hemocytes due to the opsonic activity of agglutinins (Hardy et al., 1977; Vasta & Marchalonis, 1983). Curiously, lysozymes, key lytic enzymes in the hemolymph of vertebrates and other invertebrates including insects, have not been reported in crustaceans (Smith & Chisholm, 1992).

### **Discussion Part 3: 1997 Epizootiological sampling**

*Hematodinium perezii* is present at low prevalences in the main spawning grounds of the blue crab in Chesapeake Bay; i.e., near the mouth of the bay. The parasite occurs at higher levels in the spawning grounds during the Fall. The peak season for crab reproduction is late Spring and Summer (Van Engel, 1958, 1987, Hill et al., 1989). Therefore, while crab reproduction occurs during the Fall, the parasite is not present during the peak periods of crab reproduction. Since low salinities appear to inhibit the spread of the disease, the 1996 data may represent conservatively low estimates of the prevalence and spread of the disease in the bay's fishery (Shields, 1997).

The disease should have relatively little impact on the large crab fishery in the bay and its tributaries. However, the smaller coastal fishery is at risk to the parasite as the disease occurs in high salinity waters, and epizootics can occur during the periods that may be sensitive to the region's crab stock; i.e., spring (peak molting and mating periods), and the fall (end of migration and reproductive periods) (Figure 12).

In 1997, the prevalence of the parasite was low to moderate during the ovigerous season. The increased prevalence in adult female crabs, and the heavy infections observed in some ovigerous crabs, indicated that the disease may potentially kill a modest portion of the preovigerous and ovigerous crabs, and may overwinter in the spawning grounds under favorable conditions to the parasite.

Most of the crabs collected from the lower bay (Mouth, and LB South East) were in or adjacent to the various Crab Management Areas/Sanctuaries designated by the Virginia Marine Resources Commission (Figure 13). These sanctuaries are designed to protect preovigerous and spawning populations during sensitive portions of their life cycles. *Hematodinium perezii* was found at higher prevalences in female crabs from within the sanctuary than from females taken in the tributaries. Prevalences of 1.0 to 13.3% were noted within the boundaries of the crab sanctuaries during Fall, 1997. The fall peak in prevalence in mature females, and the predilection of the disease for juvenile crabs (Messick, 1994), indicates that during epizootics the disease may threaten reproduction in the sanctuaries, and could impact on survivorship of the next season's harvest.

#### Description of need for additional work

Several lines of research would facilitate our understanding of *Hematodinium perezii* and its spread in crab populations (Figure 14). The effect of environmental factors on the fulmination of epizootics should be examined experimentally. Salinity and temperature play key roles in the spread of the disease but more data are needed to firmly establish their influences. Low level monitoring of reference sites (i.e., Fall and Spring seasons) would help to provide better

correlative data with the environmental factors, and may give early warning of an impending epizootic. Since we have established that the parasite can be maintained via serial passage, well-designed laboratory experiments on salinity and temperature should be initiated. Serial passage will also allow more life cycle studies, and should pave the way for full scale culture experiments. Molecular studies to develop a diagnostic probe for use with other dinoflagellates (e.g., *Pfiesteria piscicida*) would be quite useful.

## EVALUATION OF PROJECT

### Were the goals and objectives attained?

The goals of the project were obtained. The mortality study showed that *Hematodinium* was highly pathogenic to adult crabs at slightly lower than summer temperatures. Infections were maintained via serial passage. Pathophysiological studies showed that the crabs died from a decline in metabolic reserves, and a general decline in cellular defenses.

This research fit directly into NOAA's Strategic Plan (NOAA, 1995) by aiding in sustaining healthy fisheries (via study of the epizootics), and in sustaining healthy coasts (via a better understanding of the disease). *Hematodinium perezii* may alter the management of the coastal blue crab fishery as current fishery models do not include higher estimates of mortality due to the disease. To reiterate, the effects of *Hematodinium* spp. on the Tanner crab, Norway lobster, rock crab, and velvet crab fisheries, and our current data indicate that *H. perezii* may have a significant impact on the coastal blue crab fisheries along the Atlantic seaboard of the USA.

### Were modifications made to the goals and objectives?

Since 1996 was one of the coldest and wettest years on record (i.e., an anomalous year for studying temperature and salinity relationships), the broad scale sampling from the previous Saltonstall-Kennedy grant was continued in 1997 from the lower Chesapeake Bay and the two reference locations.

Dissemination of project results.

The mortality study is in review as a manuscript. The pathophysiology study is in preparation as a manuscript. Appendix A is in review as a manuscript. In addition, the project has generated 4 presentations:

Shields, J.D. 1999. Epizootiology of a parasitic dinoflagellate in blue crabs from Chesapeake Bay.

Invited speaker, Eastern Michigan University, January.

Shields, J.D. 1999. *Hematodinium*-induced mortalities in blue crabs in Virginia. Invited

symposium speaker: Mortality in populations of the blue crab, *Callinectes sapidus*.

Crustacean Society Annual Meeting, Lafayette, LA, May.

Shields, J.D. 1998. Diseases of blue crabs of importance to Virginia. Eastern Shore Laboratory,

Wachapreague, VA, November.

Shields, J.D. 1998. Proliferation of *Hematodinium perezii*, a parasitic dinoflagellate, in the blue

crab, *Callinectes sapidus*. 3d International Symposium on Aquatic Animal Health,

Baltimore, MD, September.



**Project Management**

Project Manager:	Dr. Jeffrey Shields
Laboratory Technicians:	Christopher Squyars
	Christine "Bean" Scanlon
Undergraduate Interns (NSF REU):	Michelle Levesque
	Erinn Newman
High School Internship:	Jennifer Lewis
Crab collectors: VIMS Trawl Survey	Pat Geer, and others
VIMS Dredge Survey	Marcel Montane, and others
Waterman	Seth Rux, Red Bank Seafood Co.

All of the work was done at the Virginia Institute of Marine Science. The histological processing was done at the Shellfish Diseases Laboratory (Nita Walker, Rita Crockett). Crab collecting was done by various groups at VIMS, and by Seth Rux, a member of the Eastern Shore Working Watermen's Association.

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The staff of the VIMS Trawl Survey, and VIMS Blue Crab Dredge Survey provided valuable time and assistance to this study. Seth Rux, Marcel Montane, Pat Geer, the staff of the Wachapreague Marine Laboratory and many others contributed to the success of the project. Gretchen Messick provided useful criticisms to the study of *Hematodinium*.

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**Table 1.** Parasite intensity ( $\times 10^5$  parasites  $\text{ml}^{-1}$  infected host $^{-1}$ ) in the hemolymph in relation to days post-inoculation. Counts were combined from Experiments I and II. Parasite density was not assessed for infected crabs that died between sampling times.  $N_{\text{plas}}$  = number of crabs with plasmodia,  $N_{\text{troph}}$  = number of crabs with trophonts,  $N_{\text{infected}}$  = number of infected crabs exhibiting parasites in the hemolymph. See Table 3 for sample sizes for hemocytometry.

Days	$N_{\text{plas}}$	Plasmodia Mean $\pm$ se	log(Plasmodia) Mean $\pm$ se	$N_{\text{troph}}$	Trophont Mean $\pm$ se	log(Troph) Mean $\pm$
7	1	0.50 $\pm$ 0.00	4.70 $\pm$ 0.00	1	0.25 $\pm$ 0.00	4.40 $\pm$
14	4	1.44 $\pm$ 0.53	5.09 $\pm$ 0.16	3	1.25 $\pm$ 0.29	5.06 $\pm$
18	1	1.25 $\pm$ 0.00	5.10 $\pm$ 0.00	2	1.00 $\pm$ 0.25	4.99 $\pm$
21	5	7.70 $\pm$ 4.61	5.61 $\pm$ 0.24	5	8.65 $\pm$ 4.17	5.87 $\pm$
26	11	14.98 $\pm$ 6.07	5.85 $\pm$ 0.17	12	8.19 $\pm$ 3.86	5.40 $\pm$
32	2	8.38 $\pm$ 1.63	5.92 $\pm$ 0.09	3	4.25 $\pm$ 1.32	5.59 $\pm$
35	4	7.88 $\pm$ 3.89	5.69 $\pm$ 0.25	4	49.81 $\pm$ 36.82	6.35 $\pm$

**Table 2.** Relative intensity of plasmodia in histological preparations of heart sections of mature, non-ovigerous female blue crabs from the Experiment III, the Early Infection Experiment. Mean intensities represent direct counts of plasmodia and are not standardized by tissue area.

<b>Day</b>	<b>No. Infected/No. Injected</b>	<b>Mean Intensity (+ sd)</b>	<b>Range</b>
Control	0/4	0.0 ± 0.0	-
3	4/5	3.6 ± 3.9	1 - 10
5	5/5	12.0 + 11.0	1 - 26
7	5/5	55.8 + 26.1	15 - 74



**Table 3.** Total hemocyte density ( $\times 10^6$  hemocytes  $\text{ml}^{-1}$ ) in relation to days post-inoculation. Hemocyte counts were combined from Experiments I and II. "Immune" crabs represent surviving hosts that successfully fought off infection in Experiment II (. = no data, no infected crabs survived to Day 40).

Days	Uninfected control crabs		Inoculated, infected crabs		Inoculated, immune crabs	
	N	Hemocyte density	N	Hemocyte density	N	Hemocyte density
		Mean $\pm$ se		Mean $\pm$ se		Mean $\pm$ se
7	18	29.17 $\pm$ 3.09	22	14.39 $\pm$ 2.05	4	22.99 $\pm$ 2.32
14	8	32.81 $\pm$ 3.01	11	16.10 $\pm$ 3.19	4	33.25 $\pm$ 2.76
18	9	32.28 $\pm$ 6.72	10	12.24 $\pm$ 1.51	.	.
21	8	23.83 $\pm$ 2.20	10	17.68 $\pm$ 4.39	4	32.72 $\pm$ 1.04
26	18	26.65 $\pm$ 2.15	16	7.64 $\pm$ 1.49	4	26.29 $\pm$ 8.00
32	12	20.97 $\pm$ 3.41	4	4.21 $\pm$ 2.04	.	.
35	8	23.37 $\pm$ 4.48	4	10.86 $\pm$ 6.73	4	23.61 $\pm$ 11.72
40	10	20.35 $\pm$ 2.12	.	All dead	4	22.53 $\pm$ 5.69

**Table 4.** Early Infection Experiment: total hemocyte density ( $\times 10^6$  hemocytes  $\text{ml}^{-1}$ ) in relation to days post-inoculation (. = not done).

Days	Uninfected control crabs		Inoculated, infected crabs	
	n	Hemocyte density	n	Hemocyte density
		Mean $\pm$ se		Mean $\pm$ se
Presample	5	15.91 $\pm$ 1.25	20	23.28 $\pm$ 2.06
3	.	.	5	12.11 $\pm$ 0.50
5	.	.	5	9.39 $\pm$ 2.94
7	.	.	5	11.40 $\pm$ 0.96
10	5	23.98 $\pm$ 4.87	.	.

**Table 5.** Differential densities of hemocytes ( $\times 10^6$  hemocytes  $\text{ml}^{-1}$ ) in relation to days post-inoculation. Hemocyte counts were combined from Experiments I and II. "Immune" crabs represent surviving hosts that successfully fought off infection in Experiment II (. = no data, no infected crabs survived to Day 40). Sample sizes are given in Table 3.

Days	Granulocyte density Mean $\pm$ se	Semigranulocyte density Mean $\pm$ se	Hyalinocyte density Mean $\pm$ se
Uninfected control crabs			
7	8.13 $\pm$ 0.97	13.88 $\pm$ 1.28	7.16 $\pm$ 1.10
14	8.34 $\pm$ 1.17	15.13 $\pm$ 1.66	9.34 $\pm$ 0.94
18	8.99 $\pm$ 1.42	15.37 $\pm$ 3.33	7.83 $\pm$ 2.18
21	5.88 $\pm$ 0.93	10.86 $\pm$ 0.93	7.09 $\pm$ 0.91
26	6.56 $\pm$ 0.73	13.06 $\pm$ 1.04	7.04 $\pm$ 0.76
32	4.10 $\pm$ 0.81	12.14 $\pm$ 2.21	4.73 $\pm$ 0.53
35	5.64 $\pm$ 1.15	11.67 $\pm$ 2.34	6.05 $\pm$ 1.37
40	4.66 $\pm$ 0.61	9.29 $\pm$ 1.13	6.39 $\pm$ 0.68
Inoculated, infected crabs			
7	5.28 $\pm$ 1.04	6.18 $\pm$ 0.71	2.92 $\pm$ 0.56
14	5.59 $\pm$ 1.34	6.90 $\pm$ 1.26	3.62 $\pm$ 0.94
18	3.29 $\pm$ 0.67	6.76 $\pm$ 0.65	2.20 $\pm$ 0.47
21	4.77 $\pm$ 1.23	8.37 $\pm$ 1.75	4.60 $\pm$ 1.54
26	2.10 $\pm$ 0.45	4.25 $\pm$ 0.83	1.28 $\pm$ 0.36
32	1.11 $\pm$ 0.57	2.19 $\pm$ 1.14	0.90 $\pm$ 0.41
35	3.11 $\pm$ 2.13	6.11 $\pm$ 3.58	1.64 $\pm$ 1.06
40	.	.	.
Inoculated, immune crabs			
7	9.73 $\pm$ 0.65	7.08 $\pm$ 1.58	6.19 $\pm$ 0.93
14	12.31 $\pm$ 0.83	11.91 $\pm$ 1.34	9.03 $\pm$ 1.00
18	.	.	.
21	11.34 $\pm$ 2.51	11.94 $\pm$ 3.44	9.44 $\pm$ 4.75
26	8.16 $\pm$ 3.73	10.00 $\pm$ 2.55	8.14 $\pm$ 2.80
32	.	.	.
35	7.98 $\pm$ 4.03	8.03 $\pm$ 3.89	7.61 $\pm$ 3.82
40	4.59 $\pm$ 1.35	10.00 $\pm$ 2.30	7.94 $\pm$ 2.71

**Table 6.** Early Life History Experiment: differential densities of hemocytes ( $\times 10^6$  hemocytes  $\text{ml}^{-1}$ ) in relation to days post-inoculation. Sample sizes are given in Table 4.

Days	Granulocyte density Mean $\pm$ se	Semigranulocyte density Mean $\pm$ se	Hyalinocyte density Mean $\pm$ se
Uninfected control crabs			
Presample	3.72 $\pm$ 0.31	7.66 $\pm$ 0.50	4.53 $\pm$ 0.79
10	7.48 $\pm$ 1.60	11.20 $\pm$ 1.94	5.30 $\pm$ 1.47
Inoculated, infected crabs			
Presample	6.88 $\pm$ 0.92	11.43 $\pm$ 1.00	4.97 $\pm$ 0.40
3	4.95 $\pm$ 0.40	4.36 $\pm$ 0.31	2.80 $\pm$ 0.15
5	2.51 $\pm$ 0.91	5.40 $\pm$ 1.60	1.49 $\pm$ 0.51
7	2.93 $\pm$ 0.68	6.92 $\pm$ 0.80	1.56 $\pm$ 0.31

**Table 7.** Acid phosphatase levels in hemolymph of blue crabs infected with *Hematodinium perezii*. Note the lack of enzyme in uninfected crabs versus that in infected hosts.

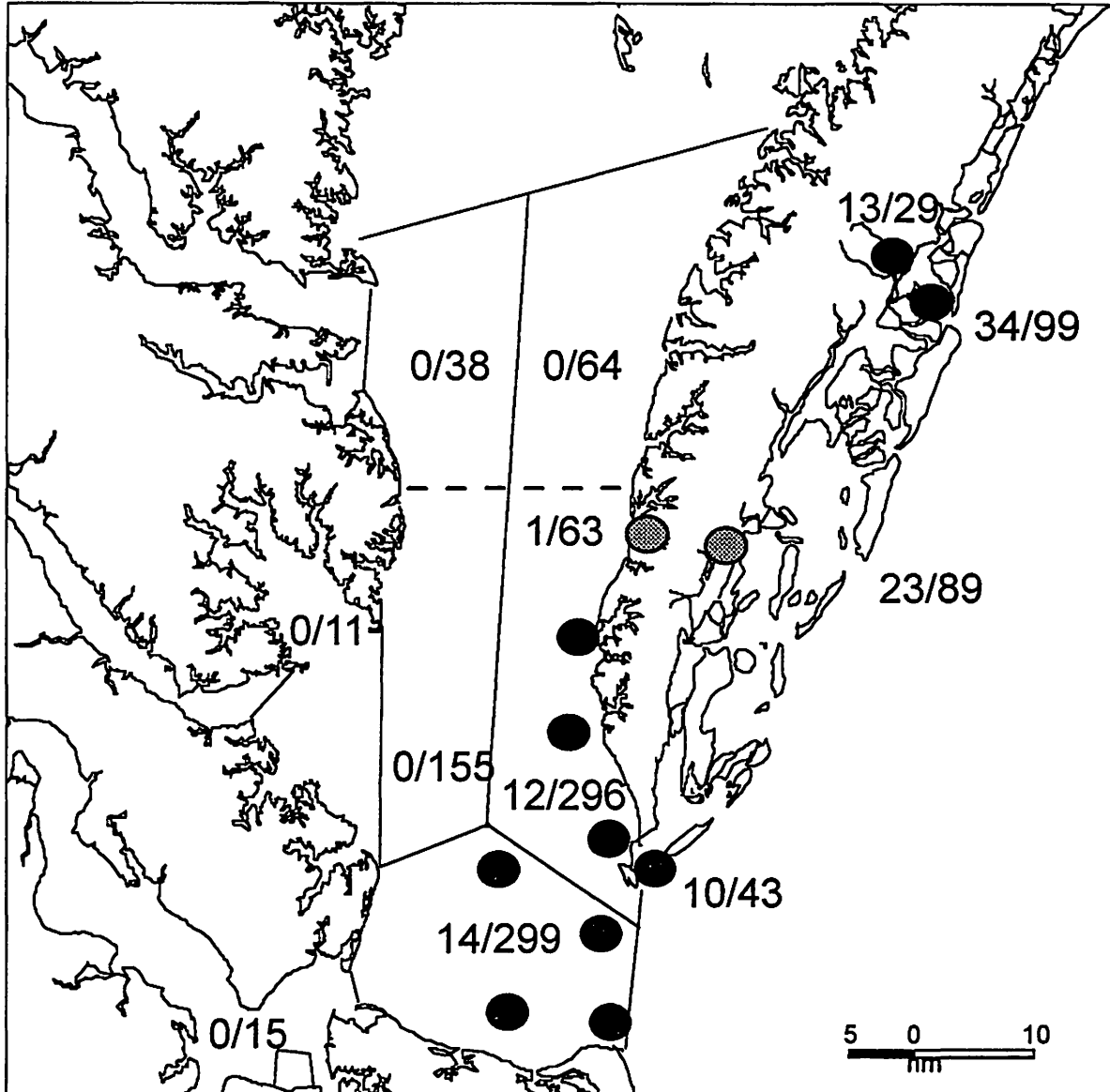
Status	Below detection (0.1 SU/ml)	Above detection
Uninfected	16	3
Light	4	4
Moderate	5	11
Heavy	2	13

Chi-square=19.03, d.f.=3, P<0.001

**Table 8.** Frequencies of minimum serum dilutions at which agglutination occurred in uninfected, lightly (0.3-3 parasites/100 host cells), moderately (3.3-10 parasites per 100 host cells), and heavily (10+ parasites per 100 host cells) infected crabs. Note that serum in sheep red blood cells showed distinctly less reactivity than chicken and rabbit red blood cells.

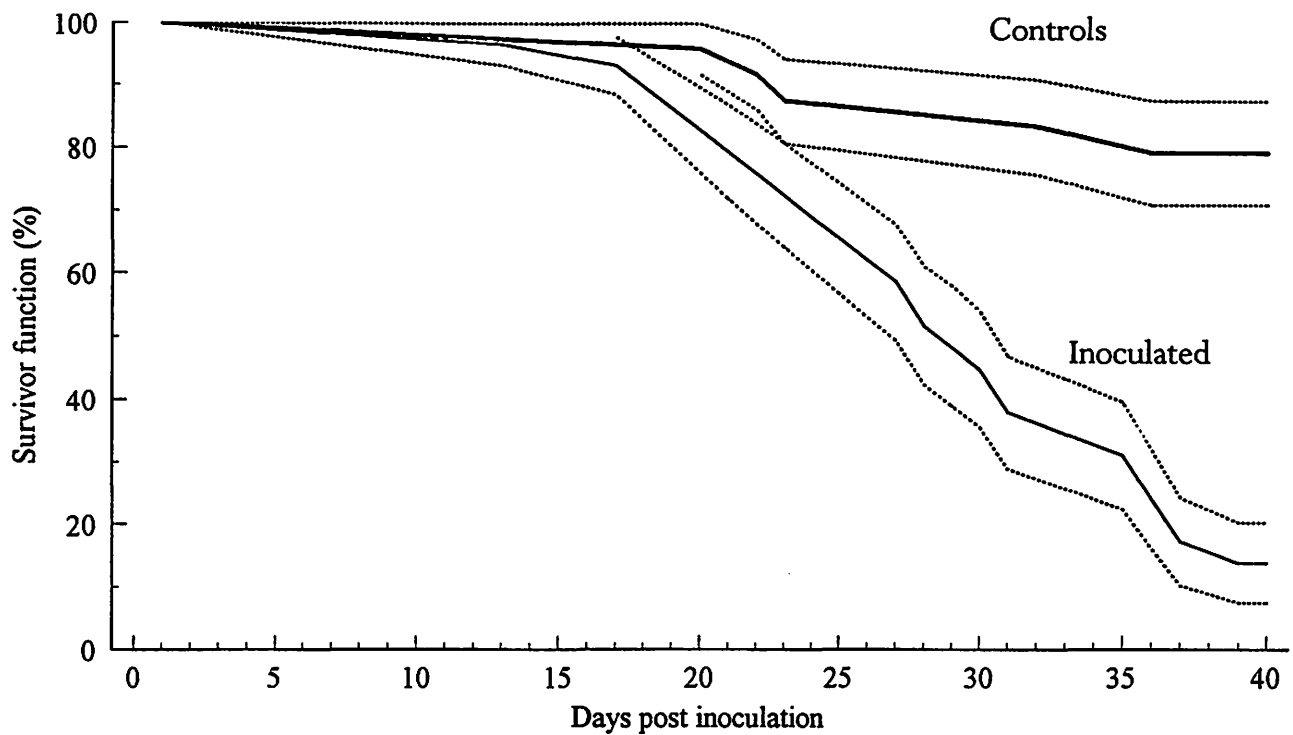
Serum dilution	Rabbit				Sheep				Chicken			
	Uninf	Light	Mod	Heavy	Uninf	Light	Mod	Heavy	Uninf	Light	Mod	Heavy
No dilution	6	5	9	4	15	13	12	12	0	6	1	2
1:1	2	1	6	5	7	1	6	5	12	4	6	10
1:2	5	3	2	2	1	1	2	0	3	2	12	3
1:4	1	3	3	2	0	4	1	0	2	3	3	1
1:8	2	3	3	3	0	2	2	1	1	3	1	1
1:16	3	5	0	2	1	0	0	1	2	4	0	3
1:32	4	2	1	1	0	1	0	3	3	0	0	2
1:64	0	0	0	3	0	0	1	0	1	0	0	0
1:128	0	0	0	0	0	0	0	0	0	0	1	0

Fall 1996



● Reference location

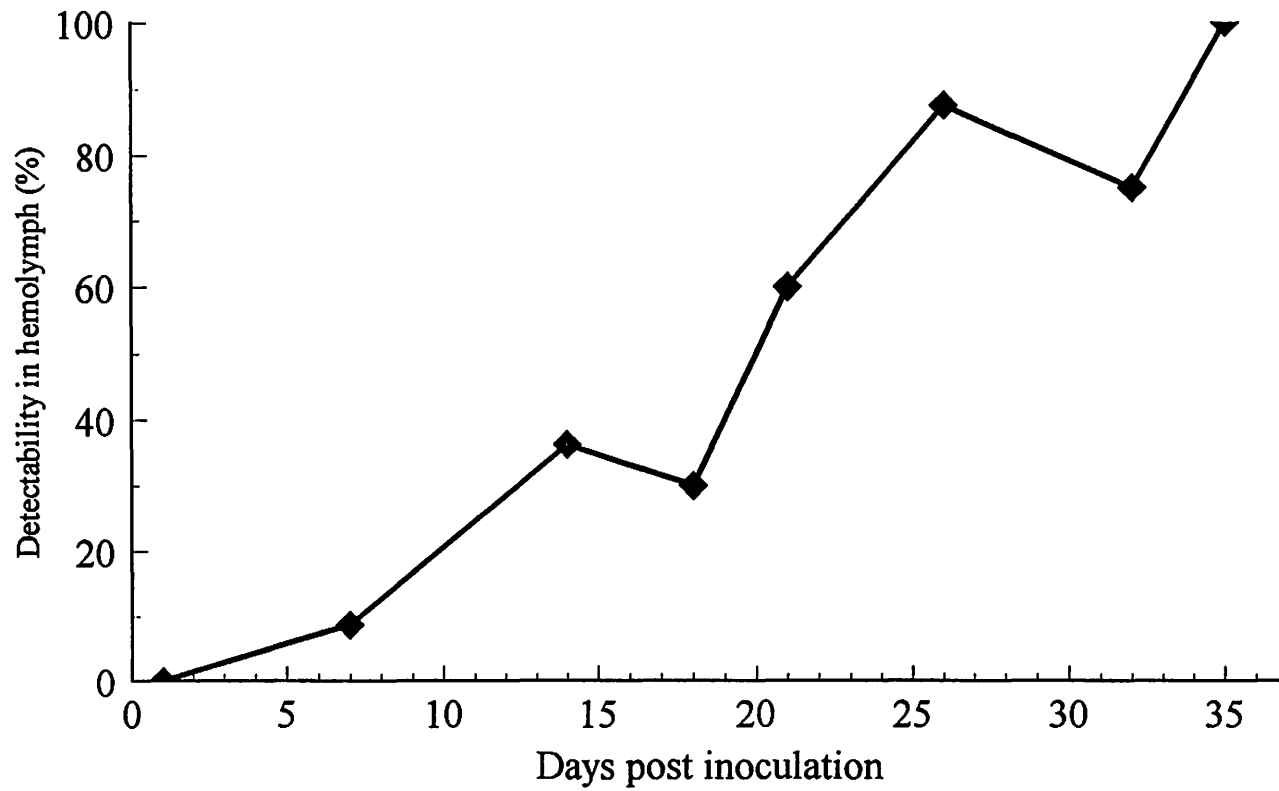
**Figure 1.** Distribution and prevalence of *H. perezi* in blue crabs from Chesapeake Bay, Fall, 1996. Black circles represent locations where the disease was found. Gray dots represent reference locations (Hungars and Red Bank Creeks). Numbers indicate infected crabs versus total number sampled.



VARIABLE	ESTIMATE	Std Error	Chi-Square	Probability
Intercept	2.933	0.106	763.33	0.0001
Uninfected	1.055	0.287	13.50	0.0002
Injected	0	0	.	.
Scale	0.517	0.083		

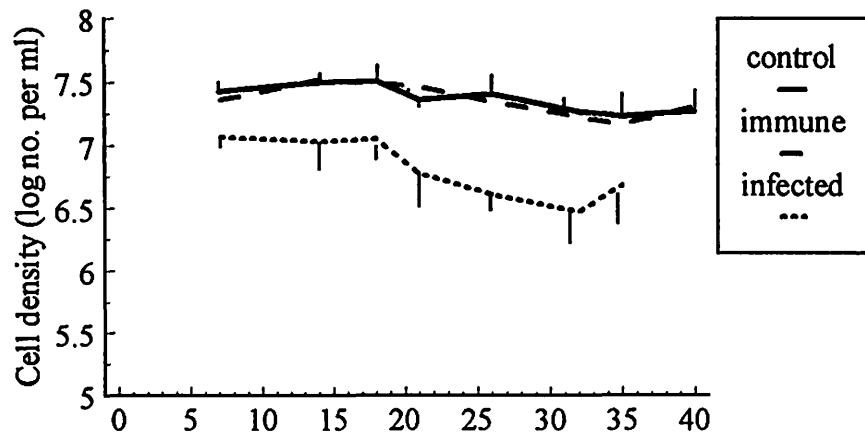
Figure 2. Survival function (Kaplan-Meier estimation) for uninfected crabs and crabs infected with *Hematodinium perezii*. Dashed lines are upper and lower standard errors. Data are grouped from Expts. I and II, and include all infected crabs. Sample sizes are given in Figure 1. Proportional hazards model (table) indicates significant differences in mortality between uninfected and infected crabs (includes a 14 day lag period).



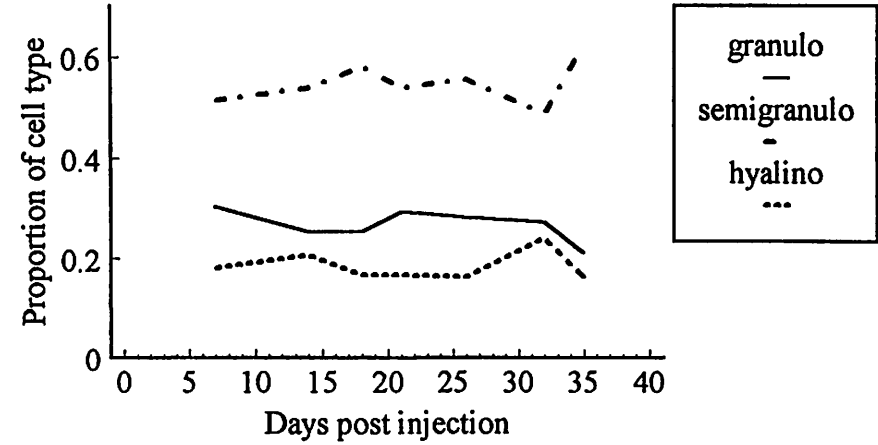


**Figure 3.** Detectability of parasites in hemolymph of infected blue crabs over the course of infection. Data combined from Experiments I and II, includes only infected crabs. Samples sizes were 21, 11, 10, 10, 16, 4, and 4 crabs on Days 7, 14, 18, 21, 26, 32, and 35 respectively.

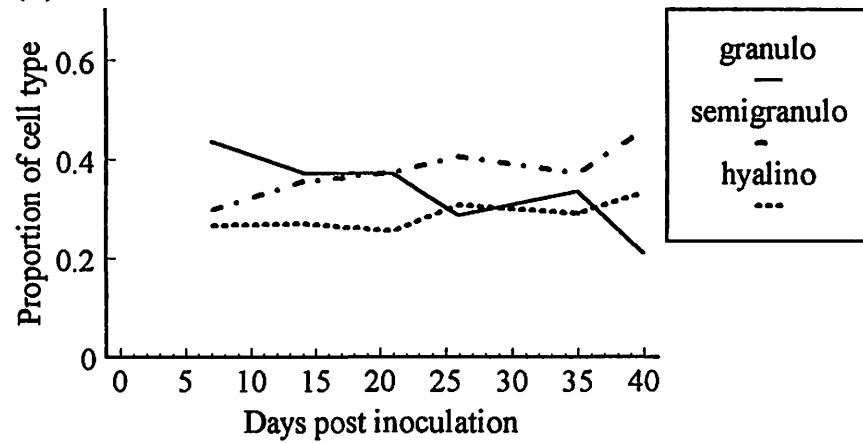
(A) Total hemocyte density



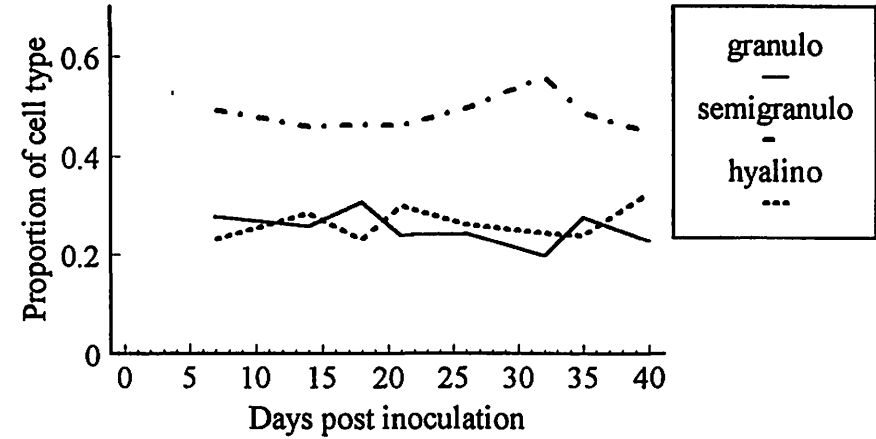
(B) Infected hosts



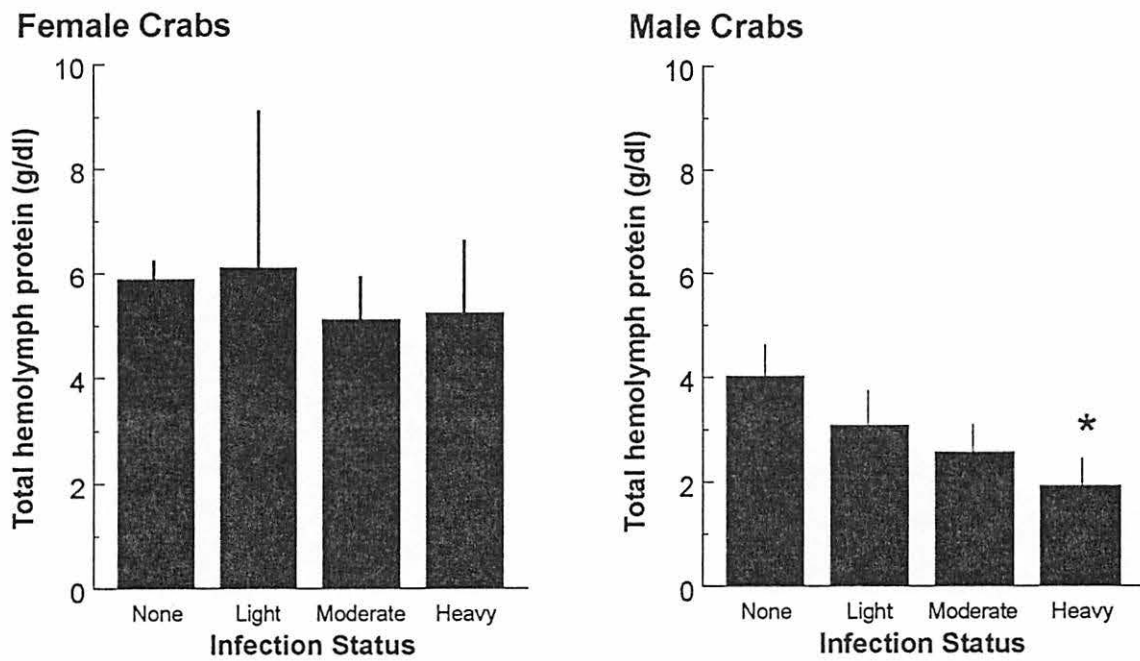
(C) "Immune" hosts



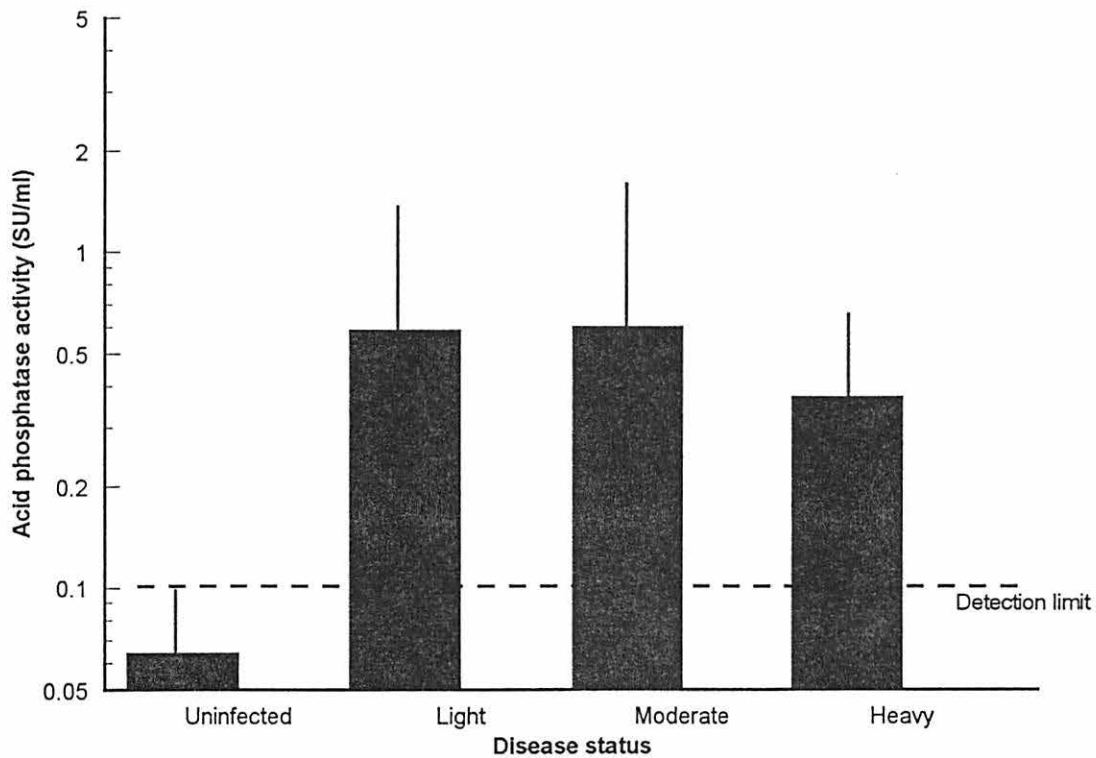
(D) Uninfected hosts



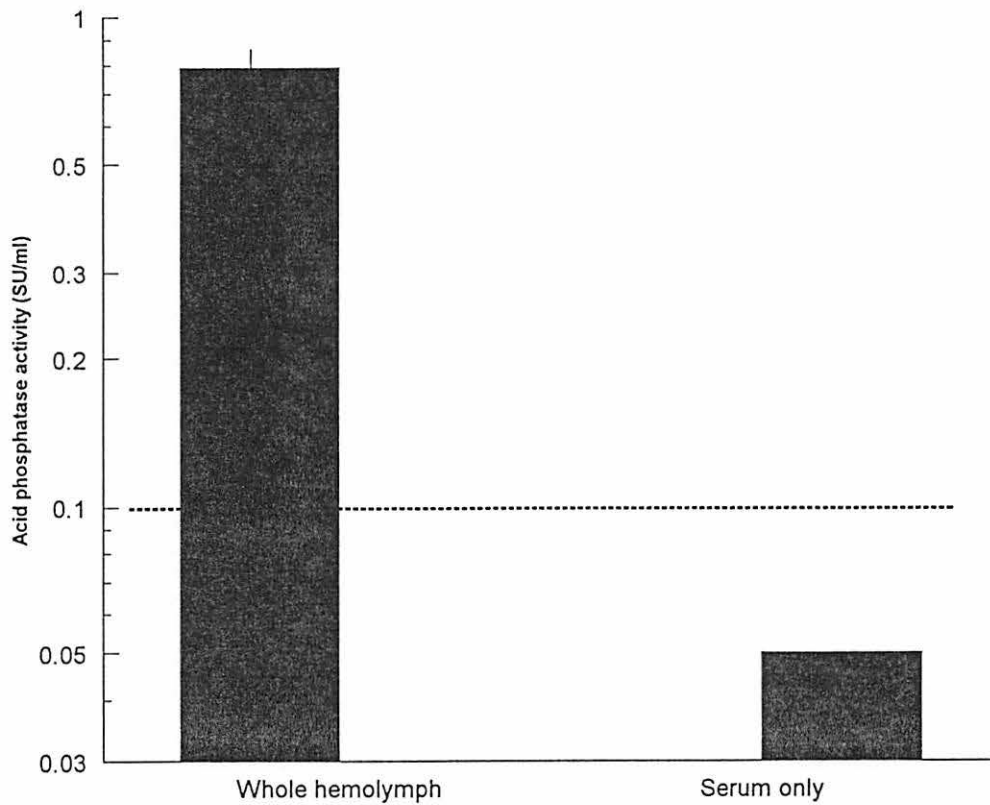
**Figure 4.** Total hemocyte densities and proportions of host cell types in uninfected, infected and "immune" crabs. Data combined from Experiments I and II. Bars = se. Standard error (not shown) for the proportion of host cell types were low, and varied from 0.02 to 0.05. "Immune" crabs were the survivors from Experiment II that never developed infections. Sample sizes given in Table 3.



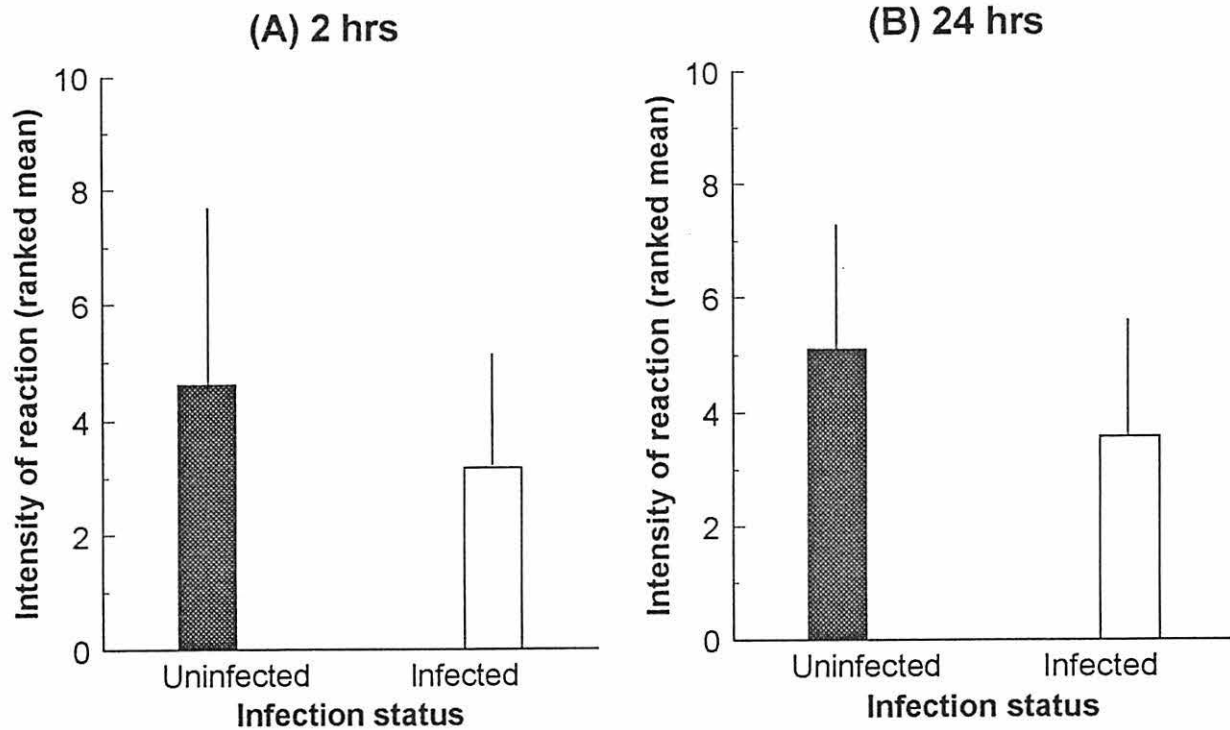
**Figure 5.** Total hemolymph proteins in blue crabs infected with *Hematodinium perezii*. Significant declines in hemolymph proteins were noted for the heavily infected male crabs (\*) (ANOVA,  $p < 0.05$ , Tukey's HSD). Sample sizes were 12 in each category (except for lightly infected females where  $n=6$ ).



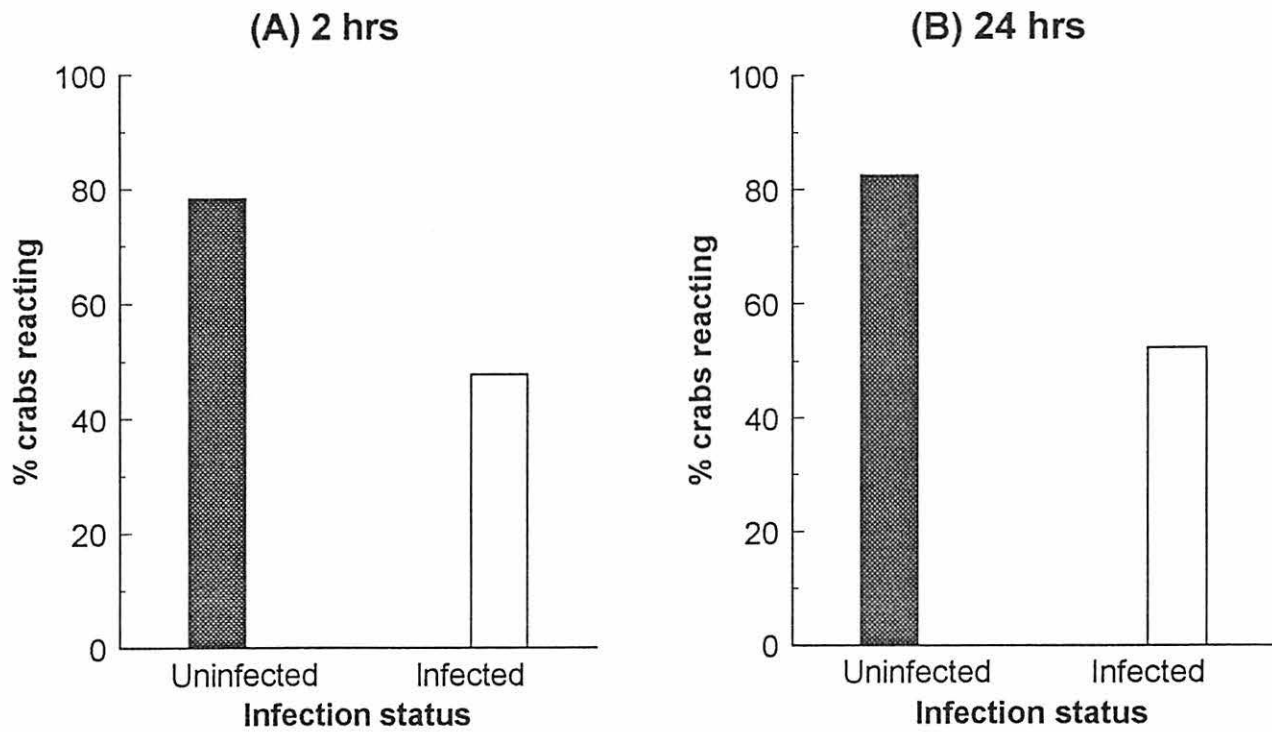
**Figure 6.** Acid phosphatase activity in crabs infected with *Hematodinium perezii*. Infected crabs had significantly higher levels of enzyme activity (ANOVA for log (SU/ml), HSD, d.f.= 3, 50,  $P < 0.001$ ). For statistical analysis, half the value for the minimum detection limit was used for readings below the detection limit. Light infections had intensities of 0.33 to 3.0 parasites/100 host cells; moderate, 3.1-33 parasites/100 host cells; heavy, >33 parasites/100 host cells.



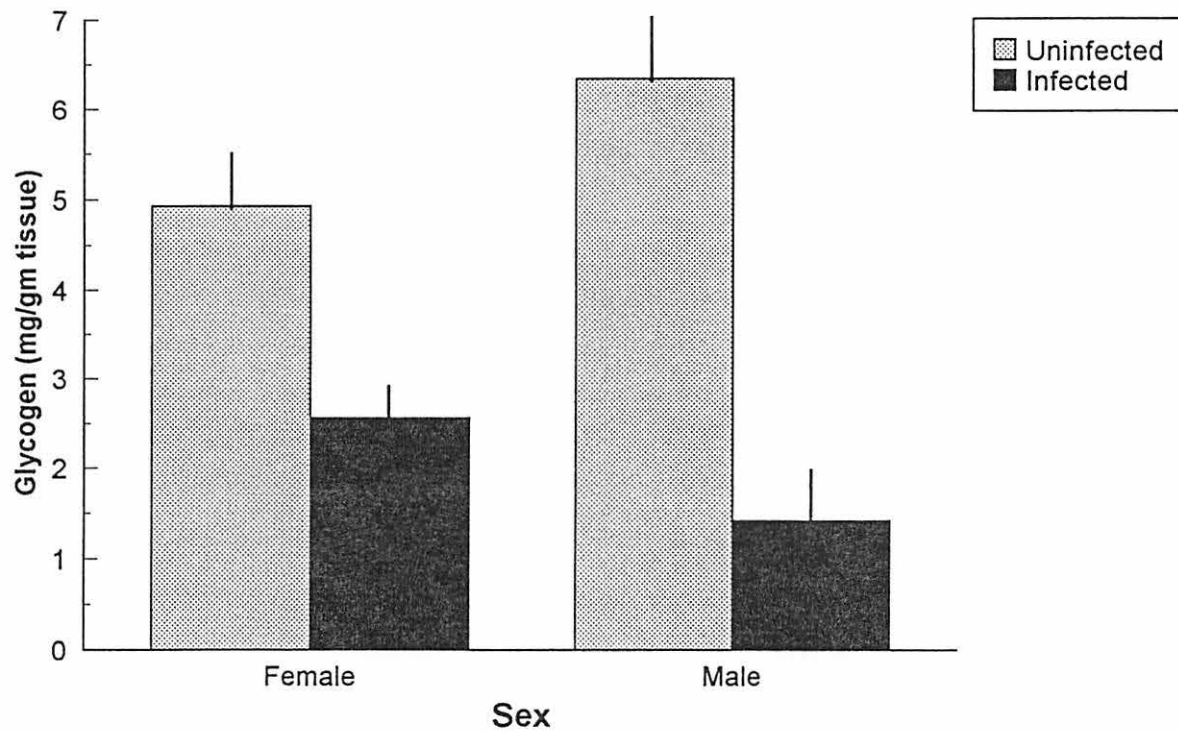
**Figure 7.** Acid phosphatase activity in a moderate infection (crab #2416). The activity is located in the cells, not the serum. Also compare the serum only value with that for the uninfected hosts in Figure 7. The minimum detection limit is shown (dashed line). Both whole hemolymph, and serum only samples were run in triplicate.



**Figure 8.** Intensity of hemagglutination reaction (mean  $\pm$  sd of ranked values) in relation to infection status over time. There were no significant differences between infection categories (t-test, d.f.=17,10,  $t=1.565$ , and  $t=1.669$ , respectively). Data from serial dilutions are typically not normally distributed. The data have been ranked for the test.

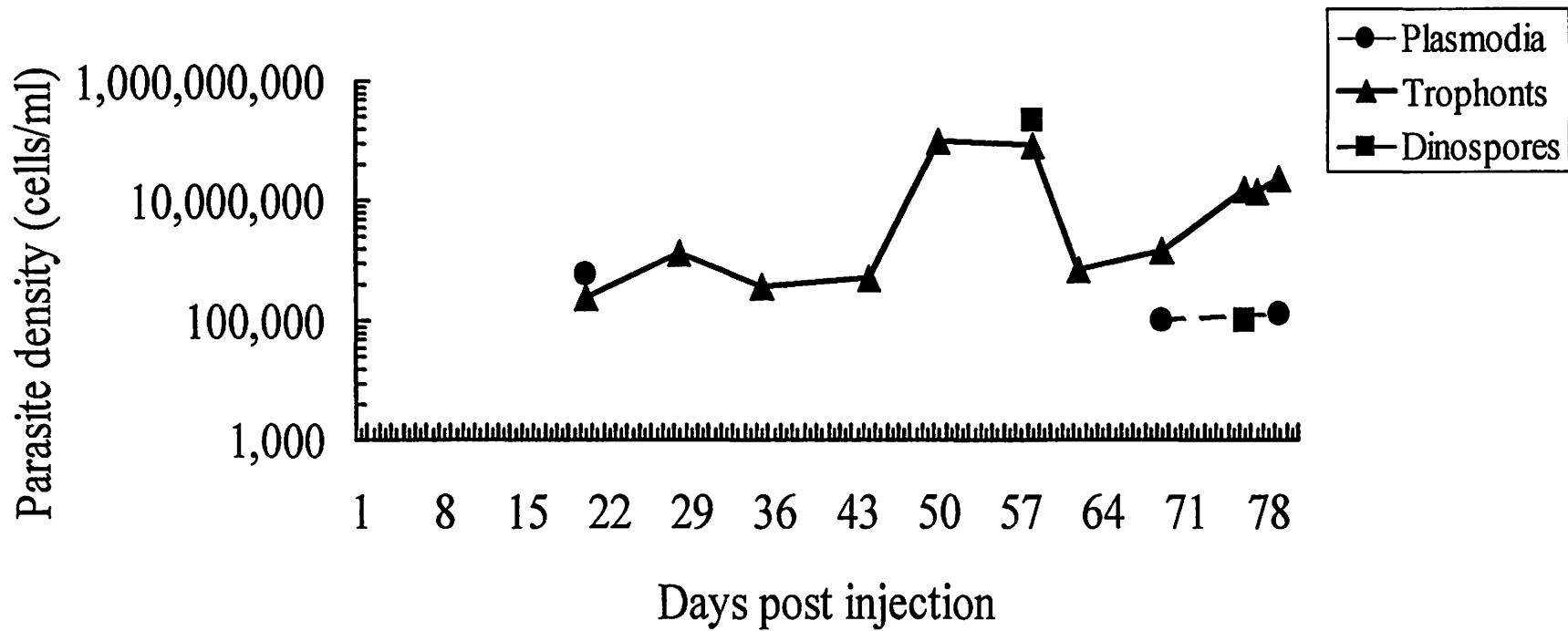


**Figure 9.** Proportion of individual crabs exhibiting hemagglutination in relation to infection status. There were 23 crabs in each treatment. (A) Hemagglutination after 2 hrs (Chi-square=4.572, d.f.=1,  $p < 0.05$ ). (B) Hemagglutination after 24 hrs (Chi-square=4.847, d.f.=1,  $p < 0.05$ ).

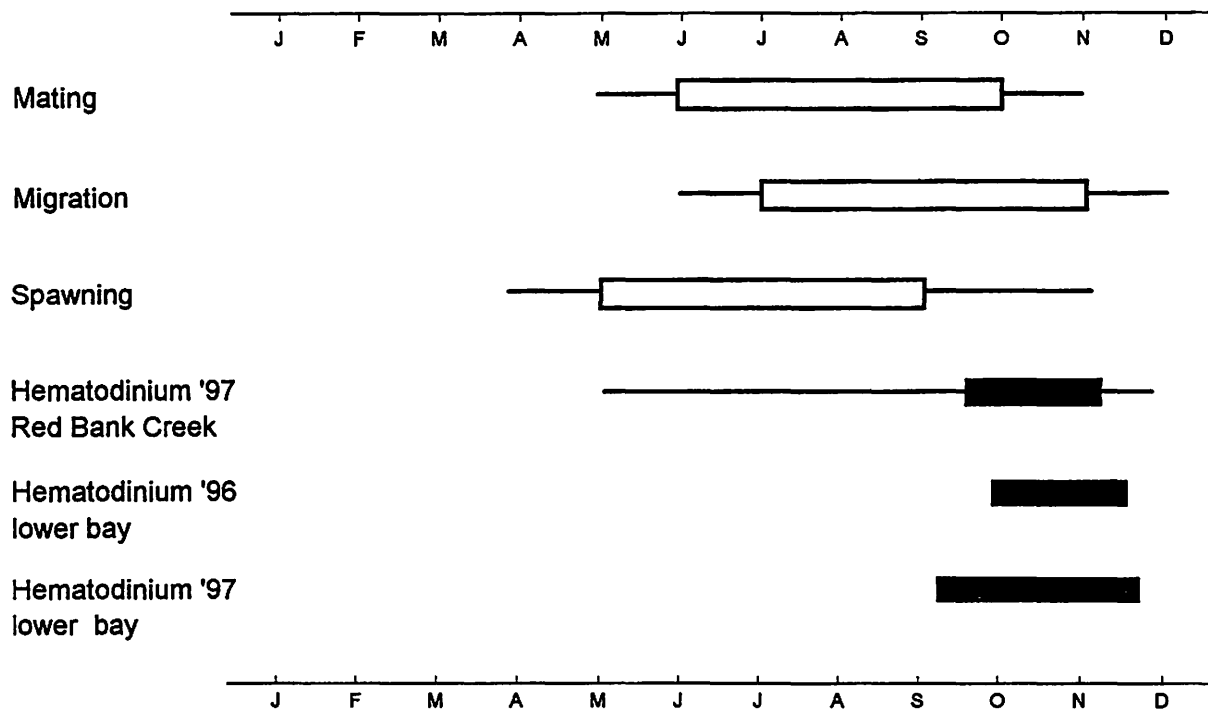


**Figure 10.** Glycogen levels in hepatopancreatic tissues of *Hematodinium*-infected and uninfected blue crabs. Levels were significantly lower for both sexes, with infected males having a significantly greater change than females (2-way ANOVA,  $P < 0.001$ ,  $n = 12, 12, 12$ , and  $9$ , respectively with triplicate sampling for each individual).

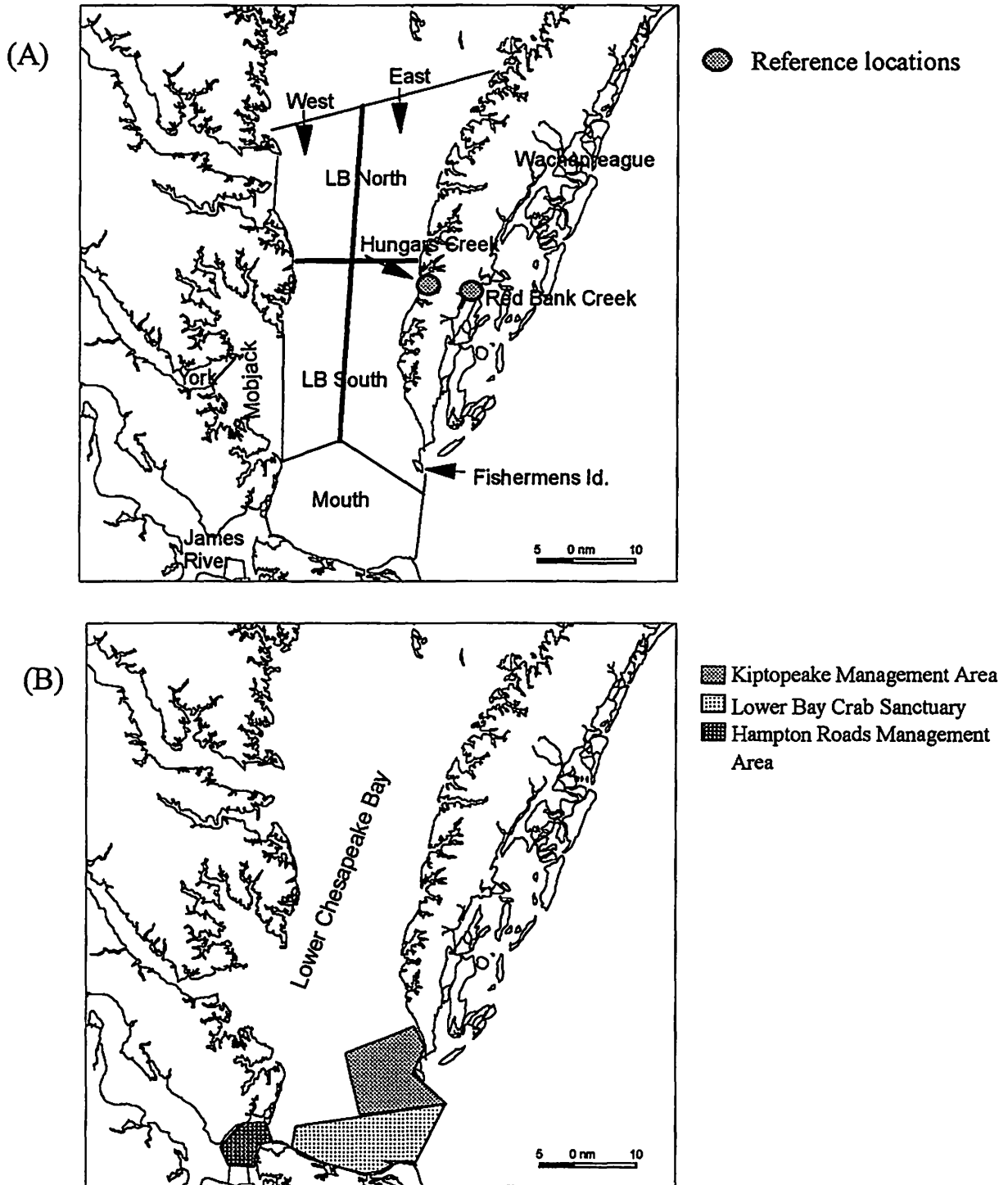




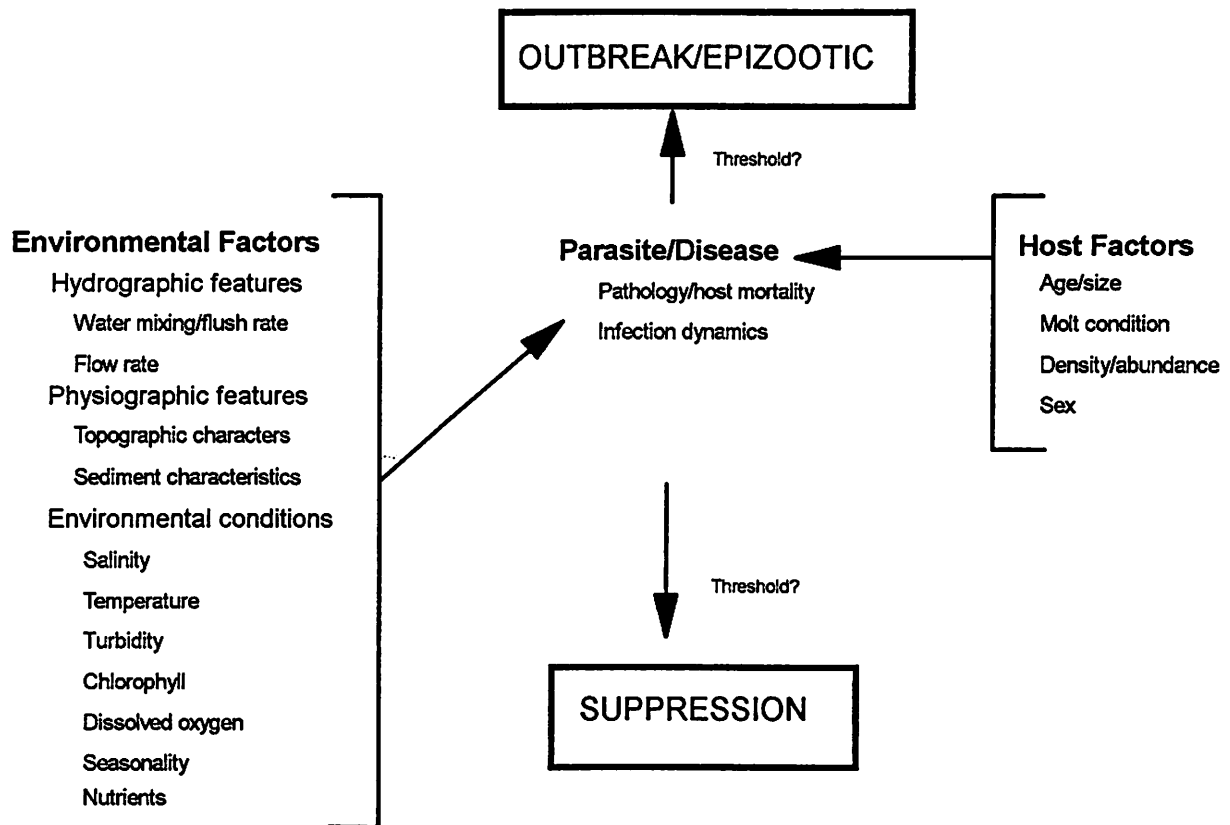
**Figure 11.** Parasite density in a blue crab (#3977) presenting a chronic, natural infection from an enzootic location (Red Bank Creek, VA). Note that the crab did not present an infection on Day 1 (collection date). It was held separately and examined intermittently. The crab died Day 80 post collection.



**Figure 12.** Temporal patterns in various reproductive patterns of female crabs shown with underlying prevalences of *Hematodinium perezii*. Bars represent peak periods of activity; lines, ranges of activity. Red Bank Creek is the "seaside" reference site; lower bay is the mouth, and adjacent strata.



**Figure 13.** (A) Schematic of aggregate locations used in the analysis (cf. strata used by VIMS Trawl Survey). (B) Crab management areas designated by the Virginia Marine Resources Commission. The Kiptopeake and Hampton Roads areas are closed to dredging in winter. The Lower Bay is closed to pot fishing but open to dredging.



**Figure 14.** Conceptual model of identified and potential factors that may contribute to epizootics of *Hematodinium perezii* in blue crabs.

culture.doc

November 18, 1998

**RH: Partial culture and cryopreservation of *Hematodinium perezii***

**PARTIAL CULTURE AND CRYOPRESERVATION OF THE PARASITIC  
DINOFLAGELLATE *HEMATODINIUM PEREZI* FROM THE BLUE CRAB  
*CALLINECTES SAPIDUS***

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Keywords: *Callinectes sapidus*, *Hematodinium perezii*, culture studies, cryopreservation, parasitic dinoflagellate

**Abstract:**

*Hematodinium perezii* is a parasitic dinoflagellate that infects blue crabs along the eastern seaboard of the United States. Recurring epizootics fulminate in late summer and autumn causing mortalities in high salinity estuaries. Currently, the parasite can only be maintained in the laboratory via serial injection. *In vitro* culture and cryopreservation of the pathogen were attempted. Isolates were held in a balanced salts buffer, a common dinoflagellate medium (f/2), and three other media. Temperatures of 4°, 15° and 20° C were examined for the balanced salts medium. The main stages in the life cycle of *H. perezii* were observed, but only partial completion of the life cycle was attained. Under culture conditions, plasmodia developed into schizonts but plasmodial budding and schizogony were not completed. Trophonts (vegetative stages) progressed to highly vacuolated presporonts which rarely sporulated to become dinospores. Aberrant trophonts and plasmodia were enlarged and highly vacuolated. Parasites survived in primary culture for up to 16 d at 4° C, 28 d at 15° C and 7 d at 20° C. Continuous culture was not successful. Naïve crabs acquired infections when inoculated with cultures that had been maintained *in vitro* for 14 d, but those inoculated with reconstituted cryopreserved samples did not acquire infections. Parasites reconstituted from cryopreservation were alive, but did not grow in culture, nor were they infectious. Recovery of live parasites was significantly higher in glycerol than in dimethyl sulfoxide. Successful culture and reconstitution of cryopreserved *H. perezii* from the blue crab will require a better support medium.

## Introduction

*Hematodinium perezii* is a parasitic dinoflagellate that infects several decapod crustaceans along the East Coast of the USA. In the blue crab, *Callinectes sapidus*, the parasite is highly pathogenic and proliferates quickly to kill its host (Shields & Squyers, in review). Heavily infected crabs are lethargic, and frequently have discolored hemolymph (brown, yellow, milky or chalky). The disease occurs primarily in high salinity bays, and has been reported from the New York Bight to the Gulf of Mexico (Newman & Johnson, 1975; Messick & Sinderman, 1992). Epizootics in blue crabs were reported in 1975 from Florida (30% prevalence, Newman & Johnson, 1975), and in 1991 and 1992 from Maryland and Virginia (70 to 100% prevalence, Messick, 1994). In 1996 and 1997, prevalences ranged from 10 to 40% in adult crabs from the eastern portions of Chesapeake Bay in Virginia (Shields, 1997).

*Hematodinium* spp. or *Hematodinium*-like species occur in a variety of different amphipod and decapod hosts (for review see Shields, 1994). At present there are only two described species of *Hematodinium*: *H. perezii* Chatton & Poisson, 1931, and *H. australis* Hudson & Shields, 1994. The parasite in the blue crab is morphologically identical with the type species. They share a motile, elongate, vermiform plasmodium (30-100  $\mu\text{m}$  in length), and the trophont (vegetative stage) is of similar size (9-22  $\mu\text{m}$ ). By convention (e.g., Newman & Johnson, 1975; MacLean & Ruddell, 1978), and from its distinct vermiform plasmodium, we concur that *Hematodinium perezii* sensu Chatton & Poisson, 1931 is the infectious species in the American blue crab.

We have maintained *H. perezii* in blue crabs for over 7 months using weekly serial injections (Shields & Squyers, in review). Such studies require considerable space, time, and logistical

planning. Thus, other methods to maintain and study the parasite would be useful. Culture attempts have recently succeeded with a related "species" of *Hematodinium*. Appleton & Vickerman (1998) used a balanced salts solution augmented with fetal bovine serum (FBS) to culture *Hematodinium* sp. from the Norway lobster, *Nephrops norvegicus*. They report several new aspects of the life cycle of the parasite, including an unusual slime-mold-like plasmodial stage (arachnoid trophont), and temperature-induced sporulation (<8° C). Previously, a partial progression of the life cycle of *Hematodinium* sp. from the Tanner crab, *Chionoecetes bairdi*, was observed using filtered host hemolymph (Meyers et al., 1987), and balanced salts augmented with fetal bovine serum (Morado, F., pers. comm.). We attempted to culture *H. perezi* in several different culture media. In addition, we tested cryopreservation of the dinoflagellate for long term storage. The second objective required trials with differing types and concentrations of cryopreservants. Lastly, we tested primary cultures and cryopreserved samples for infectivity.

### **Materials and methods**

Blue crabs were collected from the Chesapeake Bay and several of its subestuaries by the VIMS Trawl Survey using a 30 ft (9.144m) semi-balloon otter trawl (Marinivich Gulf shrimp trawl) with 1.5" (38.1mm) stretch mesh body, 0.75" (19.05mm) cod-end, and a 0.25" (6.35mm) mesh cod end liner with attached tickler chain (3/8" link). Crabs were also collected using commercial crab pots from two reference locations on the Delmarva Peninsula: Red Bank and Hungars Creeks, Virginia. During the experiments, crabs were fed fish and squid semiweekly and held individually in aquaria (5 gal., 19 L) at 20° to 21° C, and 24 ppt salinity.



*Hematodinium perezii* was maintained in the laboratory by serial passage of infected hemolymph. Naturally infected and inoculated crabs were housed separately and used as hemolymph donors to inoculate other crabs ( $10^5$  -  $10^6$  parasites per host; Shields & Squyars in review). Hemolymph was drawn with a tuberculin syringe (1 mL) and a 25½ ga. needle from the arthroal membrane at the juncture of the basis and the ischium of the 5th pereopod (swimming leg). Parasite density (cells mL<sup>-1</sup> hemolymph) was estimated with a hemocytometer (Neubauer improved, Bright Line). Densities higher than  $1.0 \times 10^7$  cells mL<sup>-1</sup> were diluted 1:5 with buffer (see below) and recounted. Permanent preparations of hemolymph were made by smearing hemolymph onto acid-cleaned, poly-L-lysine-coated microslides, allowing to gel for 2-3 minutes, then placing in Bouin's fixative (after Messick, 1994). The smears were processed through a routine Harris hematoxylin and eosin-Y procedure (Humason, 1979, p. 123 without acid destain).

### Culture methods

The primary culture medium (FMAM) consisted of 10% fetal bovine serum (FBS) in a physiological saline buffer (NaCl, 19.31 g/l; KCl 0.65 g/l; CaCl<sub>2</sub>•2H<sub>2</sub>O 1.38 g/l; MgSO<sub>4</sub>•7H<sub>2</sub>O 1.73 g/l; Na<sub>2</sub>SO<sub>4</sub> 0.38 g/l; HEPES 0.82 g/l;) adjusted to pH 7.8, with added glucose (1.0 mg/mL) (modified from Appleton and Vickerman, 1998). Penicillin (500mg/l) and streptomycin (500mg/l) were added to hinder bacterial growth. The FMAM was filter-sterilized at 0.2 um, and distributed in 10 ml aliquots into 25 or 50 ml culture flasks using sterile technique. For primary isolations, ten flasks were prepared for each crab. For later subcultures, five flasks were used. For most isolations ( $\leq 10^6$  parasites mL<sup>-1</sup>) and subcultures, a ratio of 9.0 ml FMAM to 1.0 ml infected hemolymph was used.. Heavily infected hemolymph ( $\geq 10^7$  parasites mL<sup>-1</sup>) required a ratio of 10.0 ml FMAM to 0.1 ml hemolymph. Densely packed isolates were subcultured into fresh media.

Uninfected hemolymph served as controls. Cultures were maintained at 15° C, but some were held at 4° C or 20° C. Cultures were observed regularly (every one to three days) with a Zeiss inverted microscope at 400x, and subjectively judged as exhausted or effete when cell death was high.

Five other culture media were examined albeit less rigorously: "f/2" made to 24 ppt. with artificial seawater (Forty Fathoms), basal salts (Sigma G1775) and a 0.5% vitamin solution (Sigma G1525) (Guillard and Ryther, 1962), Shields and Sang Insect Medium (Sigma S3652) with and without 10% FBS and 0.5% vitamin solution (Sigma G1525), artificial seawater (Forty Fathoms), and *Perkinsus marinus* BSA-free medium (LaPeyre and Faisal, 1997). The five media were inoculated and incubated at 15°C.

An injection experiment was performed to test the infectivity of *H. perezii* from FMAM culture. Four uninfected crabs were injected with 0.1 ml each of a primary culture that had been incubating at 15° C for 14 d. Four different crabs were injected with sterile culture medium to serve as a control. After two and three weeks the crabs were assessed for infections as described above.

### Cryopreservation

The efficacy of two cryopreservants, dimethyl sulfoxide (DMSO) and glycerol, were examined. Support media consisted of FMAM supplemented with either 5%, 10%, or 15% of either DMSO (Fisher D128) or glycerol (Fisher G33). Aliquots of 0.1 mL infected hemolymph (containing at least 10<sup>6</sup> parasites mL) were gently mixed with aliquots of 0.4 mL cryopreservant-buffer. Six to

nine samples were prepared from each infected crab at each concentration of cryopreservant. The samples were placed on ice in a foam container in a -20° C freezer for one hour, transferred from the container directly to -20 C for another hour, then moved to a -80° C freezer for one to twelve hours before being stored above liquid nitrogen for long term storage.

To assess viability of cryopreserved cells, the samples were thawed quickly in a water bath at room temperature (about 23° C). Once thawed, the density of intact cells was immediately assessed using a hemocytometer. Neutral red and trypan blue preparations (0.5%, and 0.25% in buffer solutions, respectively) were used to examine cell viability using a light microscope at 400x. Three replicate samples were thawed and assessed at a time. Depending on cell viability, the samples were either cultured in FMAM or *f*/2 media, or injected directly into crabs for infection trials. The percentage of live and dead parasites was calculated as the estimated density of parasites (live and dead) recovered divided by the estimated density in the original sample. The percentage of live parasites was calculated as the percentage of live parasites recovered from the total number of live and dead parasites in the reconstituted sample. Two-way ANOVA was used to examine differences between cell viability and concentration of cryopreservant (SYSTAT, Wilkinson, 1997). A probability level of  $p < 0.05$  was accepted as significant.

Prior to infection trials with cryopreserved samples, controls were established using parasites suspended in cryopreservant-modified FMAM. Initial controls consisted of examining cell viability in the different media (DMSO, glycerol) after 10, 15, and 30 min. None of the samples showed significant mortality with either cryopreservant. Four uninfected crabs were then inoculated with a fresh preparation (not frozen) of  $10^5$  parasites suspended in FMAM modified

with 10% DMSO. The parallel control with glycerol was not done. For the infection trials, 49 crabs were injected with 0.1 mL reconstituted sample (DMSO or glycerol). After 2 to 3 weeks, inoculated crabs were assessed for infection.

## Results

### Culture attempts

In culture, the life cycle of *H. perezii* was observed to progress from trophonts to presporonts and sporonts, then to dinospores (Figs. 1, 2). Plasmodia progressed to schizonts but division to the trophont stage was not successful. In some cases, presumptive trophonts developed into motile plasmodia 3 to 10 d after primary isolation (Tables 1, 2). A few cultures developed dinospores within the first 3 to 10 days of culture (Tables 1, 2). Dinospores freshly isolated from one crab survived for 10 d (#4611, Table 1). A presumptive cyst stage was never observed. Development and support of the parasite was not robust at temperatures of 4° C and 20° C (Table 2). Limited growth and development of the parasite was best observed at 15° C. Sporulation to dinospore stages occurred at both 15° C and 20° C.

Aberrant plasmodia and trophonts occurred in the FMAM and *f*/2 culture media. Aberrant forms were characterized by a larger size, increased vacuolization, and swollen appearance (Fig. 1e, f; Table 3); they frequently developed shortly before the cultures became exhausted.

The average duration of the primary cultures was approximately 20 d (Table 1). While proliferative growth was not evident, cells remained viable for up to 28 d. Subculturing did not extend the longevity of the cultures. Trophonts remained circular or ovoid during incubation

(Figs. 1, 2), but host hemocytes aggregated freely and flattened in large sheets on the culture flasks. In most cases, host hemocytes were well supported by FMAM; hemocytes survived for 21-24 d with a few living up to 30 d.

Two crabs in the culture inoculation trial died from bacterial infections. The remaining two crabs, inoculated with 14 day-old cultures (ex Crab 2 sub, Table 1), developed *H. perezii* infections that were evident after 3 weeks. None of the controls developed infections.

### Cryopreservation

Glycerol was a better cryopreservant than DMSO (Figs. 3, 4). The percentage of living and dead parasites recovered showed a higher trend in glycerol treatments (25-60% recovery), but the trend was not significant between treatments (vs. 20-35%;  $P=0.052$ ,  $P=0.056$ ,  $df=1,83$ ). The percentage of live parasites was, however, significantly higher in glycerol treatments compared to those in DMSO ( $P=0.009$ ), with a higher trend in recovery for parasites held in 15% glycerol (Fig. 4;  $P=0.074$ ,  $df=1,83$ ). Host cell mortality appeared to be unaffected by the type or concentration of cryopreservant (Figure 5;  $p=0.643$ ,  $p=0.285$ ,  $df=1,83$ , respectively).

Cryopreservation was unsuccessful as a storage medium using these methods. While living parasites were observed in cryopreserved samples, none of the cryopreserved parasites proliferated in the culture media. Cells which initially appeared healthy were unable to develop (e.g., Crab EJ, Table 1). Cultures were given sufficient time to recover, but parasites exposed to cryopreservation did not recover and did not progress to other life history stages.

The four control crabs inoculated with fresh, unfrozen parasites suspended in FMAM supplemented with 10% DMSO developed *H. perezii* infections. Thus, DMSO was not toxic to the parasites. None of the 49 crabs injected with the reconstituted *H. perezii* developed infections.

## Discussion

Sustained, proliferative growth of *H. perezii* was not achieved in this study. The culture media lacked an essential ingredient(s) for sustained growth of the dinoflagellate. Partial progression through different stages did, however, confirm and extend aspects of the life cycle of the parasite (Fig. 6). The multinucleate plasmodium reproduces via budding and progresses via schizogony (schizont) to produce the vegetative trophont. Amoeboid trophonts undergo successive rounds of merogony (Shields, pers. obs.) to eventually give rise to prespores (rounded trophonts) or sporonts (Fig. 2d) that undergo a rapid division to produce dinospores. Sporulation is rapid and occurs over two to four days in blue crabs (Shields & Squyars, in review); the ephemeral sporont may develop over an ever shorter time as it is rarely observed in prepared smears ("tetrad" stage, Messick, G., & Shields, J., pers. obs.).

Appleton and Vickerman (1998) achieved the continuous culture of *Hematodinium sp.* from the Norway lobster at a range from 6-10° C. The life cycle of *Hematodinium sp.* from the Norway lobster is far different than that reported for other parasitic dinoflagellates and consists of filamentous trophonts (cf. the plasmodium of Chatton & Poisson, 1931) developing into unusual colonies of plasmodia called "gorgonlocks", followed by arachnoid trophonts (filamentous forms), clump colonies, arachnoid sporonts, sporoblasts, and finally dinospores (Appleton & Vickerman,

1998). The partial life cycle of *Hematodinium* sp. from the tanner crab consists of an ovoid plasmodium that produces vegetative cells that later develop into prespores and dinospores (Meyer et al., 1987, 1990) much like that observed for *H. perezi* from the blue crab. Small, vermiform plasmodia do, however, occur in the hemolymph of infected snow crabs from Newfoundland (Shields, pers. obs.).

Cultures inoculated with presumptive trophonts of *H. perezi* occasionally developed plasmodial stages. Some blue crabs injected with only presumptive trophonts have been observed with motile vermiform plasmodia after 3 to 4 weeks of infection (Shields & Squyars, in review). Reversion to an earlier life history stage, completion of the life cycle via auto-infection, or the presence of small, undifferentiated plasmodia may explain these findings. Auto-infection (in this case completed life cycle) is unlikely in observations of cultures. Injection experiments also show that trophonts are capable of establishing infections through sustained merogony (Shields and Squyars, in review). In addition, plasmodial budding, wherein a plasmodium buds to produce further plasmodia, has been observed in *H. perezi* (cf. Fig. 1e; Shields pers. obs.). The "gorgonlocks" of *Hematodinium* sp. from the Norway Lobster resembles the arrested plasmodial budding that was observed in the present study. The development of the arachnoidal sporonts that occur in the *Hematodinium* sp. from the Norway Lobster (Field & Appleton, 1995; Appleton & Vickerman, 1998) has yet to be observed in natural infections or laboratory inoculations of *H. perezi* in blue crabs.

Primary cultures of *H. perezi* remained infectious for 2 weeks. Thus, short-term culture may be useful in studying other aspects of the parasite (e.g., release of proteases, extracellular

mechanisms of pathology). Interestingly, sustained cultures of *Hematodinium* sp. from the Norway lobster are not infectious (Appleton & Vickerman, 1998) and suggests a loss of infectivity or viability in cultured parasites. Vegetative stages (trophonts) from primary cultures (using sterile hemolymph as the medium) were successful in establishing infections in Tanner crabs, but inoculation with sporulating prespores did not produce infections (Meyers et al., 1987). *Hematodinium* spp. have been transmitted via injection into Tanner crabs (Meyers et al., 1987), blue crabs (Shields & Squyars, in review), and Australian sand crabs (*P. pelagicus*) (Hudson & Shields, 1994). Short-term culture may facilitate such infection studies by providing comparisons between succeeding stages, and as a stop-gap source of material during poor or limited collection periods.

Sporulation from the trophont stage to the dinospore stage was observed in several cultures of *H. perezi*, but it was not synchronous, nor abundant. Sporulation *in vivo* appears synchronous and can occur at least two or three times during an infection, with each event lasting from 2 to 4 days (Shields & Squyars, in review). Dinospore density can be extraordinarily high ( $1.6 \times 10^8$  dinospores mL<sup>-1</sup>) during sporulation; levels far higher for example than those reported for harmful algal blooms. However, transmission via dinospores with any species or form of *Hematodinium* has yet to be achieved.

Other dinoflagellates have been effectively cryopreserved with DMSO and glycerol.

*Amphidinium carteri* (ATCC 30829) is stored and reconstituted from 9% DMSO, while *Cryptocodinium cohnii* (e.g. ATCC 30342) is stored and reconstituted from 7.5% glycerol (Simione & Daggett, 1977; Nerad, 1991). Glycerol was the most effective for *H. perezi* with



DMSO being a poor cryopreservant. Host hemocytes did not preserve well, a fact that makes the cryopreservation process appealing in that the majority of cells being preserved are parasites. Several factors can affect viability of cryopreserved cells (Nerad, 1991). With some species, temperature control must be precise. Cells may be sensitive to the cryopreservants, or components of the medium. They may be weakened and damaged by the freezing process so that infection capability is limited. While temperature control was not ideal during cryopreservation of *H. perezii*, many parasites survived the process. Indeed, several species of microalgae have been frozen and thawed using uncontrolled freezing techniques (e.g., Simione & Daggett, 1977; Canavate & Lubian, 1995, 1997). Finding a suitable support medium for culture may help to revitalize fragile parasites after reconstitution.

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Table 1. Longevity of representative cultures (as days in culture) and various stages of *H. perezii* in FMAM held at 15° C.

Crab I.D.	Crab 1	Crab 2	Crab 2 <sup>1</sup> sub	Crab 2 <sup>2</sup> sub 2	Crab EJ <sup>3</sup>	Crab 4	Crab 5	4611 <sup>4</sup>	Crab 7
Trophont	16	28	20	14	23	26	5	0	14
Plamodia	0	15	7	0	0	6	0	0	0
Dinospore	0	4	0	5	0	0	0	10	0

<sup>1</sup> Subcultured once after 8 days.

<sup>2</sup> Subcultured twice. Once after 8 days, and again after 14 days.

<sup>3</sup> Reconstituted cells from 10% DMSO in FMAM

<sup>4</sup> Crab naturally infected, undergoing sporulation at time of culture.

**Table 2.** Longevity (as days in culture) of *H. perezii* in FMAM at different temperatures.

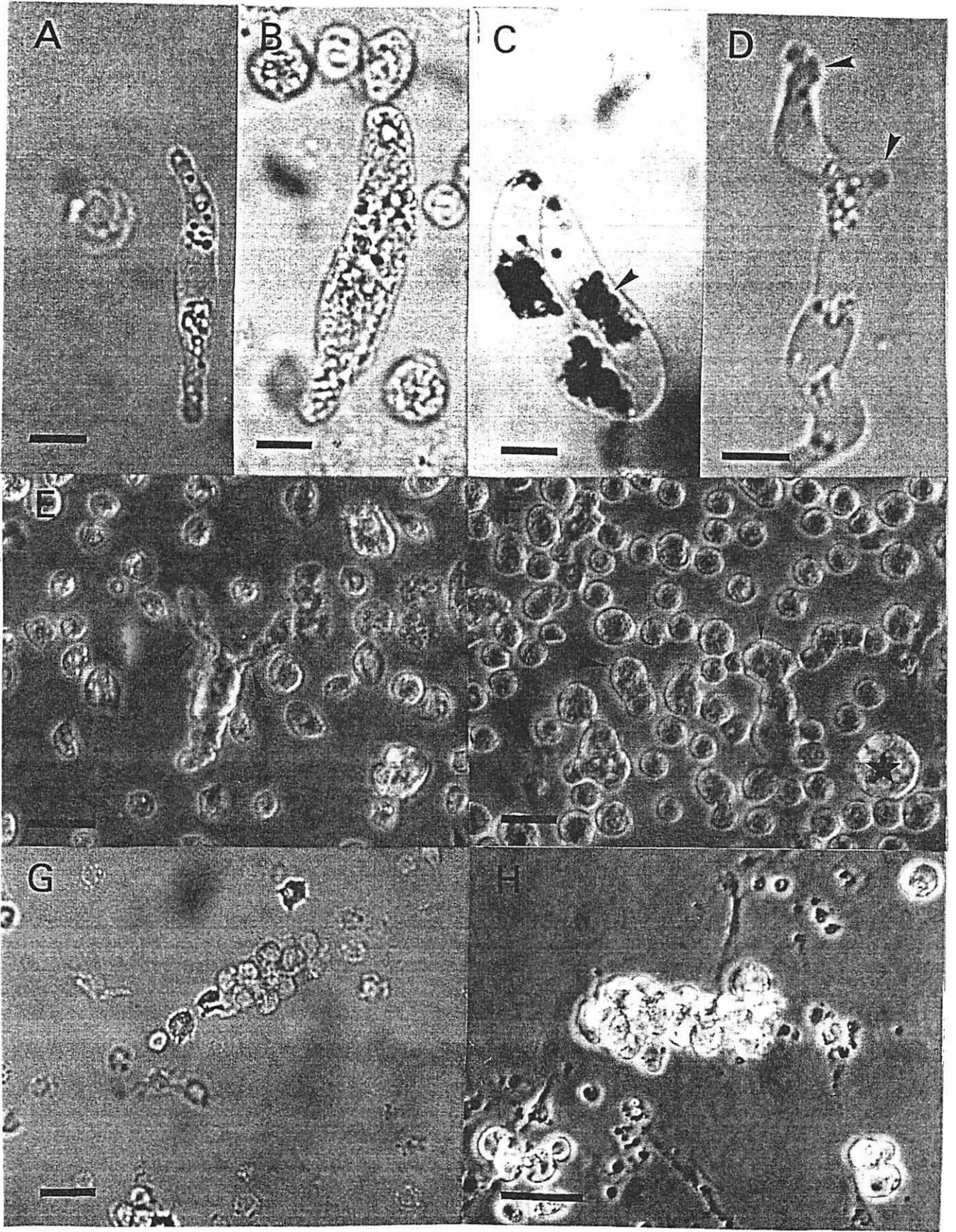
	4° C		15° C		20° C	
	C2	MT 26	C2	MT 26	C2	MT 26
Trophont	16	16	20	20	7	3
Plasmodia	3	0	2	0	3	0
Dinospore	0	0	0	0	3	0

Table 3. Lengths (in micrometers) of different life history stages of *H. perezi* observed in crab hemolymph (in vivo) and in culture (in vitro).

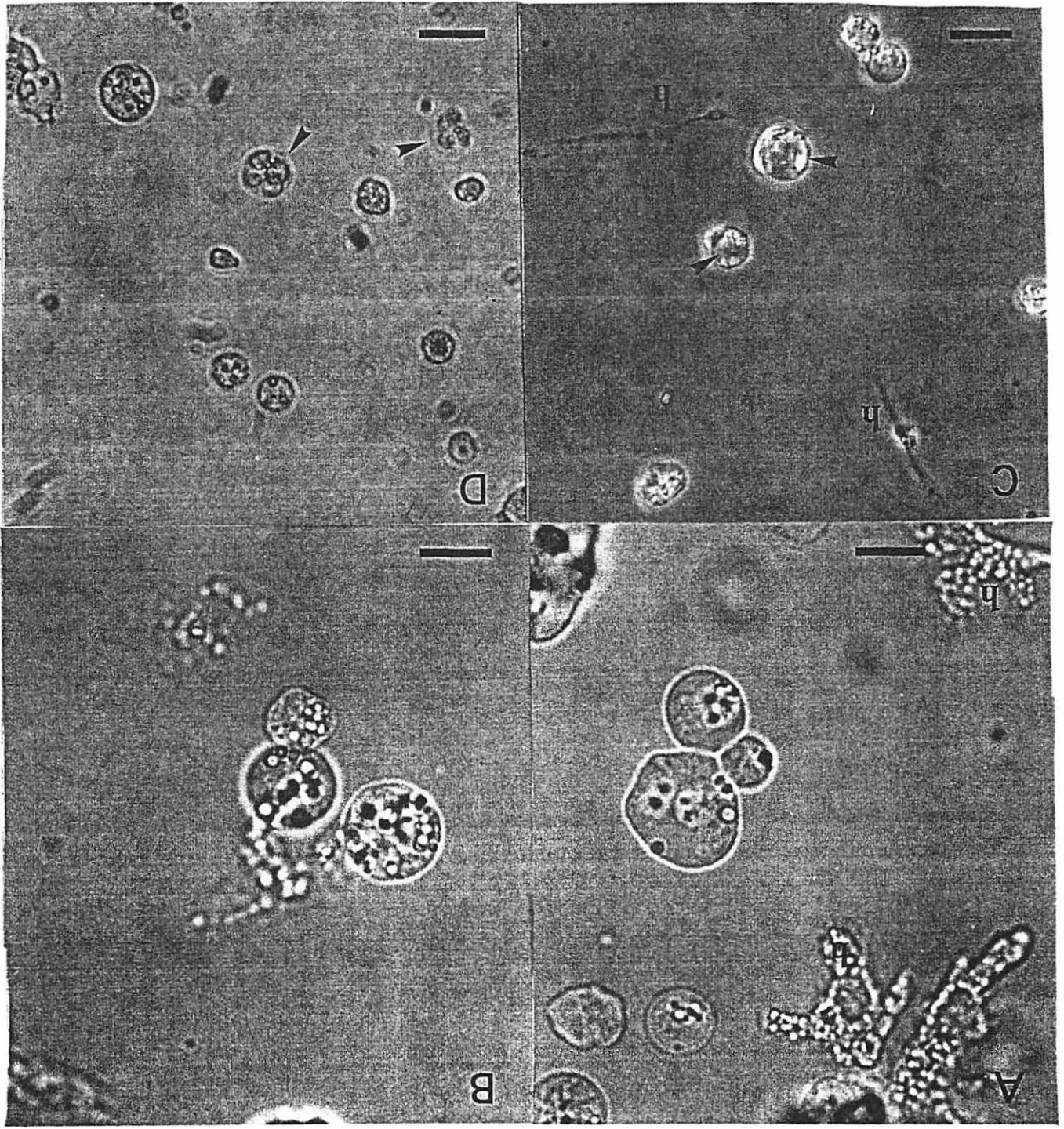
Stage	<i>in vivo</i>			<i>in vitro</i>		
	Mean	Range	SD	Mean	Range	SD
Trophont	13	9 - 22	3	13	6 - 15	3
Aberrant Trophont	--	--	--	27	18 - 83	10
Plasmodium	46	18 - 95	21	84	24 - 443	81
Dinospore	6	4 - 9	2	6	4 - 9	2

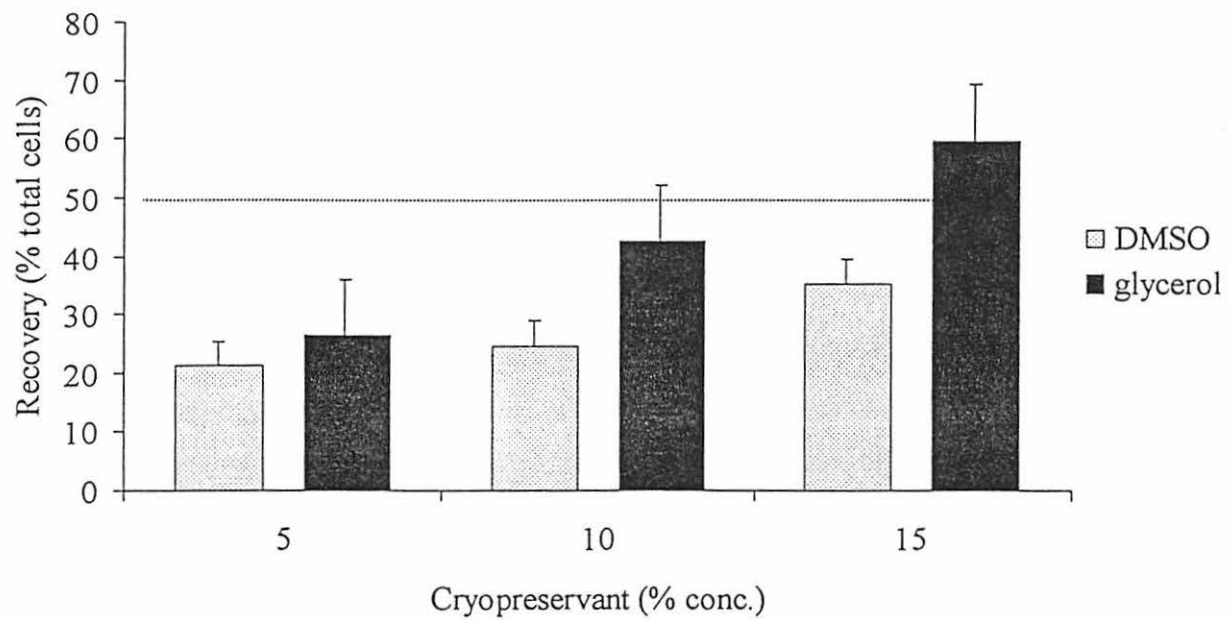
Figure 1. Natural and cultured plasmodial stages of *Hematodinium perezii* from the blue crab, *Callinectes sapidus*. (A-D) Vermiform plasmodia in hemolymph. Bar = 10  $\mu$ m. (C) Vacuoles filled with products of neutral red uptake (arrow). (D) Remnants of budding (arrows). (E) Plasmodium in culture exhibiting enlarged size and arrested budding (arrow). Bar = 20  $\mu$ m, phase contrast. (F) Parasites in culture showing enlarged plasmodia (arrows) and aberrant trophont (star). Note that other trophonts have not enlarged. Bar = 30  $\mu$ m, phase contrast (G) Plasmodium in hemolymph undergoing schizogony (schizont stage). Bar = 20  $\mu$ m. (H). Plasmodium from culture in arrested schizogony. Bar = 20  $\mu$ m, phase contrast.

Figure 2. Natural and cultured trophont (vegetative) stages of *Hematodinium perezii* from the blue crab, *Callinectes sapidus*. (A) Amoeboid trophont from hemolymph. Host granulocytes (h) have many more granules. Note the range in sizes. Bar = 10  $\mu$ m. (B) Round trophont from hemolymph. Note the larger number of refractile granules. This stage occurs later than the amoeboid form and may represent a developing sporont. Bar = 10  $\mu$ m. (C) Trophont in culture showing increased vacuolization (arrow). Hemocytes (h) have spread on the surface of the culture plate. Bar = 20  $\mu$ m, phase contrast. (D) Presumptive sporont undergoing sporogony (arrow) in culture. This stage is rarely observed in hemolymph, suggesting that it develops rapidly. Bar = 20  $\mu$ m.

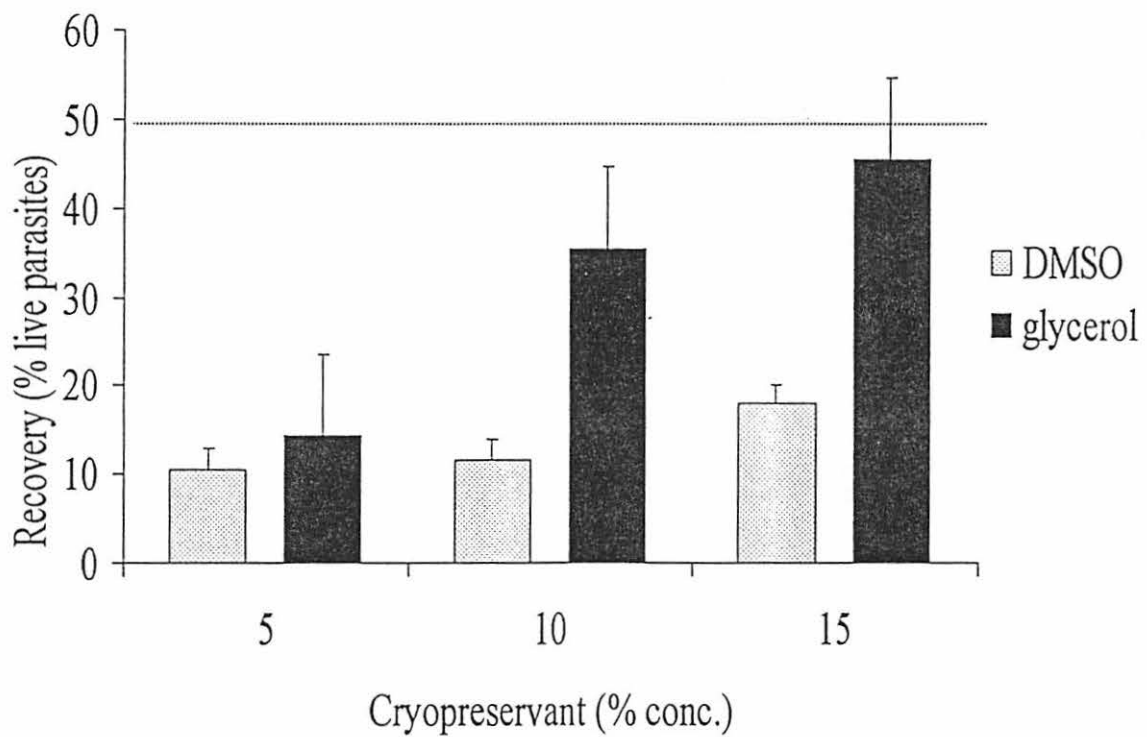






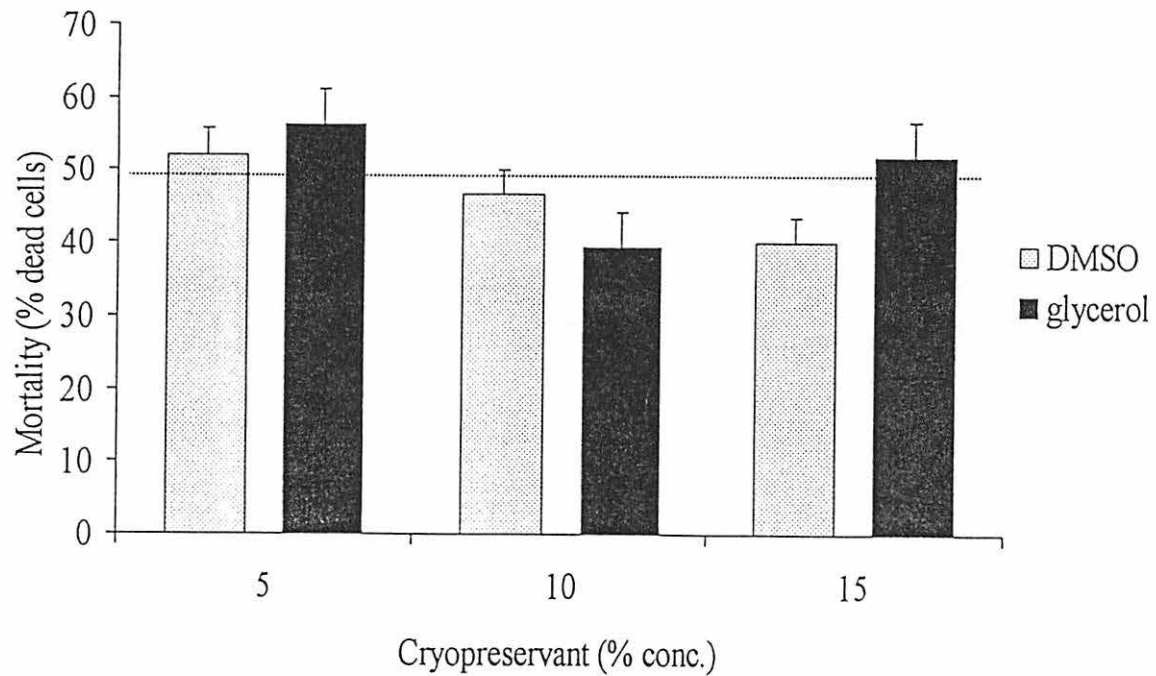


**Figure 3.** Recovery of live and dead cells of *H. perezii* reconstituted after cryopreservation. Glycerol was slightly better in terms of recovery (2-way ANOVA, cryoprotectant=0.052, concentration p=0.056, df=1,83). Dashed line represents 50% recovery of cells.



**Figure 4.** Recovery of live cells of *H. perezii* reconstituted from cryopreservation.

Glycerol was a better cryoprotectant than DMSO (2-way ANOVA, cryoprotectant  $p=0.009$ , concentration  $p=0.074$ ,  $df=1.80$ ). Dashed line represents 50% recovery of live parasites.



**Figure 5.** Hemocyte mortality in relation to cryoprotectant. Mortality was high overall and showed no differences between treatments (2-way ANOVA, cryoprotectant  $p=0.643$ , concentration  $p=0.285$ ,  $df=1,80$ ). Dashed line represents 50% mortality for reference.

Water column/Benthos

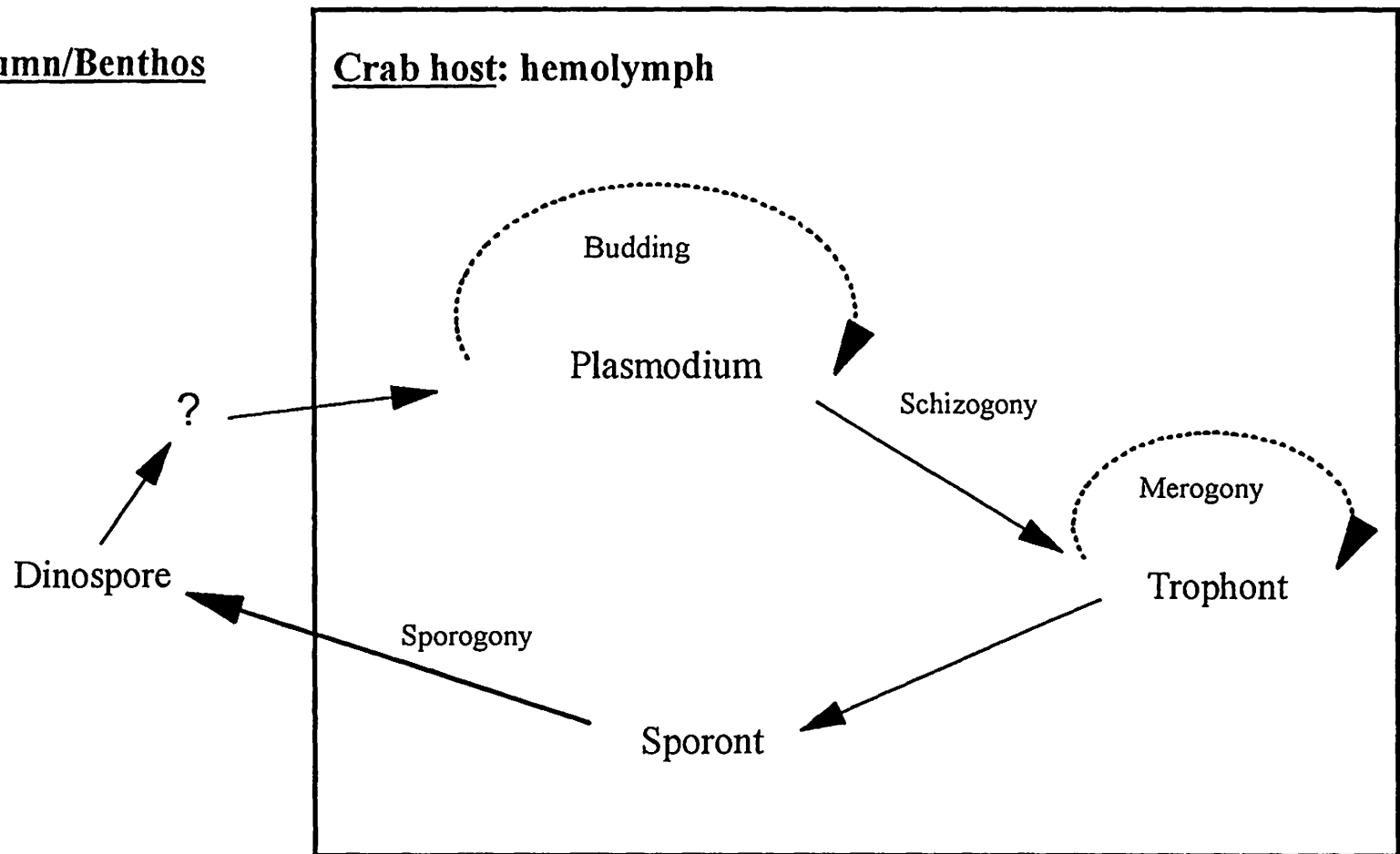


Figure 6. Known portions of the life cycle of *Hematodinium perezii* from the blue crab, *Callinectes sapidus*, as derived from culture studies and direct observation.