The Effect of Salinity on Experimental Hematodinium sp Infections in the Blue Crab, Callinectes sapidus

Anna Huntley Coffey

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The Effect of Salinity on Experimental *Hematodinium* sp. Infections in the Blue Crab, *Callinectes sapidus*

A Thesis
Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

by

Anna Huntley Coffey

2010
APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Science

Anna H. Coffey

Approved December 2010

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ABSTRACT

The blue crab *Callinectes sapidus* is a prominent and commercially important species in Chesapeake Bay. In recent years, blue crab commercial landings have declined and although fisheries managers have enforced greater restrictions, abundance is still low. The parasitic dinoflagellate, *Hematodinium* sp. has been reported at high prevalence (up to 100%) in the coastal bays of Maryland and Virginia. *Hematodinium* causes increased and rapid mortality in blue crabs experimentally infected in the laboratory. Although blue crabs are efficient osmoregulators and maintain a highly hyperosmotic internal osmolality in low salinity environments, *Hematodinium* has never been reported from crabs collected at <1 lpsu and only rarely <18psu. The lack of reported infections from low salinity areas suggests that *Hematodinium* is intolerant of low salinity. The goal of this thesis was to test the hypothesis that *Hematodinium* could survive and develop normally in blue crabs held at low salinity. The objective of the experiment described in Chapter 2 was to test whether salinity affected the mortality rate of blue crabs experimentally infected with *Hematodinium*. Blue crabs in this experiment were inoculated with the parasite, placed in one of three treatment salinities (30, 15, or 5psu), and monitored daily for mortality. Unfortunately, this experiment failed due to a delayed development of *Hematodinium* in experimentally inoculated animals and a high level of mortality in both the experimental and control groups, but the results indicated that the development of *Hematodinium* was sensitive to temperature and could be delayed by even a small change in temperature (Δ4°C). Additionally, histological examination of some of the tissues collected in this experiment provided evidence that *Hematodinium* developed to the dinospore stage in blue crabs held at 5psu. The objective of the experiment in Chapter 3 was to test whether salinity affected the progression of infection in blue crabs inoculated with *Hematodinium*. In this experiment, blue crabs were inoculated with *Hematodinium* or sham-inoculated with MAM buffer (controls) and held at either 30psu or 5psu. Crabs were necropsied at 3, 7, 10, and 15 days post-inoculation. Hemolymph and tissues were collected to determine infection status, total hemocyte counts (THC), parasite density, serum osmolality, and serum protein levels. THC was significantly lower in blue crabs infected with *Hematodinium*, but this did not differ significantly with salinity treatment. There were no significant differences in the other hemolymph parameters due to salinity or infection status. My findings indicate that *Hematodinium* can survive and develop normally in blue crabs held at a low salinity. The occurrence of *Hematodinium* in crabs at low salinity does not appear to be limited by the development of infections in the host, rather it appears that the dinospore stage has limited survival at salinities <15psu.
THE EFFECT OF SALINITY ON EXPERIMENTAL *HEMATODINIUM* INFECTIONS IN THE BLUE CRAB, *CALLINECTES SAPIIDUS*
Chapter 1 – Literature Review

The host – *Callinectes sapidus*

Commercial fishery

The blue crab *Callinectes sapidus* ranges from Cape Cod to northern Argentina (Churchill, 1919; Williams, 1974) and is a prominent benthic species in the Mid-Atlantic region of the United States. It supports the largest crab fishery in the US, representing about 50% of the total weight of all crab species commercially harvested (Hill et al., 1989). The fishery is especially lucrative along the East Coast, with the largest fishery located in the Chesapeake Bay (Van Engle, 1958; Hill et al., 1989; Sharov et al., 2003). The Chesapeake Bay fishery comprised nearly 80% of the national commercial landings in the 1950s but dropped steadily to about 30% in 2003 (Miller et al., 2005). The crab population of Chesapeake Bay experienced overfishing from 1998 to 2002, and although fisheries managers have enforced greater restrictions in recent years (for review, see Aguilar et al., 2008), abundance is still low compared to historic levels; the 2007 harvest of 43.5 million pounds is the lowest on record since 1945 (CBSAC, 2008). Unfortunately, in September 2008, the U.S. Department of Commerce declared the blue crab fishery of Chesapeake Bay a commercial fishery failure based on the drastic decline in the soft shell and peeler crab portions of the industry and placed a moratorium on the winter dredge in Virginia. (Chesapeake Bay Program 15 March 2009). In both 2009 and 2010, blue crab harvest from Chesapeake Bay increased slightly from the record-low 2007 harvest, but still remains below the long-term average of 74 million pounds (CBSAC, 2009; CBSAC, 2010).

Life cycle and ecology
In the blue crab population of Chesapeake Bay, mating occurs from May to October (Van Engl 1958) and is concentrated in the upper bay (Churchill, 1919). Although the female crab mates only once, Hines et al. (2003) estimate that a single female blue crab in Chesapeake Bay may produce a maximum of six to seven broods of about $1 \times 10^6$ eggs each. Female blue crabs migrate to higher salinity regions in lower Chesapeake Bay to brood and spawn (Jivoff et al., 2007). The migration process has two parts. The first involves inseminated females migrating to the lower bay where they produce and incubate broods. In the second part of the migration, the females migrate to the mouth of the bay or directly outside it to release the larvae (Jivoff et al., 2007). Female crabs release larvae two to 10 months after mating (Churchill, 1919). Larvae are transported offshore where they develop through 7 to 8 zoal stages and one megalopal stage in about 4-6 weeks (Costlow and Bookhout, 1959; Epifanio, 2007). The megalopae are transported back to the estuary via wind-driven currents from August through November (Lipcius et al. 2007) and molt into the first juvenile instar (J1) within the protective structured habitat of seagrass beds (Lipcius et al., 2007). Juvenile crabs remain in nursery habitats until reaching the J5-J7 instar when they disperse into unvegetated areas surrounding the nursery habitat to exploit a greater density of infaunal prey items (Lipcius et al., 2007). Juvenile crabs develop through 16 to 20 instars before reaching maturity, obtaining full adult size (~110-180mm) in about 0.5 to 1.5 years (Hines 2007). The average lifespan of a blue crab within the Chesapeake Bay is about 3 years (Hines 2007).

*Callinectes sapidus* is a euryhaline species with a distinct migratory pattern through a wide range of salinities. It is an exceptional osmoregulator and can tolerate
salinities ranging from freshwater (0 psu) to more than twice the concentration of seawater (~70 psu) in hypersaline lagoons (Mangum and Towle, 1977). Successful egg hatching and larval development require higher salinities (Van Engle, 1958; Costlow and Bookhout, 1959); hence the extensive migration of mature females to higher salinity regions after mating (Van Engle, 1958). The salinity distribution of Chesapeake Bay ranges from 0 psu in the upper reaches of the bay and tributaries to about 32-35 psu at the mouth of the estuary (Chesapeake Bay Program Water Quality Database) with blue crabs distributed throughout all regions of the bay.

Blue crab abundance and behavior varies seasonally in temperate regions. Crabs become sluggish and migrate to deeper waters during the winter in the Chesapeake Bay (Schaffner and Diaz, 1988) where they move and eat very little (Churchill, 1919; Hines, 2007). Cold temperatures may cause elevated mortality, especially during winters with very low temperatures (<3.4°C) (Sharov et al., 2003; Rome et al., 2005). Segregation of male and female crabs in the main stem of the Chesapeake Bay in the winter is determined primarily by salinity (Schaffner and Diaz, 1988). Juveniles and mature males move to deeper waters in the upper bay and tributaries and bury in the substrate to overwinter whereas mature, fertilized females migrate to the higher salinity spawning grounds of the lower estuary and remain buried in the sediment for the winter (Jivoff et al., 2007). Crab activity increases with warming temperatures and catch peaks in mid-summer (Hines et al., 1987).

Blue crab diet varies with ontogeny. Larval blue crabs are described as “encounter feeders,” not actively hunting prey items in the planktonic environment but exploiting food items, such as zooplankton, delivered via small-scale turbulence (for
review, see Epifanio, 2007). The diet of both juvenile and adult blue crabs consists of a wide array of benthic infauna and epifauna, including bivalves, shrimp, amphipods, gastropods, and polychaetes (Lipcius et al., 2007). Adult blue crabs cannibalize other blue crabs, especially juveniles, and are capable of catching some estuarine fishes (for review, see Hines, 2007).

Osmoregulation in *Callinectes sapidus*

The migrations that *C. sapidus* undertake through a wide range of salinities within estuaries (Van Engel, 1958) require exceptional osmoregulation. Blue crabs maintain their hemolymph hyperosmotic to the environment at intermediate and low salinities and are iso-osmotic at higher salinities (above about 25-27psu), (Lynch et al., 1973; Mangum and Towle, 1977). Although internal osmotic concentration drops at low salinities, Lynch et al. (1973) showed that blue crabs held at 5psu had an internal osmotic concentration comparable to about 20-23psu. The migration of blue crabs through an environment that changes in salinity and the associated changes in internal osmotic concentration within the blue crab have implications for the progression of disease and transmission of the parasitic dinoflagellate, *Hematodinium* sp., because the parasite is not found at low salinities (see below).

The pathogen - *Hematodinium* sp.

Species of the genus *Hematodinium* are parasitic dinoflagellates belonging to the order Syndinida, which includes parasites of crustaceans that invade the hemocoelom. Members of the genus *Hematodinium* are typically parasites of decapod crustaceans, many of which are commercially important species (Shields, 1994). The type species, *Hematodinium perezi*, was described by Chatton and Poisson (1931) from *Carcinus*
maenas and Portunus depurator. A second species, Hematodinium australis, was described from the sand crab Portunus pelagicus in Australia (Hudson and Shields, 1994). A species of Hematodinium was first reported from the blue crab C. sapidus in North Carolina, Georgia and Florida in 1975 (Newman and Johnson 1975). It has also been reported from blue crabs in the coastal bays of Virginia and Maryland (Messick, 1994), New Jersey, South Carolina, and Texas (Messick and Shields, 2000). A high prevalence of infection in C. sapidus (up to 100%) has been reported from the coastal bays of Maryland and Virginia (Messick, 1994; Messick and Shields, 2000).

The life cycle of Hematodinium sp. in the blue crab has not been completely described, although it includes at least six stages in vitro: multinucleate motile vermiform plasmodia (filamentous trophonts), uninucleate vegetative ameboid trophonts, arachnoid sporonts, sporoblasts, a prespore stage, and two types of motile dinospores (micro- and macrospores; Stentiford and Shields, 2005). However, not all of these stages (e.g. arachnoid sporont) have been identified in vivo in the blue crab (Stentiford and Shields, 2005).

The route of transmission of Hematodinium spp. infections is still unclear, although many hypotheses have been presented. These hypotheses include ingestion of infective trophonts through cannibalism, ingestion of dinospores in the water column, ingestion of infected alternate hosts (i.e., amphipods), and direct penetration of the host by the dinospores (Meyers et al., 1987; Hudson and Shields, 1994; Shields et al., 2007). Attempts to transmit Hematodinium to uninfected C. sapidus via the water column, direct contact with infected crabs, and exposure to crab feces from an infected host were all unsuccessful, although observation of an infective stage in each of these attempts was
never ascertained (Messick et al., 1999). More recently, researchers have been successful at transmitting *Hematodinium* sp. via exposure to dinospores in the water column (Frischer et al., 2006) and through cannibalism in blue crabs (Walker et al., 2009). Shields and Squyars (2000) transmitted *Hematodinium* sp. to uninfected blue crabs via inoculation with both the vegetative trophonts and plasmodial stages and suggested that inocula containing plasmodia may produce more pathogenic infections, though this is not a feasible route for natural infection.

**Pathology in *Callinectes sapidus***

Blue crabs with heavy *Hematodinium* infections exhibit signs of lethargy, opaque muscles, milky hemolymph, and occasionally some discoloration of the carapace (Messick, 1994). Shields and Squyars (2000) suggested that infected blue crabs die before developing the bitter flavored meat found in infected Tanner and snow crabs. The median time to death in experimentally infected blue crabs was 30.3 days (Shields and Squyars, 2000) compared to 91.4 days in snow crabs (Shields et al., 2005). Shields and Squyars (2000) reported that infected blue crabs were seven to eight times more likely to die than uninfected hosts. Although the cause of death is not clear, studies on infections in *C. sapidus* and in the Norway lobster (*Nephrops norvegicus*) have indicated that respiratory dysfunction and starvation are contributing factors to mortality (Field et al., 1992; Taylor et al., 1996; Shields et al., 2003).

Shields and Squyars (2000) reported that *Hematodinium* plasmodia were found in the heart tissue of 93% of the infected crabs three days post-inoculation and uninucleate trophonts were found after only seven days. Infected crabs exhibited significant decreases in hemocyte density, and mortality associated with the decreased hemocyte
density rather than the increase in parasite density. They described three possible outcomes for blue crabs contracting *Hematodinium* infections based on their observations: (1) acute infections in which crabs exhibit rapid mortalities without developing heavy infections and do not lead to mass sporulation of the parasite, (2) chronic infections in which crabs survive the acute stage, live longer with the infection, and the parasite sporulates, and (3) resistance to infection (four crabs inoculated with the parasite in their challenge studies did not develop infections).

**Effects of Salinity on Distribution and Prevalence of *Hematodinium* sp.**

The distribution and prevalence of *Hematodinium* infections in blue crabs is related to salinity. Infected blue crabs have not been reported from waters with salinities less than 11 psu and rarely in waters less than 18 psu (Newman and Johnson, 1975; Messick and Shields, 2000). In addition, *Hematodinium* sp. infections found in the coastal bays of Maryland were significantly associated with high salinities, with prevalence highest in waters of 26 to 30 psu (Messick and Shields, 2000). In a laboratory study, Messick et al. (1999) found that, though all naturally infected crabs held at two salinity treatments (29psu and 10psu) had decreases in parasite intensity, the crabs in the 10 psu treatment experienced a greater decrease in parasite intensity compared to the crabs at 29 psu. Since the parasite densities in both treatments declined, the conclusions regarding the effect of salinity on *Hematodinium* sp. infections in blue crabs are still uncertain.
Chapter 2 – The effect of salinity on the mortality rate of blue crabs experimentally infected with *Hematodinium*

**Abstract** *Hematodinium* sp. is a parasitic dinoflagellate that infects the hemocoelom of decapod crustaceans, including the blue crab, *Callinectes sapidus* and causes significant pathology and mortality. *Hematodinium* infections have not been reported from blue crabs in salinities <11 psu and rarely <18 psu, although blue crabs are distributed throughout regions of these salinities. The objective of this study was to investigate the effect of salinity on mortality in blue crabs infected with *Hematodinium*. Crabs were inoculated with *Hematodinium*, acclimated and held in one of three treatment salinities (5, 15, or 30psu), and monitored for mortality. High levels of control mortality and a delayed development of *Hematodinium* at 15°C caused this study to fail. However, infections developed in crabs held at 5psu, the first evidence that dinospore production can occur at a low salinity in a blue crab. This finding supports the hypothesis that *Hematodinium* can survive in a blue crab host at low salinity and successfully develop to the end stage of the *Hematodinium* life cycle.

**Introduction**

*Hematodinium* spp. are parasites of many commercially important crustaceans, including the blue crab, *Callinectes sapidus* (for review, see Stentiford and Shields, 2005). Blue crabs with heavy *Hematodinium* infections exhibit signs of lethargy, opaque muscles, milky hemolymph, and occasionally some discoloration of the carapace (Messick, 1994). In the lab, the median time to death in infected blue crabs was 30.3 days and infected crabs are seven to eight times more likely to die than uninfected crabs.
Although the cause of death is not clear, studies on infections in *C. sapidus* and in the Norway lobster (*Nephrops norvegicus*) have indicated that respiratory dysfunction and starvation are contributing factors to mortality (Field et al., 1992; Taylor et al., 1996; Shields et al., 2003). Temperature and salinity are thought to be important moderators of *Hematodinium* infection in blue crabs. The prevalence of *Hematodinium* sp. infections in *Callinectes sapidus* peaks in late autumn with a near disappearance of the parasite in late winter to early spring (Newman and Johnson, 1975, Messick, 1994, Messick and Shields, 2000). Sheppard et al. (2003) reported an occurrence of *Hematodinium* sp. infection in *C. sapidus* in Georgia during an unusually warm winter (2001-2002) although the parasite had been absent in previous winters (1999-2001). The strong seasonality in the prevalence of *Hematodinium* indicates that temperature is a possible factor contributing to the transmission and proliferation of the parasite.

Salinity is an important component in the natural distribution of *Hematodinium* infections in blue crabs. Blue crabs regularly migrate through a wide range of salinities in the Chesapeake Bay, from high salinity ocean water to freshwater (Mangum and Towle, 1977). However, infections have never been reported from blue crabs in areas with salinities less than 11 psu and rarely from areas with waters less than 18 psu (Newman and Johnson, 1975; Messick and Shields, 2000). The absence of reported *Hematodinium* infections in blue crabs in low salinities may indicate that the parasite is intolerant of low salinity. However, blue crabs in nearly freshwater environments maintain an internal osmolality highly hyperosmotic to the environment (Lynch et al.,
1973; Mangum and Towle, 1977) and well within the salinity range in which

*Hematodinium* is found (i.e., 23psu).

The fact that blue crabs osmoregulate at low salinities supports the hypothesis that

*Hematodinium* could survive in a blue crab migrating to a low salinity environment. Although this does not explain the absence of *Hematodinium* infections in low salinity regions, it suggests that perhaps an external stage of *Hematodinium* is intolerant of low salinities and, thus may limit transmission in these environments. The life cycle of *Hematodinium* from *C. sapidus* has not been fully described and there are still many hypotheses regarding transmission. Some blue crabs infected with *Hematodinium* shed free-swimming dinospores through a process known as sporulation (Shields and Squyars, 2000) and this stage may be involved in transmission of infections (Stentiford and Shields, 2005). Frischer et al. (2006) reported successful transmission of *Hematodinium* in blue crabs via dinospores in the water column. Many other marine parasitic dinoflagellates are transmitted via the free-swimming dinospore stage (Coats, 1999). Walker et al. (2009) also reported successful transmission of *Hematodinium* in blue crabs via cannibalism of tissues containing the ameboid trophont stage, though this mode of transmission has not been supported by recent work (Li et al., unpub. data).

The objectives of my study were to observe the effects of salinity on the mortality rate of blue crabs infected with *Hematodinium* in the laboratory; and examine the effect of salinity on the development of *Hematodinium* within infected hosts. This experiment examined the effect of three salinity treatments on the mortality rates of blue crabs experimentally infected with *Hematodinium*. Although the blue crab is an exceptional osmoregulator, the process of maintaining an internal osmolality hyperosmotic to the
external environment may be energetically costly (Piller et al., 1995; Kinsey and Lee, 2003). I hypothesize that animals held in the lower salinity treatments would experience a higher mortality rate due to the increased metabolic stress of osmoregulation.

**Materials and Methods**

Blue crabs were collected in commercial crab pots from Wachapreague and the York River, VA, in September and October, 2009, and were transported in coolers to the Virginia Institute of Marine Science in Gloucester Point, VA. Animals were held in flow-through aquaria containing York River water at ambient temperature and salinity until used in the experiment. Crabs were transferred into individual tanks in 3 recirculating aquarium systems (Figure 2). The systems were individually fitted with heat exchangers, and each system was filled with artificial seawater (ASW) and held at 25psu and 15°C. Ammonia, pH, phosphates, nitrites, and nitrates were monitored and water changes were performed every 3-4 days.

A total of 112 crabs were used in the experiment; system A held 32 animals (16 control and 16 experimental crabs) and systems B and C each held 40 (20 control and 20 experimental crabs). The salinity of each system was adjusted by no more than 5psu per day and reached the target salinities of 30, 15, and 5psu salinity for systems A, B, and C, respectively, within seven days. Prior to inoculation with Hematodinium, the crabs were acclimated at the treatment salinities for at least seven days after the target salinity was reached. Crabs were fed thawed squid twice weekly and monitored daily for mortality. Crabs that died during this period were replaced with acclimated crabs from the recirculating system sumps (Figure 1).
The *Hematodinium* inoculum was prepared from 14mL of infected hemolymph drawn from a heavily infected crab. The hemolymph was mixed with 10mL MAM buffer (described in Appleton and Vickerman, 1997) and glucose in a 50mL culture flask and the parasite cells were counted using a hemocytometer. The resulting concentration was $2 \times 10^6$ parasites/mL. The inoculum was diluted 1:1 with MAM and glucose and the cells were counted again on the hemocytometer. The resulting concentration was $1 \times 10^6$ parasites/mL. The inoculum was placed on ice for subsequent inoculation into experimental animals.

Crabs were processed and screened for disease after the previously mentioned acclimation period. The carapace width of each crab was measured and external conditions were assessed. Crabs were screened for *Hematodinium* sp. and other pathogens by preparing hemolymph smears with 0.3% Neutral red, which were then examined immediately. Animals with any signs of disease were replaced with healthy, acclimated individuals from the recirculating system sumps (Figure 1). Hemolymph was also screened for bacterial infections on marine agar plates which were examined after 24 and 48 hours. Crabs were inoculated with either 0.1mL of MAM buffer (control animals) or 0.1mL ($1 \times 10^5$ parasites/crab) of prepared *Hematodinium* sp. inoculum (experimental animals), then returned to aquaria. Ten additional animals were inoculated with *Hematodinium* and placed in individual glass aquaria at 19°C and 30psu to serve as positive controls for the inoculum.

Crabs were monitored twice daily for mortality. Infection status was assessed with a hemolymph smear when mortality occurred and the time to death was recorded. Moribund crabs were considered dead if they could not right themselves when turned
over onto their dorsal carapace. Tissues (heart, hepatopancreas, epidermis, leg muscle, and gills) were collected for histology if the infection status could not be determined from the hemolymph smear. Animals that died within the first seven days of the experiment were excluded from the analysis due to handling stress associated with the initial processing.

The experiment ran until 50% mortality had occurred in at least 2 of the salinity treatments. Crabs inoculated with *Hematodinium* sp. that survived through the end of the experiment were removed from the recirculating systems and maintained in static tanks containing ASW at 19°C and maintained at their treatment salinities until death. These animals were screened for infection status upon death.

Tissues were preserved in Bouin’s fixative for at least 48 h, rinsed in tap water, and preserved in 70% EtOH. Gills were decalcified for 2 hours using the formic acid-sodium citrate method and all tissues were processed following paraffin histological techniques and stained with Mayer’s hematoxylin and eosin (Luna, 1968). Slides were examined and infection status and life stage were assessed and recorded for each animal.

The statistical program, R, was used to run a survival analysis on the mortality data. T-tests were run in Minitab.

**Results**

Mortality was steady over time in all three salinity treatments (Figure 2). Mortality was low initially in the 30psu treatment, but increased by day 23. Crab mortality was not significantly different among salinity treatments (Table 1). Crabs inoculated with *Hematodinium* experienced higher mortality than control crabs in all 3 treatments, but a survival analysis showed that this difference was not significant (Table 2).
A total of eight positive control crabs died within the first seven days of the experiment, presumably from handling stress; they did not have *Hematodinium* present in the hemolymph at the time of death. The two remaining positive controls lived 15 and 24 days, respectively. Both of these animals died with heavy infections of filamentous trophonts (vermiform plasmodia) in the hemolymph, indicating that the experimental inoculum was infectious at the 19°C holding temperature.

Although the crabs held in the recirculating systems experienced steady mortality, *Hematodinium* was not observed in the hemolymph smears of experimental crabs until day 35. At this time, one crab in the 30psu treatment was found dead with a moderate plasmodia infection in the hemolymph. The infection was confirmed with histology (Figure 3). This was the only experimental animal to develop a detectable infection before the end of the experiment on day 37.

Several of the surviving inoculated animals developed infections after being transferred to individual glass aquaria at the end of the experiment (Table 3). The three animals held at 30psu developed *Hematodinium* infections detectable in the hemolymph smears. None of those held at 15psu developed detectable infections. Two held at 5psu developed *Hematodinium* infections which were detectable in the hemolymph. Although these animals had filamentous trophont infections in the hemolymph at the date of death, surprisingly, they also had dinospores present in histological samples (Figure 3).
Table 1. Survival of blue crabs in 3 salinity treatments (Log-Rank Test)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Observed</th>
<th>Expected</th>
<th>$\chi^2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>28</td>
<td>19</td>
<td>18.2</td>
<td>0.3</td>
<td>0.875</td>
</tr>
<tr>
<td>15</td>
<td>28</td>
<td>18</td>
<td>17.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>24</td>
<td>14</td>
<td>15.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. A comparison of blue crab survival with sham or *Hematodinium* inoculations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Alive</th>
<th>Dead</th>
<th>$\chi^2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43</td>
<td>19</td>
<td>24</td>
<td>2.6</td>
<td>0.109</td>
</tr>
<tr>
<td>Inoculated</td>
<td>37</td>
<td>10</td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Infection status of inoculated animals transferred to static tanks after day 37

<table>
<thead>
<tr>
<th>Salinity Treatment</th>
<th># Survivors</th>
<th># Infected</th>
<th># Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>30psu</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>15psu</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>5psu</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
Discussion

Although this experiment failed due to high mortality in the negative control crabs (Figure 2) and delayed development of *Hematodinium* at all treatment salinities, valuable information was obtained. This is the first evidence of a *Hematodinium* infection developing in a blue crab at a salinity outside the pathogen’s reported natural salinity range. The parasite not only survived in crabs at 5 psu, but developed to the dinospore stage in both of the animals that became infected in this treatment. The dinospore stage has been suggested as a possible transmissive stage that occurs toward the end of advanced infections (Stentiford and Shields, 2005). Admittedly, the observations reported for this study are based only on 2 animals, but demonstrates that *Hematodinium* is capable of normal development within blue crabs held in low salinities. This finding is indirect support of the hypothesis that infected crabs have not been reported from low salinity environments because transmission does not occur there, and not because development of the pathogen within the host is hampered at low salinity.

Another interesting observation in this experiment was that the development of *Hematodinium* in the positive control animals held in separate glass aquaria at 19°C was more rapid than that of the experimental animals held in the recirculating systems. The two surviving positive control crabs had detectable infections by days 15 and 24, respectively, while the earliest detectable infection in any of the salinity treatments was on day 35. All positive control and experimental animals were handled in exactly the same manner, inoculated with the same inoculum. The only noticeable difference among these groups was the difference in temperature. The experimental animals in the
recirculating systems were held constant at 15°C while the positive controls were held in glass aquaria with an average temperature of 19°C.

Shields and Squyars (2000) found that *Hematodinium* was detectable in 100% of the infected animals by day 35. These animals were held between 20-21°C throughout the experiment. Although a direct comparison to the data from this experiment cannot be made, an animal with no detectable infection by day 37 at 15°C was later found to be infected with *Hematodinium* on day 63 of the experiment, after it had been moved to 19°C.

Temperature has been suggested to be an important factor determining *Hematodinium* infection prevalence (Messick et al., 1999; Messick and Shields, 2000). *Hematodinium* exhibits strong seasonality, including a peak in prevalence in summer and autumn months and a steep decline in winter months (Messick and Shields, 2000). Messick et al. (1999) reported a decline in infection intensity in blue crabs naturally infected with *Hematodinium* at temperatures lower than 9°C. Although 4°C may not be a seemingly large difference in temperature (between 15°C and 19°C), it was apparently enough to delay the development of *Hematodinium* in the animals held at the lower temperature in this study. Based on the previously reported seasonal trends, an experiment should be conducted regarding the effect of temperature on the development of *Hematodinium* infections in blue crabs.

In summary, it appears that *Hematodinium* develops in blue crabs held in low salinity seawater when the parasite is directly inoculated into the osmoregulating host. The parasite can develop to the prespore and dinospore stages, which may be the stages important to transmission of the parasite. Further study is needed to determine whether
Hematodinium dinospores are intolerant of low salinity water outside of the crab host. It is this stage that exits the host in certain circumstances, and it may be affected by a sudden drop in salinity upon an exit from a hyper-osmoregulating crab into a low salinity environment.
Figure 1. Diagram of recirculating aquarium systems used in salinity experiments. A. Front view of a system. B. Side view of a system. Arrows indicate water flow. Black arrows indicate flow from sump into individual tanks. White arrows indicate flow from individual tanks into the common system sump.
Figure 2. Mortality curves for blue crabs inoculated with *Hematodinium* in three salinity treatments. The dashed line is a reference line for 50% mortality. Mortality in the sham-inoculated controls is shown as a dotted line, and that for the experimentally infected crabs are shown as a solid line.
Figure 3. *Hematodinium* sp. dinospores in the hepatopancreas of an experimentally infected blue crab held in a salinity treatment of 5psu. Arrows indicate *Hematodinium* dinospore cells. Note the small, compact nucleus in individual dinospores. Trophic stages have a larger nucleus in the dinokaryon state.
Chapter 3 – The effect of salinity on the progression of disease in blue crabs infected with *Hematodinium* sp., Dinoflagellata: Syndinida

**Abstract**  *Hematodinium* sp. is a parasitic dinoflagellate of marine crustaceans, including the blue crab, *Callinectes sapidus*. *Hematodinium* infections in blue crabs have only been reported from waters >1 lpsu salinity (Newman and Johnson, 1975; Messick and Shields, 2000). However, blue crabs maintain a hyperosmotic internal concentration in low salinity environments (0-5 lpsu) and should be capable of maintaining an infection in low salinity waters, even if *Hematodinium* cells are intolerant of low salinities. This experiment was designed to observe the effect of low salinity on the progression of disease in crabs experimentally infected with *Hematodinium*. Blue crabs were acclimated to either 5 lpsu or 30 lpsu salinity treatments. They were inoculated with *Hematodinium* sp. and necropsied at 3, 7, 10, and 15 days post-inoculation. Overall, salinity did not have an effect on the proliferation or development of *Hematodinium* infections in blue crabs, and infections in the low salinity treatment developed normally. Infected animals exhibited a significant decline in total hemocyte counts (THC), but other hemolymph parameters were not affected. This experiment indicates that *Hematodinium* can survive and develop in a blue crab at low salinity, but the parasite may still be incapable of transmission in this environment, which would explain the lack of infections in crabs at low salinities.

**Introduction**

*Hematodinium* is an important disease of many marine crustaceans, including the blue crab, *Callinectes sapidus*. In certain areas, prevalence of the parasite in blue crab populations has approached nearly 100% (Messick, 1994; Messick and Shields, 2000). Infected animals show signs of hemocytopenia and increased mortality in the laboratory.
(Shields and Squyars, 2000), suggesting that this parasite has the potential to seriously impact infected blue crab populations.

Migrations through wide ranges of salinities occur regularly throughout the life cycle of the blue crab. Larval blue crabs require higher salinities (>15psu) to hatch properly and survive (Van Engle, 1958; Costlow and Bookhout, 1959) and since mating typically occurs in brackish waters, mature female blue crabs must migrate to higher salinity waters to spawn (Tankersley, et al. 1998). Juvenile blue crabs also migrate, post-settlement, from ocean water into the low salinity and brackish waters within an estuary and its tributaries (Van Engle, 1958). Salinity may limit where Hematodinium infections are found (Newman and Johnson, 1975, Messick and Shields, 2000). Despite the presence of blue crabs throughout a variety of salinities, there have been no reports of Hematodinium infections occurring in blue crabs in areas at <11psu (Newman and Johnson, 1975, Messick and Shields, 2000). The reason behind this trend has not been well examined, but suggests either that Hematodinium can develop in crabs living at low salinities or is not transmitted among crabs at low salinities.

The blue crab is an exceptional osmoregulator, and as such, is capable of maintaining an internal osmolality highly hyperosmotic to its environment (Lynch et al., 1973; Mangum and Towle, 1977). For example, a crab held in an environment at 5psu salinity has a serum osmolality equivalent to about 23psu (Lynch et al., 1973). This indicates that a migrating blue crab infected with Hematodinium should be able to sustain the infection upon reaching a low salinity environment, provided that Hematodinium infections can tolerate osmolalities as low as 23psu.
The life cycle of *Hematodinium* in the blue crab has not been fully described, but early infections are characterized by a filamentous trophont (vermiform plasmodia) stage in the hemolymph, followed by a vegetative ameboid trophont stage, then a transformative prespore stage, and ending with a free-swimming dinospore stage (Stentiford and Shields, 2005). The free-swimming dinospore stage of *Hematodinium* sp. may be involved in transmission (Stentiford and Shields; 2005, Frischer et al., 2006; Li et al. 2010). The dinospore stage in other species of marine parasitic dinoflagellates is widely accepted as the infective agent (Coats, 1999). Dinospores of *Hematodinium* spp. have been observed exiting other crustacean hosts in mass sporulation episodes through the gills (for review, see Stentiford and Shields 2005). If the dinospore is the primary transmissive stage for *Hematodinium*, then the lack of reported infections at low salinities may be due to an intolerance of the dinospore stage to low salinities.

The goal of this study was to understand how salinity affects the development and progression of infection in blue crabs experimentally infected with *Hematodinium*. The primary objective was to determine if *Hematodinium* infections progress normally in blue crab hosts held in a low salinity treatment. A secondary objective was to examine whether there were differences in infections between animals held at low vs. high salinities.

**Materials and Methods**

**Animal Collection and Maintenance**

Crabs were collected in commercial crab pots from Wachapreague, VA, in May, 2010, and transported in coolers to the Virginia Institute of Marine Science in Gloucester Point, VA. The crabs were placed in flow-through tanks containing York River water at
ambient salinity and temperature for 24 hours. Forty animals were placed into each of two recirculating aquaria, fitted with heat exchangers, which contained artificial seawater (ASW) at 26 ± 2psu and 15°C. An additional fifteen animals were placed in prepared static glass aquaria which contained ASW at 26 ± 2psu and 22°C ± 1°. All animals were fed thawed squid three times a week.

Animals were acclimated to treatment salinities (5psu and 30psu) by adjusting the salinity in the recirculating systems and static tanks by no more than 5psu per day. The temperature in the recirculating systems was gradually raised, 1-2°C per day until both systems were at 20°C, during this acclimation period. Salinity was held constant once the target salinity was reached, and animals were maintained at a steady salinity for seven days prior to inoculation. Temperature was held constant at 20°C in the recirculating aquaria throughout the experiment; however, the temperature in the static aquaria fluctuated between 20°-24°C. Water quality was monitored and water changes were performed twice weekly to maintain appropriate water quality parameters.

**Inoculum Preparation**

The inoculum was prepared following the method described in Shields and Squyars (2000). Hemolymph was drawn from two donor crabs naturally infected with *Hematodinium*, which had infections in the ameboid trophont stage. The raw hemolymph was diluted with 10mL MAM buffer separately in 25cm² polystyrene culture flasks and placed on ice. The mixtures were transferred into sterile 15mL polystyrene centrifuge tubes, centrifuged at 4000rpm at 4°C for 10 minutes, and the supernatant was decanted with a pipette. The culture flasks were rinsed with 5ml MAM buffer, transferred to centrifuge tubes, centrifuged and decanted as above. The contents of all centrifuged
tubes were combined, centrifuged again, and parasite suspensions were counted with a hemocytometer (Neubauer bright line). The stock inoculum was at a concentration of $1.7 \times 10^5$ parasite cells/ml.

Due to some clotting in the inoculum, hemolymph was drawn a second time from the same two donor crabs. These samples were centrifuged and washed as described above, then combined with the primary inoculum described above. Parasite cells were counted and the resulting inoculum contained $1 \times 10^6$ parasites/ml or $1 \times 10^5$ parasites/dose. The inoculum was placed on ice and used immediately.

**Animal Processing and Inoculation**

Animals were individually transported from aquaria to the processing room in buckets fitted with portable air pumps and filled with ASW at the appropriate treatment salinity. Carapace width was measured and the general external condition of each crab was assessed. About 300 μl of hemolymph was drawn from each animal. Aliquots of 100 μl of hemolymph were fixed in a 10:1 dilution with MAM buffer and 5% cacodylate in an Eppendorf tube and refrigerated. Another 100μl sample was frozen in an Eppendorf tube to be used in measuring the osmolality and serum protein of the hemolymph of each animal. Several drops of hemolymph were used immediately to prepare a hemolymph smear with Neutral red stain to screen each crab for existing *Hematodinium* infections or other pathogens. Any naturally infected animals were replaced with healthy individuals that had been acclimated as above, in the recirculating system sumps. Several drops of the remaining hemolymph were dotted onto marine agar to screen for bacterial infections. The agar plates were examined after 24- and 48-hour
periods, and presence or absence of bacteria in the hemolymph was recorded for each animal.

Experimental animals were injected with 100μl of the prepared *Hematodinium* inoculum at a dose of $1 \times 10^5$ parasites per individual. Control animals were given a sham inoculation of 100μl of MAM buffer. The inoculation site was sterilized with 70% EtOH before and after the injection. Animals were then immediately returned to aquaria.

**Data Collection**

Each animal was randomly assigned an inoculation treatment (either control or experimental) and a day of necropsy before the beginning of the experiment. Assignment to salinity treatments was haphazard. Animals were necropsied 3, 7, 10, and 15 days post inoculation, with five exposed animals from each salinity treatment killed and examined for infection on each necropsy day. On day three, five control animals were necropsied and on day 15, four and three control animals were necropsied from the 5psu and 30psu treatments, respectively. The smaller sample sizes from the control groups on day 15 were due to the loss of animals from natural mortality during the experiment. After 15 days, four inoculated animals (one high salinity, three low salinity) were maintained to observe disease progression beyond 15 days. Due to the small sample size, these animals were excluded from the analyses.

Hemolymph and tissues (heart, epidermis, midgut, backfin muscle, hepatopancreas, and gills) were collected at the time of death for all animals. Final hemolymph samples collected at the time of necropsy were preserved as described above. Hemolymph smears were examined with Neutral red stain to determine infection status at the time of necropsy.
Histology

Tissues were preserved in Bouin’s fixative for at least 48 h, rinsed in freshwater, and saved in 70% EtOH. Gills were decalcified using the formic acid-sodium citrate method and all tissues were processed following paraffin histological techniques and stained with Mayer’s hematoxylin and with eosin (Luna, 1968). Slides were examined and infection status, life stage, and intensity were assessed for each tissue type. Assessment of infection intensity in the tissues was semi-quantitative with three levels: light (1-5 parasite cells visible at 40x), moderate (5-20 parasite cells visible at 40x), and heavy (>20 parasite cells visible at 40x).

Total hemocyte counts (THC), Hemolymph Osmolality and Serum Proteins

Hemolymph samples fixed in MAM buffer and 5% (v/v) cacodylate were mixed in a 1:1 dilution with Janus green stain and loaded onto a hemocytometer. Hemocytes and parasite cells were counted twice for most animals, and three counts were taken for samples that had more than a 10% difference between the first two counts. Cell concentrations were calculated from the average of all counts.

Serum osmolality was measured with a VAPRO 5520 vapor pressure osmometer. Serum protein was measured using a Westover Model RHC-200 hand-held veterinary refractometer.

The programs R and Minitab were used to perform statistical analyses. Due to the high level of variability in some of the hemolymph parameters, THC and parasite density were log transformed and serum protein was square root-transformed for the analyses.
Results

The proportions of crabs that became infected with *Hematodinium* were similar (Chi-square test, $\chi^2=0.575$, df=1, $p=0.448$) between both salinity treatments, with 33.3% of the inoculated animals in the high salinity treatment (30psu) and 38.9% in the low salinity treatment (5psu) becoming infected. For the analyses, all inoculated crabs that did not develop histologically observable infections were described as exposed, but otherwise uninfected.

The presence of bacteria in the hemolymph prior to inoculation did not affect crab survival (Chi-square test, $\chi^2=0.153$, df=1, $p=0.696$), nor did it affect the susceptibility of crabs to infection with *Hematodinium* (Chi-square test, $\chi^2=0.651$, df=1, $p=0.420$).

THC decreased significantly ($t=-6.69$, df=19, $p<0.01$) post-mortem; therefore, animals that died naturally during the experiment were excluded from further statistical analysis involving this parameter. Serum protein was unaffected by recent post-mortem change ($t=-0.58$, df=15, $p=0.570$).

**Total Hemocyte Counts (THC)**

The 3-way ANOVA described in the following analyses appears as: $\text{Log}_{10}\text{THC}=\text{Sample Date}+\text{Salinity}+\text{Infection Status}$. The sample date refers to the number of days post-inoculation, or day 0 for the initial samples. Infection status refers to the categories infected, uninfected, and control animals. Initial samples showed that all animals had similar THC values pre-inoculation (3-way ANOVA, $F=0.365$, df=87, $p=0.779$). Infected animals experienced significant decreases in THC compared to control animals (3-way ANOVA, df=55, $F=6.241$, $p<0.01$). Mean THC tended to decline in infected animals in the low salinity treatment compared to the mean THC in crabs in
the high salinity treatment (Figure 4), but THC was not statistically different in crabs held at different salinities (3-way ANOVA, F=6.241, df=53, p=0.450), nor was there an interaction between salinity and infection status (3-way ANOVA, df=53, F=4.717, p=0.844). The mean THC of the infected animals in the low salinity treatment remained consistently lower than that in the uninfected and control crabs from day 7 to day 15 (Figure 4). The drop in THC in the infected animals in the low salinity treatment was significantly greater than the controls and uninfected animals in the same treatment (3-way ANOVA, df=26, F=5.392, p=0.009). However, there was no significant difference in the mean THC of the infected crabs compared to the control crabs in the high salinity treatment alone (3-way ANOVA, df=19, F=1.79, p=0.168).

**Serum Osmolality**

The 3-way ANOVA used in this analysis appears as: Serum Osmolality=Sample Date+Infection Status+Salinity. There was an expected difference in hemolymph osmolality between salinity treatments (3-way ANOVA, df=60, F=9.69, p<0.01). Infected animals in the low salinity treatment maintained osmoregulatory capabilities similar to uninfected and control animals, and there was no difference in hemolymph osmolality based on infection status (3-way ANOVA, df=30, F=2.079, p=0.138).

**Serum Protein**

The 3-way ANOVA used in this analysis appears as: Square root(Serum Protein)=Sample Date+Salinity+Infection Status. Initial samples showed that serum protein levels were similar in all animals prior to inoculation (3-way ANOVA, df=81, F=1.973, p=0.125). Serum protein levels were variable among all control, infected, and uninfected animals (Figure 5). Mean serum protein levels were not significantly affected
by infection status (3-way ANOVA, df=61, F=2.553, p=0.059, Figure 6). The possible outlier shown in Figure 6 represents the serum protein levels for five animals. The high serum protein levels in these animals could not be explained by sex (Table 4) or molt status; thus, they were included in the analyses. Mean serum protein levels were not different between salinity treatments (3-way ANOVA, df=61, F=2.553, p=0.452). The 2-way ANOVA used in this analysis appears as: Square root(Serum Protein)=Sample Date+Infection Status. Infection status (infected, uninfected, or control) did not affect serum protein levels in either the low salinity (2-way ANOVA, df=31, F= 1.897, p=0.824) or the high salinity treatments (2-way ANOVA, df=24, F= 0.4614, p= 0.536).
Table 4. Mean serum protein levels in blue crabs inoculated with *Hematodinium* in relation to crab sex.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Control</th>
<th>Infected</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>6.04 ± 3.2</td>
<td>5.94 ± 3.2</td>
<td>6.09 ± 3.1</td>
</tr>
<tr>
<td>Female</td>
<td>5.8 ± 3.2</td>
<td>5.8 ± 3.2</td>
<td>6.0 ± 3.2</td>
</tr>
</tbody>
</table>

Parasite Density

The 2-way ANOVA used in this analysis appears as: $\log_{10}\text{Parasite Density}=\text{Sample Date}+\text{Salinity}$. Mean parasite density was not different between the salinity treatments (2-way ANOVA, df=17, $F=0.390$, $p=0.683$, Figure 7). Mean parasite densities in the animals from the high salinity treatment had larger standard errors than those from the lower salinity treatment, indicating greater variability in density (Figure 7).

Histology

Of the 12 infected animals in the high salinity treatment, 50% had light infections, 25% had moderate infections, and 25% had heavy infections in their tissues. Of the 14 infected animals in the low salinity treatment, 42.9% had light infections, 42.9% had moderate infections, and 14.3% had heavy infections in their tissues (Figure 8). In the low salinity treatment, 42.9% of the infections had mostly plasmodia, 7.1% had ameboid trophonts, and 50% had prespores or dinospores. In the high salinity treatment, 66.7% of the infections had plasmodia, 25% had ameboid trophonts, had 8.3% were prespores or dinospores (Figure 9).

Several animals in the low salinity treatment developed moderate to heavy levels of prespore and dinospore cells, apparent in the hemolymph and other tissues, in a relatively short time period (5-6 days). Initially, the majority of these infections were
mistaken for ameboid trophont infections due to the ameboid-like movements of the parasite cells in the hemolymph smears taken at the time of death (Figure 9). However, the parasite cells present in the preserved hemolymph and histological sections exhibit the keel-like shape and different staining patterns characteristic of *Hematodinium* dinospores (Figure 11). In contrast, only one animal in the high salinity treatment showed this rapid development to the prespore stage and it died 5 days post-inoculation. Only one other animal in the high salinity treatment had an infection that developed to the prespore stage and it died naturally, 18 days post-inoculation.

Six crabs from the low salinity treatment and one from the high salinity treatment had prespores or dinospores present in their tissues at the time of death, ranging from days 5 to 15. However, the proportion of crabs that developed prespore or dinospore cells in the low salinity treatment by the time of death was not significantly greater than that proportion of crabs in the high salinity treatment (Fisher’s Exact Test, p=0.078). Although the concentrations of prespores and dinospores were relatively high in some of these animals, a mass sporulation episode was not observed. Additionally, there were no obvious signs of tissue destruction in the gills, which would have indicated a release of parasite cells into the environment.

One control animal in the high salinity treatment died naturally on day 1 of the experiment, and was excluded from the analyses. This animal had a very light *Hematodinium* plasmodia infection in the heart, hepatopancreas, muscle, and epidermal tissues upon histological examination. This infection was so light that it was undetectable in the initial and final hemolymph smears. The infected control animal indicates a background natural infection prevalence of 4% in the animals used in this
experiment. No other control animals were found with parasite cells in hemolymph smears or histological preparations.

Nine experimental crabs had infections that were undetectable in the hemolymph; four from the low and five from the high salinity treatments. All nine of these infections were found as light infections in the tissues and were found on day 7 or later of the experiment. Although some of these infections were found only in the heart, others had parasite cells in the muscle, epidermis and gill tissues. All of the infections that were undetectable in the hemolymph were in the plasmodia stage and two of these infections also had ameboid trophont cells present in the tissues (Table 5). Parasite intensity was similar in hemolymph and tissue samples (Figure 12). Typically the infections that were not apparent in the hemolymph had very light infections in the tissues. Parasite intensity did not differ between the salinity treatments (Chi-square test, $\chi^2 = 1.206$, df=2, p=0.547).

Two animals recorded as having light to moderate densities of *Hematodinium* ameboid-like cells in the hemolymph did not have visible parasites in their tissues. One of these animals died naturally, but the tissues were in very poor condition and did not fix well; it is possible the parasite was present in the tissues but undetectable. It is unclear why the parasites are not visible in the tissues of the other crab.

**Discussion**

In this study, I show that low salinity has no significant impact on the development of *Hematodinium* within experimentally infected blue crabs. Similar proportions of inoculated crabs became infected in both salinity treatments and *Hematodinium* in crabs from both salinities exhibited similar growth and development. An important finding in this experiment was the rapid development of *Hematodinium* to
prespore and dinospore stages in the tissues, especially in the animals in the low salinity treatment. Dinospores are often implicated as the transmissive stage of infection in the host and reportedly develop over a longer period of time (Shields and Squyars, 2000, Stentiford and Shields, 2005, Frischer et al., 2006). A greater proportion of infected crabs in the low salinity treatment exhibited prespore or dinospore stages in their tissues than those in the high salinity treatment. This finding was not statistically significant, but it may indicate an effect of salinity on the development of *Hematodinium* life stages.

I originally hypothesized that *Hematodinium* infections would develop at a more rapid rate in a low salinity environment due to the additional physiological demand of osmoregulation imposed on the host (Findley et al., 1981, Sabourin, 1984). However, many of the signs of host stress associated with low salinity, including increased heart rate, decreased oxygen content of the hemolymph, and lowered oxygen affinity of the hemolymph do not last more than four days after acclimation to low salinity (Sabourin, 1984). My experiment included a seven day acclimation period, and it is unlikely that osmoregulatory host stress would have been a factor for *Hematodinium* development. Salinity did not have a significant effect on any of the hemolymph parameters measured in this experiment, but the rapid development of prespore and dinospore stages in the crabs held in the low salinity treatment may be an indication that the *Hematodinium* life cycle progresses more quickly in a hyper-osmoregulating host.

I found that infections can develop rapidly in intensity. Two animals in the high salinity treatment exhibited a rapid proliferation of ameboid trophonts in just three days. This was an unexpected finding based on Shields and Squyars (2000), who reported that crabs developed moderate to heavy infections over 14-30 days. However, it is not the
first report of such rapid proliferation of *Hematodinium* cells as Frischer et al. (2006) and Walker et al. (2009) reported that crabs obtained and developed fatal infections within four days. It is possible that the hosts that exhibited such rapid development or proliferation were more susceptible to infection or compromised in some way, thereby facilitating reproduction by the parasite. Given the background natural infection prevalence of 4% in animals used in this experiment, it is possible, though unlikely, that these animals obtained the infections prior to the experiment, although no *Hematodinium* was detected during the initial hemolymph screening process in these crabs.

Whereas this is the first report of *Hematodinium* infection in blue crabs held at such a low salinity, it is not the first report of *Hematodinium* infections occurring in low salinity (Li et al., 2008). Li et al. (2008) reported *Hematodinium* infections in the mud crab, *Scylla serrata* in aquaculture ponds in water less than 9psu. However, the details of how and where these crabs were originally collected were not reported. The majority of crustaceans infected by *Hematodinium* are stenohaline species that rarely experience a significant fluctuation in salinity (Stentiford and Shields, 2005). Both *S. serrata* and *C. sapidus* are efficient osmoregulators and experience a wide range of environmental salinities when they migrate through estuaries. This may affect the dynamics of *Hematodinium* infections in these hosts, especially during migration to low salinity regions. Although dinospores were found in the blue crab tissues in this study and in *S. serrata* tissues (Li et al., 2008), there was no evidence that transmission occurred at low salinity in either study. Additional work is needed to determine whether dinospores are intolerant of low salinity, though it seems likely that a free parasite stage would have difficulty adjusting to a rapid salinity change upon exiting the host.
Hematodinium dinospores are likely to encounter osmotic shock upon exiting the hyperosmotic internal environment of an osmoregulating host into a low salinity environment. This would explain the rare reports of infections from blue crab populations in low salinity areas (Newman and Johnson, 1975, Messick and Shields, 2000). Although a crab that obtained an infection in high salinity waters may migrate to low salinity areas, if the dinospore cannot survive or swim actively enough to transmit the infection to a naïve host, outbreaks of Hematodinium sp. in these low salinity areas would be non-existent. Therefore, the chances of finding an infected crab in a low salinity area would be unlikely. Many species of dinoflagellate dinospores have a specific salinity range in which they exhibit optimal growth and swimming rates (Hand et al., 1965, White, 1978, Noga and Levy, 2006). Even if the low salinity water does not kill Hematodinium sp. dinospores by osmotic shock, they may still be negatively affected by the low salinity and thus, incapable of transmitting the infection.

The hemocyte densities reported in this study support the findings from Shields and Squyars (2000). In both studies, the THCs of the infected crabs were significantly lower than those of the control crabs, and there was no change in hemocyte density inoculated crabs that did not develop infections. This may indicate that some animals are more susceptible to Hematodinium infection, although initial hemolymph samples showed that THC and serum protein levels were not significantly different between inoculated animals that became infected and those that did not.

Serum protein levels were also unchanged in infected crabs tested in my study, whereas Shields et al. (2003) reported a significant decline in serum protein in male blue crabs with heavy Hematodinium infections. The majority of the infections sampled in
this study were light or moderate infections, and it is likely that the infections had not progressed enough to elicit a change in the serum protein levels of the infected hosts at the time of necropsy.

The heart appears to play an important role in the establishment of *Hematodinium* infections as it was the first tissue infected and nearly all of the infected animals had parasites present in the heart, even when there were no parasite cells in any other tissue. No other tissue was as consistently positive for infection. Shields and Squyars (2000) also found plasmodia in the heart tissue of crabs from an early infection trial, as early as three days post-inoculation.

The high number of infections that were undetectable in the hemolymph was unexpected. Although latent *Hematodinium* infections have been reported previously in naturally infected blue crabs (Messick et al., 1999), this is the first report of such a high prevalence of undetected infections based on hemolymph smears (38%). All of the infections from this experiment that were detectable only in the tissues were light infections, indicating that the density of *Hematodinium* cells in the host was too low to be found in the volume of hemolymph (< 50μL) collected for microscopic examination. Additionally all of these infections had parasite cells in the plasmodia stage in the tissues, indicating that this is likely the initial stage of infection (for review, see Stentiford and Shields, 2005).

The relatively low proportion of inoculated crabs that became infected with *Hematodinium* from this experiment was also unexpected. Shields and Squyars (2000) reported 86% of the crabs inoculated with *Hematodinium* became infected with the parasite. The remaining 14% were described as being immune or refractory to infection.
In this experiment, only about 36% of the inoculated crabs became infected over both treatments. However, Shields and Squyars (2000) noted that detectability of *Hematodinium* in the hemolymph did not reach 100% until 35 days post-inoculation and was only about 38% by day 15. Detectability of *Hematodinium* in the hemolymph of the crabs from this study was 63% over 15 days and was defined as:

\[
\frac{\text{# of infected crabs positive for } Hematodinium \text{ in hemolymph}}{\text{# of infected crabs positive for } Hematodinium \text{ in histology}}
\]

A direct comparison of the results between this study and the Shields and Squyars (2000) study is difficult due to differences in dosage and life stage in the inoculum. Shields and Squyars (2000) used an inoculum containing mostly plasmodia while the inoculum used in this study was made up primarily of ameboid trophont cells. It is possible that the plasmodia life stage is more virulent or more successful in initializing *Hematodinium* infections, which has been previously suggested based on anecdotal evidence (Shields and Squyars, 2000).
Figure 4. Mean total hemocyte count ($\log_{10}$ THC) in blue crabs experimentally infected with *Hematodinium* sp. in crabs held at two salinities. Sham inoculated controls are shown in the dashed line. Experimentally infected crabs are shown in the solid line. Experimentally inoculated crabs that did not obtain infections are shown in the dotted line. Bars are SE.
Figure 5. Mean serum protein levels (sq. root g/100mL) in blue crabs experimentally infected with *Hematodinium* and held in two salinity treatments. Sham inoculated controls are shown in the dashed line. Experimentally infected animals are shown in the solid line. Experimentally inoculated animals that did not obtain infections are shown in the dotted line. Bars are SE. (B) Graph including only control animals and infected animals in the 5psu salinity treatment.
Figure 6. Serum protein levels (square root transformed) in blue crabs experimentally infected with *Hematodinium* in two salinity treatments. The outlier shown represents five animals.
Serum Protein (sq. root(g/100 ml))

- Control
- Infected
- Uninfected

Infection Status

n=5
Figure 7. Mean parasite density ($\log_{10}$ cells/mL) in the hemolymph of blue crabs experimentally infected with *Hematodinium* sp. in two salinity treatments over a 15 day period. Infected crabs held in the 5psu treatment are shown in the solid line. Infected crabs held in the 30psu treatment are shown in the dashed line. Bars are SE.
Parasite Density (log₁₀ cells/mL) vs. Time (Days Post-Inoculation)

- 5psu
- 30psu
Figure 8. Proportion of blue crabs experimentally infected with *Hematodinium* sp. that developed light, moderate, or heavy infections in two salinity treatments.
Parasite Intensity

- Heavy
- Moderate
- Light

Percent Infected Animals (%)
Figure 9. The proportion of blue crabs experimentally infected with *Hematodinium* sp. that developed plasmodia, amoeboid trophont, or prespore and dinospore stages of the parasite.
Life Stage

- Prespores/Dinospores
- Ameboid Trophont
- Plasmodia
Figure 10. Ameboid cells of *Hematodinium* sp. stained with Neutral red. The parasites exhibited ameboid-like movements in the hemolymph of an experimentally infected blue crab five days post-inoculation.
Figure 11. *Hematodinium* sp. dinospores in the hemal sinus of the hepatopancreas in an experimentally infected blue crab five days post-inoculation. Dinospores indicated by black arrows. Note the small, compact nucleus in individual dinospores. Trophic stages have a larger nucleus in the dinokaryon state.
Figure 12. Mean parasite density (log cells/mL) in the hemolymph and intensity of parasites in tissues in blue crabs experimentally infected with *Hematodinium* sp. Assessment of parasite intensity in histological sections was semi-quantitative: 1=light, 1-5 parasite cells at 40x magnification; 2=moderate, 5-20 parasite cells at 40x; 3=heavy, >20 parasite cells at 40x. Crabs represented in these graphs were held at either 5psu or 30psu salinity treatments. (A) Mean parasite density in the hemolymph is shown along the primary y-axis on the left and is represented in the dashed line. Mean parasite intensity in the tissues is shown along the secondary y-axis to the right and is represented in the solid line. Bars are SE. (B) The marker at zero represents nine experimentally infected animals that did not have observable infections in the hemolymph, but had light infections in the heart tissue.
A. Parasite Density and Intensity over Days Post-Inoculation

B. Parasite Intensity in Histology with n=9
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