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Lack of transmission of *Hematodinium* sp. in the blue crab *Callinectes sapidus* through cannibalism

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ABSTRACT: *Hematodinium* spp. are parasitic dinoflagellates of marine crustaceans. Outbreaks of *Hematodinium* sp. have impacted commercial landings of the blue crab *Callinectes sapidus* in the coastal bays of Virginia and Maryland (USA), with seasonal peaks in prevalence reaching 85%. The life cycle and transmission routes of the parasite in blue crabs are poorly understood. Cannibalism and waterborne transmission may be routes of transmission, although little conclusive evidence has been reported for these modes. We examined cannibalism as a route by a series of experiments wherein we repeatedly fed adult and juvenile crabs the tissues of crabs infected with *Hematodinium*. In each experiment, feeding was done 3 times over the course of 1 wk. Only 2 of 120 crabs were infected within 7 to 9 d after feeding, and these 2 were likely infected prior to the experimental exposures. Crabs inoculated with hemolymph from infected donors served as positive controls. They developed infections over 11 to 21 d, indicating that the *Hematodinium* sp. used in the cannibalism trials was infectious at the time of inoculation. Because amphipods also harbor *Hematodinium*-like infections, we fed tissues of infected crabs to the estuarine amphipod *Leptocheirus plumulosus*. *Hematodinium* DNA was detected in amphipods shortly after feeding, but not in animals held for longer periods, nor was it observed in histological preparations. Amphipods did not obtain infections by scavenging infected crab tissues. Our results show that *Hematodinium* sp. is not effectively transmitted through ingestion of diseased tissues, indicating that cannibalism may not be a major route of transmission for *Hematodinium* sp. in blue crabs.

KEY WORDS: Parasite · Dinoflagellate · Disease · Amphipod · Crustacea · Scavenger

INTRODUCTION

The parasitic dinoflagellate *Hematodinium* sp. infects the blue crab *Callinectes sapidus* along the east and Gulf of Mexico coasts of the USA (Newman & Johnson 1975, Messick 1994, Shields 1994, Messick et al. 1999, Messick & Shields 2000, Shields & Squyars 2000, Sheppard et al. 2003). Trophic stages of the parasite proliferate in the hemocoel of their crustacean hosts, often reaching high densities of $>10^8$ cells ml^{-1} (Newman & Johnson 1975, Messick & Shields 2000, Shields & Squyars 2000). Infected hosts experience dramatic pathological alterations to their organs and tissues, and eventually die from malfunction of the hepatopancreas, degradation of muscle tissue, and respiratory dysfunction (Stentiford & Shields 2005). Outbreaks of the disease have impacted stocks of the blue crab in the coastal bays of Maryland and Virginia, and the lower Chesapeake Bay, but infections are limited to high salinities and have not been reported in low salinity subestuaries of the western portions of the bay (Messick 1994, Messick & Shields 2000).

Little is known about how *Hematodinium* sp. is transmitted to crabs in their natural environment. The *in vitro* life cycle for the parasite in blue crabs has only recently been described (Li et al. in press). In controlled laboratory studies, the disease has been successfully transmitted to blue crabs by injection of hemolymph containing filamentous trophonts (also

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known as vermiform plasmodium) or ameboid trophonts of the parasite (Messick & Shields 2000, Shields & Squyars 2000). In 2 independent studies, experimentally infected blue crabs began dying 14 to 17 d post inoculation (dpi), with cumulative mortalities of 86% by 40 dpi (Shields & Squyars 2000) and 100% by 55 dpi (Messick & Shields 2000). Naturally infected crabs held in captivity died over a period of 14 to 35 d, depending on the severity of the infections (Messick & Shields 2000).

As with the parasites in the Microsporidia and *Paramoeba perniciosa*, cannibalism has been speculated to be a possible mode of transmission for *Hematodinium* to blue crabs (Shields & Overstreet 2007). Blue crabs are aggressive and become cannibalistic as they grow; cannibalism can contribute 15 to 25% of the normal diet of adult crabs (Virnstein 1977, Laughlin 1982, Moksnes et al. 1997, Hines 2007). Several groups have examined this mode of transmission with *Hematodinium*, with varying results. Sheppard et al. (2003) anecdotally reported that the disease was transmitted to juvenile *Callinectes sapidus* via ingestion of infected crab tissues. However, Hudson & Shields (1994) were not able to transmit a closely related congener, *H. australis,* to the crab *Portunus pelagicus* via ingestion, but samples sizes were small in their study. More recently, Walker et al. (2009) reported transmission via cannibalism in short-term trials with *C. sapidus*. They found *Hematodinium* infections developing only 16 h after feeding crabs with infected tissues, with 63% (7 of 11) of the crabs showing evidence of infection 24 to 48 h after feeding. However, it took significantly longer to establish heavy infections of *Hematodinium* in inoculated crabs, i.e. from 7 to 30 d (Messick & Shields 2000, Shields & Squyars 2000), than the time frame of hours observed by Walker et al. (2009), leading us to explore this route of transmission in more detail.

Cannibalism is essentially no different from predation, scavenging, or ingestion as a route of transmission. Predation is an attractive hypothesis as a possible mode of transmission because *Hematodinium* infections have been reported from several other crustacean species that occur in the same habitat with blue crabs (Shields 1994, Sheppard et al. 2003, Stentiford & Shields 2005, Pagenkopp Lohan et al. in press) and likely serve as their prey (see Hines 2007). For example, amphipods make up a sizeable component (2 to 35%) of the diet of juvenile blue crabs (Laughlin 1982, Meise & Stehlik 2003, Dittel et al. 2006) and several species of amphipods carry *Hematodinium*-like infections (Johnson 1986, Messick & Shields 2000). Amphipods have been speculated to serve as alternate or reservoir hosts for *Hematodinium* (Hudson & Shields 1994, Shields 1994, Sheppard et al. 2003, Stentiford & Shields 2005). However, the role of amphipods in the transmission of the pathogen has not been investigated.

The primary objective of our study was to experimentally determine whether cannibalism is an effective route of transmission of *Hematodinium* sp. to blue crabs. We conducted a series of cannibalism experiments by repeatedly feeding *Hematodinium*-infected tissues to juvenile and adult crabs. Histological assessments were done to evaluate the development of *Hematodinium* infections in crabs exposed via cannibalism. In addition, infected tissues were fed to the amphipod *Leptocheirus plumulosus* to determine whether scavenging might serve as an effective route of transmission for the pathogen. This amphipod was selected because it is easily cultured, commercially available, and indigenous to Chesapeake Bay, USA (DeWitt et al. 1992, McGee et al. 1993). It is also a close congener of *L. pinguis*, which is known to harbor *Hematodinium*-like infections (Johnson 1986). Histological examinations and polymerase chain reaction (PCR) diagnostics were used to investigate scavenging as a transmission pathway in amphipods.

MATERIALS AND METHODS

Blue crabs *Callinectes sapidus*

Naïve, juvenile blue crabs, 2 to 5 cm in carapace length (CL), were collected from York River, Virginia, USA, during the annual Virginia Institute of Marine Science (VIMS) Winter Dredge Survey in 2010. *Hematodinium* infections have not been reported in blue crabs from the York River (Messick & Shields 2000, J. Shields et al. unpubl. data). Crabs were transported to VIMS in coolers and held together in a recirculating 1000 gal (~3750 l) system for 3 to 7 d prior to experiments. Crabs were then transferred into 10 gal (~37.5 l) aquaria (salinity = 24 ± 3 ppt, temperature = 20 ± 3°C), equipped with preconditioned biological filters (Whisper) and a crushed coral substrate.

Adult crabs (10 to 15 cm CL) were collected from the Delmarva Peninsula, Virginia, during routine field surveys for *Hematodinium* in 2010, using commercial traps baited with menhaden. Prior to their transport to VIMS, these crabs were screened for *Hematodinium* using hemolymph smears and neutral red as described by Stentiford & Shields (2005).

The intensities and stages of infection were categorized as described by Shields & Squyars (2000, see their Table 4). Infected crabs were held separately in 10-gal aquaria or a recirculating trough; they were used as donors for the experiments. Presumptively uninfected crabs were housed together in the recirculating 1000 gal system for 3 to 7 d prior to experiments for acclimation in the adult cannibalism experiments. Uninfected crabs were transferred to a recirculating system (salinity = 24 ± 2 ppt, temperature = 22 ± 2°C, equipped with preconditioned biological filters, UV light, and a crushed coral substrate) for experimental treatments. Note that the crabs from the Delmarva Peninsula were screened for infection, but they were collected from an area with endemic disease caused by *Hematodinium*. Additional uninfected crabs (naïve to infection) were collected off the VIMS pier on the York River using commercial traps baited with menhaden; these crabs were used in the long-term adult cannibalism experiment. All crabs were pre-screened for infection using hemolymph smears as above, 7 to 10 d prior to their use in experiments.

Susceptibility of juvenile crabs via cannibalism

To gauge whether juvenile crabs might be more susceptible to infection via cannibalism, juvenile crabs were allowed to feed on naturally infected crab tissues. Fresh tissues of various donors, with light, moderate, or heavy infections, were mixed together and fed to experimental crabs. Tissues from uninfected crabs served as controls. Pieces of gills, muscle, epidermis, heart, hepatopancreas, and other internal organs were dissected from infected or uninfected crabs and fed to juvenile crabs. Prior to feeding, all juveniles were fasted for 2 to 3 d. In the first week, crabs in the negative control group (17 crabs) and experimental group (36 crabs) were fed 3 times with fresh tissues of uninfected crabs and infected crabs (~1.0 g crab^{-1}), respectively. The estimated exposure of crabs to *Hematodinium* cells is listed in Table 1. The estimates were based on the wet weight of tissues fed to crabs, infection status of donor crabs (see Table 4 in Shields & Squyars 2000), and the mean hemolymph volume of a blue crab (Gleeson & Zubkoff 1977). Thereafter, crabs were fed semi-weekly with thawed squid. For positive controls, 18 juvenile crabs (6 crabs donor^{-1}) were injected with 50 µl (approximately 5×10^4 parasites) of hemolymph from the infected donor crabs. All of the crabs were monitored daily for mortality. Dead or moribund crabs were assessed for *Hematodinium* infections using hemolymph smears as above, and various tissues were dissected and fixed in Bouin's solution and processed for histology according to Wheeler et al. (2007) for later diagnosis. At the end of the experiment (21 d after the first feeding), surviving crabs were killed and processed as above to determine the status of *Hematodinium* infections. Water quality was monitored and maintained within reasonable limits (ammonia: 0 to 0.3 ppm, nitrite: 0 to 0.6 ppm, pH: 7.4 to 8.2). Over the time course of the experiment, salinity ranged from 24 to 27 ppt and temperature ranged from 17 to 21°C.

Susceptibility of adult crabs via cannibalism

Cannibalism in adults was tested in 3 separate experiments, a pilot study, an early time course study, and a long-term study. Water quality in the pilot study was not strictly monitored, but it was monitored during the early time course study and twice per week in the long-term study over the time course of that experiment (19 d). Prior to feeding, all adult crabs were fasted for 2 to 3 d and then fed fresh tissues of uninfected or infected crabs. The estimated exposure of crabs to *Hematodinium* cells is listed in Table 1.

In the pilot study, 35 adult crabs were fed 3 times a week (every other day) with fresh tissues (~3 to 5 g crab^{-1}, Table 1) from infected crabs that were mixed together prior to feeding. Thereafter, crabs were fed thawed squid 3 times a week. Crabs were processed as above to determine if they had acquired *Hematodinium* infection. In addition, 11 crabs were injected with 100 µl (approximately 10^5 parasites) of hemolymph from infected donors with heavy ameboid trophont infections and used as positive controls.

In the early time course study, 29 adult crabs were fed once with pieces of tissues (approximately 3 to 5 g, Table 1) from donor crabs with heavy infections consisting of ameboid trophonts as described by Walker et al. (2009). At 1, 3, 6, 16, 24, and 48 h after feeding, 4 to 5 crabs were randomly collected and processed to assess the status of *Hematodinium* infections using hemolymph smears. These crabs were then dissected and processed for histology as per Wheeler et al. (2007).

In the long-term study, crabs in the negative control group (11 crabs) and experimental group (20 crabs) were fed 3 times (every other day) during the first week with approximately 3 to 5 g of uninfected

Table 1. Exposure of *Callinectes sapidus* to *Hematodinium* trophonts in separate feeding trials. The estimated no. of *Hematodinium* cells to which crabs were exposed was based on wet weight of tissues fed to crabs, infection status of donor crabs (see Table 4 in Shields & Squyars 2000) and mean hemolymph volume of a blue crab (Gleeson & Zubkoff 1977)

Cannibalism trial	Feeding	Approx wet wt of tissues fed each crab (g crab^{-1})	Est. no. of *Hematodinium* cells crab^{-1}
Juvenile crabs			
	1st	1	$10^4 - 10^5$
	2nd	1	$1 \times 10^5 - 5 \times 10^5$
	3rd	1	$> 5 \times 10^5$
Adult crabs			
Pilot study	1st	3–5	$1 \times 10^6 - 1.5 \times 10^6$
	2nd	3–5	$1 \times 10^6 - 1.5 \times 10^6$
	3rd	3–5	$1.5 \times 10^6 - 2.5 \times 10^6$
Early time course study	1st	3–5	$1.5 \times 10^6 - 2.5 \times 10^6$
	2nd	3–5	$1 \times 10^6 - 1.5 \times 10^6$
	3rd	3–5	$1.5 \times 10^6 - 2.5 \times 10^6$
Long-term study	1st	3–5	$1.5 \times 10^6 - 2.5 \times 10^6$
	2nd	3–5	$1.5 \times 10^6 - 2.5 \times 10^6$
	3rd	3–5	$1 \times 10^6 - 1.5 \times 10^6$

and infected tissue, respectively, as in the experiment with juveniles (see 'Susceptibility of juvenile crabs as cannibals'). The relative amount of tissue consumed was estimated 12 h after feedings, and any uneaten tissues were removed. Thereafter, crabs were fed thawed, diced squid every other day. During the course of the experiments, crabs were monitored daily for mortality, with dead or moribund crabs processed as above to determine their infection status with *Hematodinium*. At the end of the experiment (19 d after the first feeding), all remaining crabs were examined using hemolymph smears and then processed for histology. Water quality was monitored 2 to 3 times per week, and water changes were made to ensure that various water quality parameters remained within acceptable limits (ammonia: 0 to 0.3 ppm, nitrite: 0 to 0.6 ppm, pH: 7.4 to 8.2). Over the time course of the experiments, salinity ranged from 21 to 23 ppt and temperature ranged from 21 to 23°C.

Susceptibility of the amphipod *Leptocheirus plumulosus*

Leptocheirus plumulosus were purchased from Chesapeake Cultures, Inc. Three exposure trials were conducted. For the first 2 trials, adult amphipods were housed in 500-ml aerated plastic containers, with 10 animals in each container. In Trial 1, 4 containers with 10 animals each were used as control tanks, and the amphipods were fed tissues from uninfected blue crabs screened for *Hematodinium* using hemolymph smears as above. The estimated exposure of amphipods to *Hematodinium* cells is listed in Table 2. The experimental treatment consisted of 6 containers with 10 amphipods each that were fed infected tissues as in the experiment with juvenile crabs (see 'Susceptibility of juvenile crabs via cannibalism' above).

In Trial 2, 40 amphipods were used, with 20 animals for each treatment. During Trials 1 and 2, animals were fed 3 times over the course of 1 wk (every other day), with feedings consisting of approximately 1 ml of macerated blue crab tissue (gills, heart, hepatopancreas, and muscle) and hemolymph from *Hematodinium*-infected or uninfected crabs. Water changes were conducted 24 h after each feeding. After 1 wk, amphipods were switched to a diet of liquid invertebrate food or ground Tetramin™ pellets. Trials were run for 21 d. For infection analysis, amphipods from the first 2 trials were cut in half into head and tail sections, with one half randomly assigned for preservation in 10% neutral-buffered formalin for histological analysis as above, and the other half preserved in 95% ethanol for later processing for detection of *Hematodinium* DNA.

In Trial 3, amphipods were housed individually in 6-well plates containing approximately 12 ml of filtered York River water (YRW; 24 ppt, 22 ± 2°C) in each well. Control animals (72 amphipods in 12 plates) and experimental animals (72 amphipods in 12 plates) were housed in separate rooms. Well plates were used to prevent cannibalism among amphipods and also to track the molting of each amphipod, as molting has also been speculated as a mode of transmission for *Hematodinium.* Amphipods were fed 3 times over the course of a week with macerated blue crab tissue (gills, heart, hepatopancreas, and muscle) and hemolymph from *Hematodinium*-infected or uninfected crabs, respectively. Infected tissues contained a variety of stages of *Hematodinium*, including ameboid and filamentous trophonts. To maintain water quality, 90% water changes were completed 6 h after each feeding during the exposure periods and every 48 h thereafter.

Table 2. Exposure of *Leptocheirus plumulosus* to *Hematodinium* trophonts in separate feeding trials. The estimated no. of *Hematodinium* cells to which amphipods were exposed was calculated as in Table 1

Amphipod scavenging trial	Feeding	Vol. of minced tissues (ml container^{-1})	Est. no. of *Hematodinium* cells ml^{-1} tissue
Trial 1	1st	1	$10^4 - 10^5$
	2nd	1	$1 \times 10^5 - 5 \times 10^5$
	3rd	1	$1 \times 10^5 - 5 \times 10^5$
Trial 2	1st	1	$1 \times 10^5 - 5 \times 10^5$
	2nd	1	$10^4 - 10^5$
	3rd	1	$1 \times 10^5 - 5 \times 10^5$
Trial 3	1st	0.1	$1 \times 10^5 - 5 \times 10^5$
	2nd	0.1	$1 \times 10^5 - 5 \times 10^5$
	3rd	0.1	$> 5 \times 10^5$

After 1 wk, amphipods were fed ground Tetramin™ pellets. Two days after the last exposure, half of the amphipods from each group were preserved intact for PCR and histological examination. At the end of Trial 3 (14 d after first exposure), all remaining amphipods were preserved for molecular and histological examination. Animals that died during the experiment were also preserved in ethanol and processed for PCR.

PCR diagnostics

For molecular diagnosis in all experiments, DNA was extracted using a Qiagen tissue kit according to the manufacturer's instructions, and then processed for PCR using a specific ITS1 PCR assay as described by Small et al. (2007) with minor modifications (Pagenkopp Lohau et al. in press). Positive amplicons were sequenced using the assay as described by Li et al. (2010), to confirm the presence of *Hematodinium* DNA in amphipods. Not all amphipods were available for analysis as some animals died during the experiment and were too degraded for analysis. Additionally, some were lost during the first 2 trials due to cannibalism.

Viability of *Hematodinium* cells in seawater

To test the viability of *Hematodinium* cells exposed to seawater, suspensions of ameboid trophonts from Trial 3 were placed in seawater and monitored over time using 0.3% trypan blue and 0.3% neutral red in invertebrate saline. Cell suspensions were prepared by mixing infected hemolymph 1:1 with sterile-filtered YRW (23.5 ppt), allowing the suspension to rest for a few minutes, then washing the preparation through 2 successive low speed centrifugations in YRW (3600 rpm [2000 × *g*] 5 min, 6°C; CL3R, Thermo Scientific). Cell density was estimated with a Neubaur hemacytometer, and aliquots were dispensed into culture well plates (3 to 5 wells per time treatment) to give estimated densities of approximately 1.0×10^6 parasites ml^{-1}. At 30 min, 1, 3, 6, 24, and 50 h, aliquots of the suspensions were examined separately for viability using 0.3% trypan blue and 0.3% neutral red. Cells with neutral red uptake or the lack of uptake of trypan blue were judged as viable. In addition, aliquots from these suspensions were fixed in 95% ethanol and assessed for PCR as above.

RESULTS

Susceptibility of juvenile crabs fed infected tissues

All juvenile crabs ingested the tissues of infected donor crabs within 3 h after feedings. No *Hematodinium* infections were found in the hemolymph or tissues of the 36 juvenile crabs after 3 feedings with infected tissues (Table 3). No infections developed in the negative control group that was fed uninfected tissues, nor did juvenile crabs serving as negative controls die during the experiment. Several juvenile crabs in the positive control group started dying after 11 dpi, with a cumulative mortality of 33% by the end of the experiment (21 dpi). *Hematodinium* cells (filamentous or ameboid trophonts) were observed in the hemolymph of dead or moribund crabs in the positive control group 11 dpi, and *Hematodinium* infections were observed in the tissues of all but 2 of the crabs used as positive controls (Fig. 1A).

Table 3. *Callinectes sapidus*. Susceptibility of juvenile crabs to infection by *Hematodinium* via ingestion of infected tissues

	Hemolymph smear		Histology	
	Pos.	Neg.	Pos.	Neg.
Negative control	0	17	0	17
Positive control	15	3[a]	16	2
Experimental	0	36	0	36

[a]One crab was negative by hemolymph smears, but had a light infection in the heart tissue observed in histology. Note the absence of infections in the experimental group

Susceptibility of adult crabs via cannibalism

In the pilot study, 3 crabs in the experimental group did not feed or only partially fed on tissues of infected crabs during the 3 feeding periods; none of these 3 crabs developed infections. However, 1 crab from the experimental treatment was identified with a moderate infection of *Hematodinium*. This crab died on Day 9 after the first feeding (Fig. 1B). None of the remaining experimental crabs that had ingested infected tissues presented with *Hematodinium* infections via hemolymph smears or histological analysis (Table 4). One crab in the negative control group died on Day 5 and was diagnosed with a heavy ameboid trophont infection. This crab likely had an occult infection that was not detectable when pre-screened by hemolymph smear assay. Three crabs in the positive control group developed infections by the end of the experiment; other injected crabs died with bacterial infections shortly after injection (within 5 d).

In the early time course study, all of the crabs ingested the tissues of infected donors within 3 h after each feeding period. No *Hematodinium* infections were found in the 29 crabs from the early time course study. Tissue residues were observed in the foregut of crabs within 6 h of feeding but not thereafter (Fig. 1C). No obvious pathological changes were observed in histology.

In the long-term study, 1 crab was identified with a moderate to heavy *Hematodinium* infection after 7 d after the first feeding (Fig. 1D). This crab died with a bacterial infection in the hemolymph. This crab ingested approximately one-third of the infected

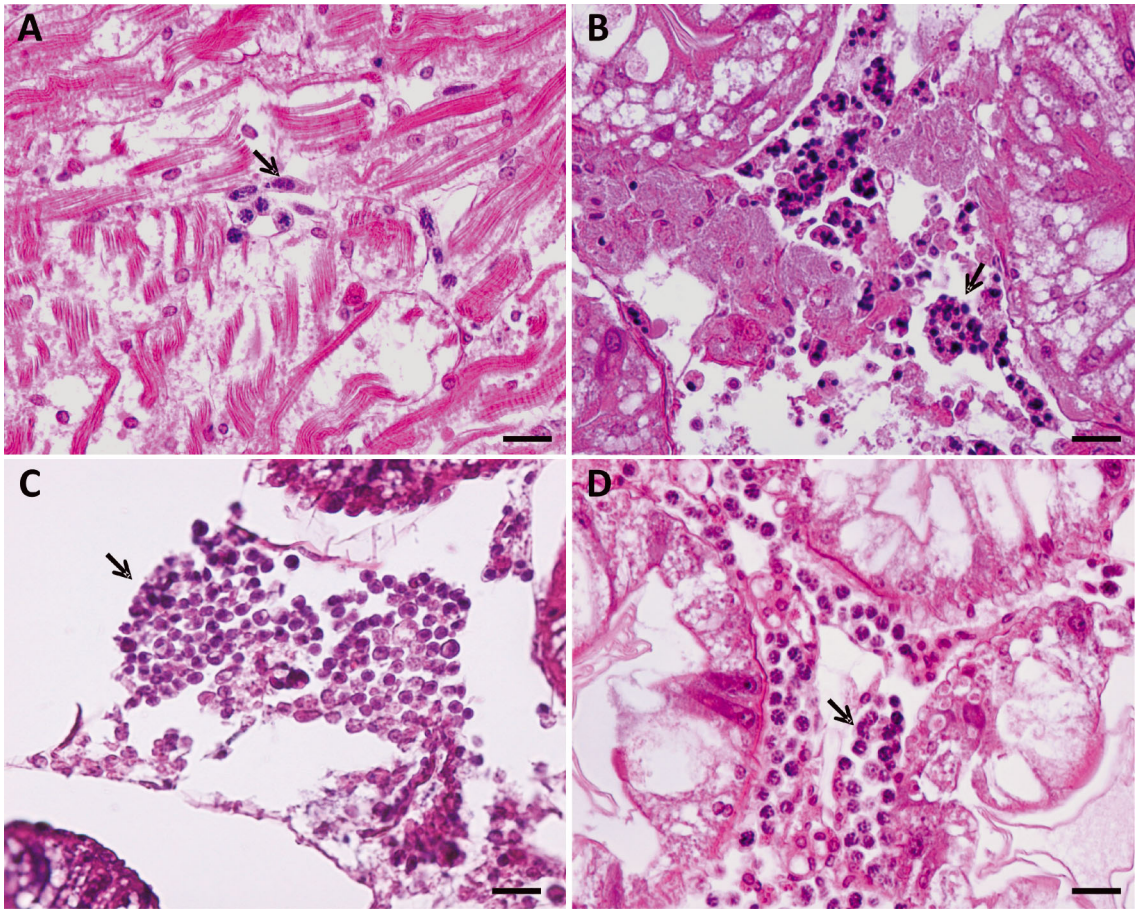

Fig. 1. *Hematodinium* sp. in various tissues of *Callinectes sapidus* used in the experiments. (A) Filamentous trophont (arrow) in the heart of a moderately infected juvenile crab. (B) Uninucleate ameboid trophonts (arrow) in the hepatopancreas of the heavily infected adult crab from the pilot study. (C) Tissue residues in the foregut of a crab collected at 1 h post feeding with pieces of *Hematodinium*-infected tissues. Note ameboid trophonts (arrow) in the hemal spaces among hepatopancreas tubules. (D) Multinucleate clump colony (arrow) in hepatopancreas tissue from the infected adult crab from the long-term study. Scale bars = 40 µm

Table 4. Susceptibility of adult *Callinectes sapidus* crabs to infection by *Hematodinium* via ingestion of infected tissues

Study	Hemolymph smear		Histology	
	Pos.	Neg.	Pos.	Neg.
Pilot				
Negative control	1[a]	16	1[a]	16
Positive control	2	9	3	8
Experimental	1	34	1	34
Early time course	0	29	0	29
Long-term				
Negative control	0	11	0	11
Experimental	0	20	1[b]	19

[a]One crab serving as a negative control had a heavy infection confirmed by hemolymph smear and histology (see 'Results')

[b]One crab had a moderate to heavy infection in the heart, hepatopancreas, and gills, but no *Hematodinium* cells were observed in hemolymph

tissues during the second feeding and fed little if any during the other 2 feedings. No infections were observed in other crabs (n = 19) fed with *Hematodinium*-infected tissues. Other crabs in these studies were active feeders and ingested most or all of the food within 1 to 3 h of feeding.

Susceptibility of amphipods fed infected tissues

None of the 129 amphipods exposed to *Hematodinium*-infected crab tissues and hemolymph developed infections that were detectable with histology (Table 5). *Hematodinium* DNA was detected via PCR in several amphipods (n = 7) that were processed shortly after being fed infected tissues. In Trial 1, 3 amphipods were PCR positive for *Hematodinium* within 2 to 7 d after the last feeding, and in the subsequent trials, 4 amphipods were positive within 2 to 5 d after the first feeding. In all 3 trials, only 7 amphipods were positive via PCR presumably from the presence of *Hematodinium* cells in the water column (see section below) or in their gut contents. Positive bands were sequenced and confirmed to be the same species of *Hematodinium* as in naturally infected blue crabs. None of the amphipods processed at the end of each experiment were positive via PCR, and no histological signs of *Hematodinium* infection were observed in any amphipods (Table 5). In addition, there was no correlation between amphipods that had molted and those that were positive for *Hematodinium* via PCR analysis during Trial 3.

Table 5. Susceptibility of the amphipod *Leptocheirus plumulosus* to infection by *Hematodinium* sp. via ingestion of infected crab tissues

Treatment	PCR		Histology	
	Pos.	Neg.	Pos.	Neg.
Trial 1				
Control	0	34	0	34
Experimental	3[a]	60	0	63
Trial 2				
Control	0	14	0	14
Experimental	1[a]	13	0	14
Trial 3				
Control	0	37	0	16
Experimental	3[a]	33	0	16

[a]Seven amphipods were positive via PCR analysis within 2 to 7 d after a feeding period. No infections were identified in amphipods held for longer periods

Viability of *Hematodinium* cells in seawater

Ameboid trophonts remained viable in seawater for at least 6 h as shown by the uptake of vital stains and by positive PCR results (Fig. 2). However, at 24 h, uptake of the vital stains had diminished with only a few viable cells showing dye uptake. Suspensions were positive for *Hematodinium* DNA through 24 h, but only 1 of 3 samples was positive via PCR at 50 h. These results indicate that positive PCR samples from amphipods could have arisen from dead or dying *Hematodinium* cells at least 24 to 50 h after feeding.

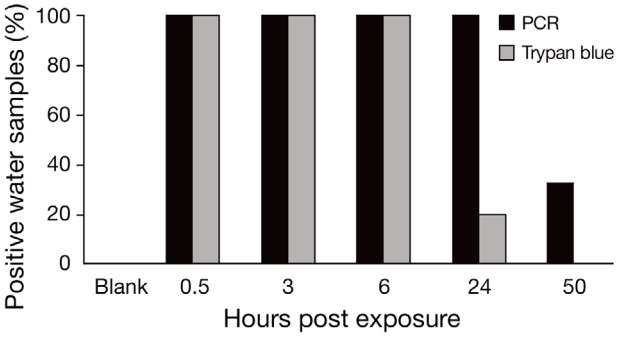

Fig. 2. *Hematodinium* sp. *In vitro* viability of cells in seawater. The exclusion of trypan blue was used to test the viability of the parasite in sterile seawater. Uptake of neutral red was identical to the exclusion of trypan blue. Samples that were positive for PCR were not necessarily positive for viable cells after 24 h. The blank is a negative control

DISCUSSION

In controlled experiments, we provide evidence that *Hematodinium* sp. in the blue crab *Callinectes sapidus* was not effectively transmitted through ingestion of diseased tissues, indicating that cannibalism was not a major route of transmission for the parasite in these crabs. Of the 36 juvenile blue crabs and 84 adult crabs fed 3 times with fresh tissues from *Hematodinium*-infected crabs, only 2 adults (2 of 120 exposed crabs, 1.7%) had infections by the end of the experiments. These 2 crabs had moderate to heavy *Hematodinium* infections 7 to 9 d after feeding, and such infections typically take longer than 1 wk to develop in naturally infected or inoculated animals (Messick & Shields 2000, Shields & Squyars 2000). They likely carried occult infections that were not detected prior to the beginning of the experiments. The development of the disease from occult infections is consistent with other studies that have shown an increase in detectability of infections over time in experimental infections (Shields & Squyars 2000).

Our results were different from those of Walker et al. (2009), who were able to infect 63% of 11 blue crabs 24 h after a single feeding on infected tissues. This discrepancy between studies is difficult to explain. It is possible that there are different strains of *Hematodinium* sp. infecting blue crabs in Georgia versus Virginia, but there is no evidence to support different strains of the parasite in blue crabs at present. A more likely explanation is that the blue crabs used by Walker et al. (2009) possessed occult infections. As mentioned previously, *Hematodinium* infections in blue crabs can develop moderate infections relatively rapidly when inoculated directly into crabs (Messick & Shields 2000, Shields & Squyars 2000), but it seems unlikely that heavy infections could develop so rapidly (24 h) with ingestion as a mode of transmission. Moreover, given a transmission rate of 63% via cannibalism, the disease should be more prevalent in adult crabs than in juveniles (because adults have high predation rates on juveniles), a fact not supported by published field studies (Messick 1994, Messick & Shields 2000). Finally, there is little other evidence to support transmission via cannibalism in blue crabs. The only other report is an anecdotal account by Sheppard et al. (2003), but no experimental details of their study were given. Attempts to transmit infection of *H. australis* using ingestion as a mode of transmission were not successful, but the sample sizes were low (Hudson & Shields 1994). Our experiments and those of other researchers highlight the need for appropriate identification of naïve crabs for experiments where occult infections can occur.

Hematodinium and *Hematodinium*-like organisms infect a broad range of crustacean hosts worldwide, suggesting that these parasites are host generalists (Stentiford & Shields 2005). In addition to brachyuran crabs, gammarid amphipods are reported to harbor *Hematodinium*-like infections (Johnson 1986, Messick & Shields 2000). Hudson & Shields (1994) and Shields (1994) speculated that amphipods may act as alternate or reservoir hosts and transmit infections to crabs via predation. In our feeding experiments, the gammarid amphipod *Leptocheirus plumulosus* ingested tissues and hemolymph of *Hematodinium*-infected crabs, but *Hematodinium* was only detectable by PCR within 2 to 7 d after feeding, and no infections were detected histologically. This finding was further substantiated by viability tests of parasite cells in sterile seawater. An alternate hypothesis is that *L. plumulosus* is not a suitable host for *Hematodinium* sp. because it is a detritus and filter feeder (DeWitt et al. 1992), but *Hematodinium*-like infections are known from a number of other amphipod species, including a close congener, *L. pinguis* (Johnson 1986). Moreover, under laboratory conditions, the amphipods were capable of cannibalism. In our study using histology, none of the amphipods obtained infections from scavenging *Hematodinium*-infected tissues. Our results indicate that at least the blue crab *Callinectes sapidus* and the amphipod *L. plumulosus* are not likely to develop infections through ingestion.

The primary mode of transmission of *Hematodinium* is still unknown, but cannibalism appears unlikely given our findings. There is evidence that transmission of *Hematodinium* and *Hematodinium*-like infections occur in association with host molting. Alaskan tanner crabs *Chionoecetes bairdi*, snow crabs *C. opilio,* and Norway lobsters *Nephrops norvegicus* have distinct cycles of infection with a *Hematodinium*-like organism in relation to molting, but the mode of entry into the host is not clear (Meyers et al. 1990, Eaton et al. 1991, Stentiford et al. 2001, Shields et al. 2005, 2007). Seasonal molting periods are known to occur in these hosts as well as in blue crabs (Van Engel 1958). Infections are much more prevalent in juvenile blue crabs than in adults (Messick 1994, Messick & Shields 2000), and juvenile crabs are known to molt up to 18 to 23 times over the course of 12 to 18 mo before reaching maturity, when they effectively stop molting (Tagatz 1968). The sharp autumnal peaks in the prevalence of *Hemato-*

dinium overlap with settlement and molting of juvenile crabs (Messick & Shields 2000, Shields 2003). Thus, it is likely that the primary mode of transmission of *Hematodinium* sp. to *Callinectes sapidus* occurs in association with host molting.

In our laboratory studies, ameboid trophonts of *Hematodinium* in the blue crab showed reduced viability after 24 h in seawater, whereas dinospores from naturally sporulating crabs can survive up to 7 d at 21 to 23°C in aquaria (Li et al. 2010). Dinospores are thought to be a transmissive stage of the parasite (Frischer et al. 2006), but until experiments are done with molting hosts, that mode of transmission remains undetermined. Lastly, sexual transmission has been posited as a mode of transmission in tanner crabs based on the finding of parasites in the seminal fluids of a few males (Meyers et al. 1996); however, this finding needs clarification to determine whether it is indeed a mode of transmission or a consequence of tissue degradation during late stages of infection. Given the importance of the commercial fisheries impacted by these parasites, clearly the modes of transmission of *Hematodinium* sp. and *Hematodinium*-like species require further attention.

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