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

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Mortality and hematology of blue crabs, *Callinectes sapidus*, experimentally infected with the parasitic dinoflagellate *Hematodinium perezi**

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Hematodinium perezi is a parasitic dinoflagellate that proliferates in the hemolymph of several crab species. In the blue crab, *Callinectes sapidus*, *H. perezi* is highly pathogenic and usually kills the host. The main symptom of the infection is lethargy. Heavy infections are characterized by discolored (brown, yellow, milky or chalky) hemolymph that does not clot. The disease occurs in blue crabs in high-salinity (>11‰) waters from Delaware to Florida, and in the Gulf of Mexico (Newman and Johnson, 1975; Messick and Sinderman, 1992). In 1975, Newman and Johnson (1975) reported a prevalence of 30% in blue crabs from Florida; the effect of this disease on the blue crab population was thought to be high.

In 1991 and 1992, prevalences of infection up to 100% were found in blue crabs (mean prevalence=43%, several locations from 70% to 100%, n=971) from coastal bays in Maryland and Virginia (Messick, 1994). Commercial watermen reported reduced catches, lethargic and moribund crabs in pots and shedding facilities, and crabs that died soon after capture (Rux, Oesterling[1]). In 1996 and 1997, 10% to 40% of adult crabs from the eastern portions of Chesapeake Bay in Virginia were infected.[2] The disease has a low prevalence or does not occur in the larger, riverine ("bayside") fishery; it appears most detrimental to the coastal ("seaside") crab fisheries.

Outbreaks of infestation by *Hematodinium* spp. have caused concerns to several major crustacean fisheries. Significant population declines and economic losses have been reported for the Tanner (*Chionoecetes bairdi*) and snow (*C. opilio*) crab fisheries of Alaska and Newfoundland (Meyers et al., 1987, 1990; Taylor and Khan, 1995),[3] the Norway lobster (*Nephrops norvegicus)* fishery of western Scotland (Field et al., 1992), and the velvet crab (*Necora puber*) fishery of western France (Wilhelm and Miahle, 1996). The parasite causes a condi-

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1 Rux, S. 1993. Red Bank Seafood Co., Box 37 Marionville, VA 23408. Personal commun.; Oesterling, M. 1993. VASG, Virginia Inst. Marine Science, Gloucester Point, VA 23062. Personal commun.

2 Shields, J. D. 1997. An investigation into the epizootiology of *Hematodinium perezi*, a parasitic dinoflagellate in the blue crab, *Callinectes sapidus.* Saltonstall-Kennedy Program, National Marine Fisheries Service, NOAA. Final Report.

3 Prevalences in Newfoundland are now at 1–15% in the northern bays. Taylor, D. 1998. DFO, CP 5567, White Hills, St. Johns, Newfoundland, Canada, A1C 5X1. Personal commun.

tion known as bitter crab disease in snow and Tanner crabs (Meyers et al., 1987). Low prevalences (1–4%) of another species, *H. australis,* have been reported in sand (*Portunus pelagicus)* and mud (*Scylla serrata)* crabs from Australia (Shields, 1992; Hudson and Shields, 1994).

Infections of *Hematodinium* spp. or *Hematodinium*-like species have been reported from a variety of different hosts (see Shields, 1994, for review). There are, however, only two described species of *Hematodinium*: *H. perezi* Chatton and Poisson, 1931*,* and *H. australis* Hudson and Shields, 1994. By convention (Newman and Johnson, 1975; MacLean and Ruddell, 1978) and from its distinct morphological features, we concur that *Hematodinium perezi* is the infectious species in the American blue crab.

Blue crabs sustain one of the largest fisheries in Chesapeake Bay. Current management plans and state regulations are based on population assessments that include numbers of juvenile and adult crabs found during winter, spring, and summer surveys (Lipcius and Van Engel, 1990; Abbe and Stagg, 1996; Rugolo et al., 1998). Although these projections include estimates of natural mortalities, they do not account for the potential epizootics and mortalities caused by *Hematodinium perezi.* In this study, we examined host mortality in controlled laboratory experiments and documented changes in the hemograms (total cell counts, and differential counts) of inoculated crabs versus uninfected crabs. We also examined proliferative growth of *H. perezi* at approximately weekly intervals and made observations on the biology and life history of the parasite.

Materials and methods

Blue crabs were collected from Chesapeake Bay and several of its subestuaries during the annual VIMS Winter Dredge Survey (part of the Chesapeake Bay Stock Assessment Program) with a 1.83-m-wide Virginia crab dredge fitted with 0.5-inch (1.25-cm) Vexar mesh dragged on the bottom for one minute at three knots. Crabs were also taken with commercial crab pots from two reference locations on the Delmarva Peninsula, Red Bank and Hungars Creeks, Virginia. Uninfected crabs were housed together for three to seven days prior to treatment to ensure acclimation and absence of overt bacterial or protozoal diseases (as assayed below). During the experiments, crabs were fed fish and squid semiweekly and held individually in aquaria (5 gal., 19 liter) at 20° to 21°C, and 24 ppt salinity. Although *H. perezi* infects both sexes, only mature, nonovigerous female crabs (healthy, orange maturing gonads, little to no shell damage, 120–160 mm carapace width including epibranchial spines) were used in the experiments. Females were used to limit the number of treatment effects (e.g. potential differences between sexes) and to improve sample sizes given the laborious nature of the experiments.

Hematodinium perezi was maintained in the laboratory by serial passage of infected hemolymph. Hemolymph from naturally infected crabs was injected directly into uninfected crabs. Naïve (unexposed) crabs and crabs used for inoculation experiments were obtained from low-salinity non-enzootic locations. Infected and inoculated crabs were housed separately and used as hemolymph donors to inject naïve hosts (10^5–10^6 parasites per host). Injections were given in the arthrodial membrane of the fifth leg at the juncture of the basis with the carapace. We have maintained *H. perezi* for over seven months using this method with no apparent loss from pathogenicity.

Two mortality experiments and one early life history experiment were undertaken. The mortality-I experiment used raw, infected hemolymph as the inoculant. Although appropriate for maintaining infections in the laboratory, raw hemolymph cannot be adjusted to manipulate parasite densities without the use of physiological buffers, nor can it be guaranteed as sterile without appropriate assessment (see Welsh and Sizemore, 1985). Preliminary experiments with sterile sea water, physiological buffers, and infected hemolymph indicated that buffer-washed parasites remained infectious, and could, therefore, be adjusted to consistent densities appropriate to controlled experiments. The mortality-II experiment used buffer-washed parasites adjusted to a density similar to that used in the mortality-I experiment. Mortality-II experiment closely resembled mortality-I experiment except for 1) handling (buffer washes with centrifugation) and 2) the use of plasmodial versus uninucleate stages of the parasite. Uninfected crabs served as controls in both experiments. Controls were used to assess handling effects and to establish baseline densities of hemocytes. The early infection experiment was designed to examine the effects of early infections on the hematology of the host and the early life history of the parasite. Experimental densities in the early infection experiment were four times higher than those in the previous experiments (4.1×10^5 vs. approx. 1.0×10^5 parasites/crab, respectively) and were arbitrarily higher to insure observation of parasites prior to their proliferation.

In the mortality-I and mortality-II experiments different proportions of trophonts and plasmodia were used (for definitions see below). The mortality-I experiment consisted of a control group of uninfected crabs (n=22) injected individually with 100 µL

of hemolymph from an uninfected donor crab and an experimental group (n=20) injected individually with 100 µL of infected hemolymph from a donor crab containing an estimated 1.3×10^6 trophonts/mL (1.3×10^5 trophonts per crab).

The mortality-II experiment consisted of a control group (n=8) injected individually with 100 µL of physiological saline buffer (modified from Appleton and Vickerman, 1998; NaCl, 19.31 g/L; KCl 0.65 g/L; $CaCl_2 \cdot 2H_2O$ 1.38 g/L; $MgSO_4 \cdot 7H_2O$ 1.73 g/L; Na_2SO_4 0.38 g/L; HEPES 0.82 g/L;) adjusted to pH 7.8, with added glucose (1.0 mg/mL) and two experimental treatments (high dose=1.0×10^5 parasites/crab; low dose=1.0×10^3 parasites per/crab, n=10, 10 respectively). To prepare the inoculum for the experimental treatments, 2.0 mL of infected hemolymph were drawn from a donor crab infected with 6.15×10^7 parasites/mL (comprising 97% plasmodia; 3% trophonts). The infected hemolymph was diluted 1:1 with buffer, centrifuged at 4000 rpm for 10 minutes, the supernatant was decanted, and the cells were resuspended in buffer. The cells were then adjusted to 1.0×10^7 parasites/mL, centrifuged through two more washes, and serially diluted to attain densities of 1.0×10^6 parasites/mL and 1.0×10^4 parasites/mL (for inoculum of 100 µL, 1.0×10^5 parasites/crab and 1.0×10^3 parasites/crab, respectively).

In both experiments, crabs were monitored daily for mortalities. Deaths within the first nine days of each experiment were excluded because of handling stress arising from infrequent, bacterial infections (e.g. Johnson, 1976). None of the crabs in the experiments were infected with amoebae, microsporans, or overt bacterial infections (but see Welsh and Sizemore, 1985 for background levels of *Vibrio* spp. in hemolymph of *C. sapidus*). Ten crabs from each treatment in the mortality-I experiment, and all of the crabs in the mortality-II experiment were bled approximately weekly to assess infection status. In the mortality-I experiment, the same ten crabs were bled approximately weekly until they died; other crabs from within the experiment were added as replacements.

Crab hemolymph was taken by using a tuberculin syringe (1 mL) with a 25.5-ga. needle from the arthrodial membrane at the juncture of the basis and the ischium of the 5th pereopod (swimming leg). Ethanol (70%) was used to sterilize the site of inoculation and blood letting. Total and differential counts of host hemocytes and estimates of parasite density were obtained from individual crabs with a hemocytometer (Neubauer improved, Bright Line, two counts per crab) with phase contrast microscopy at 400×. Host hemocytes were identified as granulocytes, semigranulocytes (intermediate cells with relatively few granules, Bodammer, 1978; Johnson, 1980) and hyalinocytes (cell types defined in Söderhäll and Cerenius, 1992). Hemocyte and parasite densities higher than 1.0×10^7 cells/mL were diluted 1:5 with buffer and recounted to provide better estimates of cell density. For comparative purposes, total hemocyte densities and differential counts from naturally infected male and female crabs were also obtained.

Parasites were easily distinguished from host cells by using phase contrast microscopy (Fig. 1): uninucleate trophonts (9–15 µm) possessed few small, refractile vacuoles and were rounded or amoeboid, without filopodia; multinucleate plasmodia (20–100 µm) were slender, vermiform, and motile. The density of infection refers to the number of parasites per mL of hemolymph. Total hemocyte density refers to the number of hemocytes per mL of hemolymph. Mean intensity refers to the mean number of parasites per quantity of *infected* host tissue (Margolis et al., 1982).

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

The early infection experiment consisted of a control group (n=5 crabs) injected individually with 100 µL of hemolymph from an uninfected donor crab and an experimental group (n=20) injected with 100 µL of hemolymph from a donor crab containing an estimated 4.1×10^6 parasites/ml (4.1×10^5 parasites per crab; comprising 79% plasmodia, 21% trophonts). Three days prior to infection, cell counts were conducted on all crabs to serve as a benchmark (presample) for before-after comparisons. On days 3, 5, and 7 after inoculation, five infected crabs were bled and dissected. Differential cell counts were conducted and tissue samples taken for histological analysis. Tissue samples were processed through a routine hematoxylin and eosin procedure and included muscle, hepatopancreas, heart, and, in some cases, foregut. The control crabs were bled and tissue samples taken 10 days after injection.

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

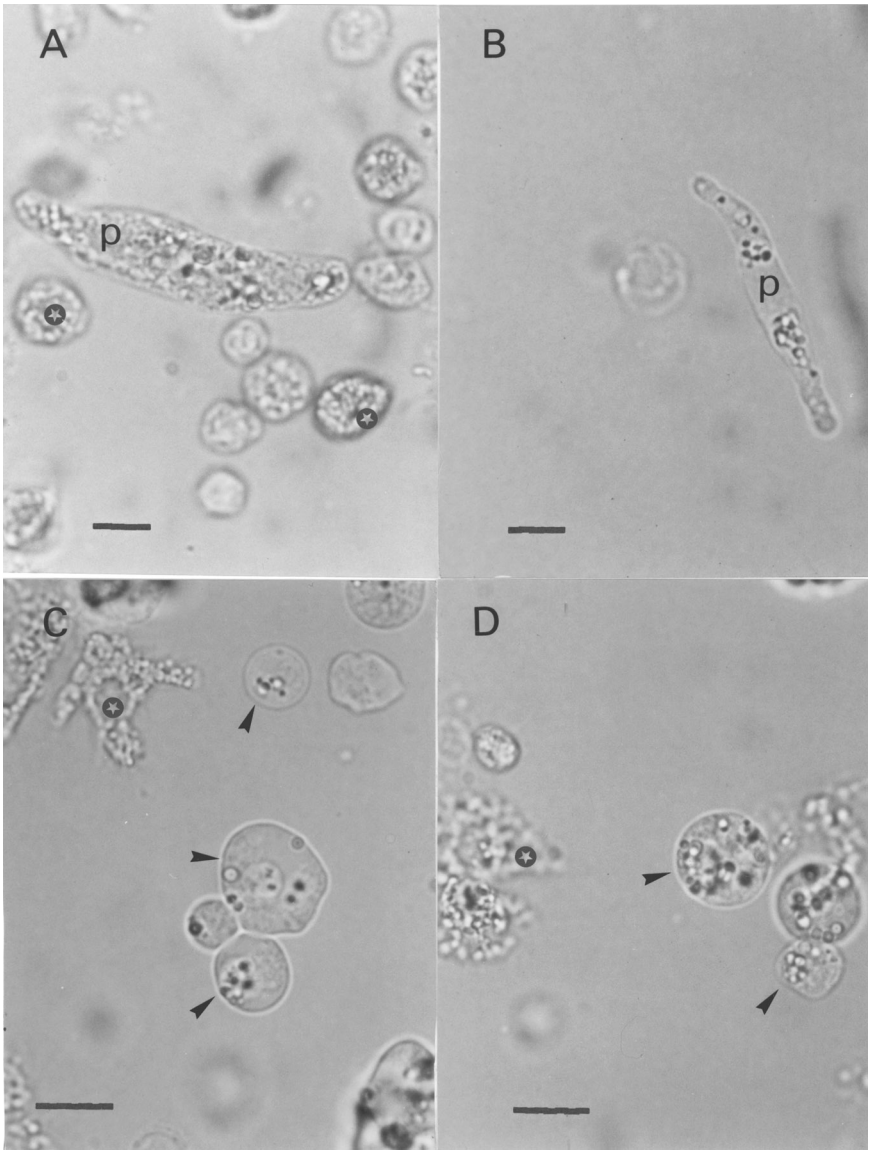


Figure 1

Hematodinium perezi from the blue crab, *Callinectes sapidus*. (**A and B**) Vermiform plasmodia (p) in hemolymph (granulocytes, star). Bar = 10 µm. (**C**) Amoeboid trophonts (arrows) with few refractile granules. Bar = 10 µm. (**D**) Round trophonts (arrows) with many refractile granules. Bar = 10 µm.

lar densities and proportions of cell types were noted in hematology and survival between the mortality-I and mortality-II experiments; hence, data were combined *a posteriori* for the analyses. Where similar trends were noted between statistics for injection dosage (10^3 vs. 10^5), data were also combined for the analysis (i.e. survivorship, hematology). SYSTAT (Wilkinson, 1997) and SAS (SAS, 1988) were used for the analyses. A probability level of $P < 0.05$ was accepted as significant.

Results

Inoculated crabs that became infected with *Hematodinium perezi* began dying two weeks after inoculation (Fig. 2). Mortalities peaked at three weeks after injection and continued to accumulate from weeks 3 through 5. The mortality rate of the infected crabs was 86%, whereas less than 20% of the controls died. Crab mortalities were similar over the time course of infection between mortality-I (infected

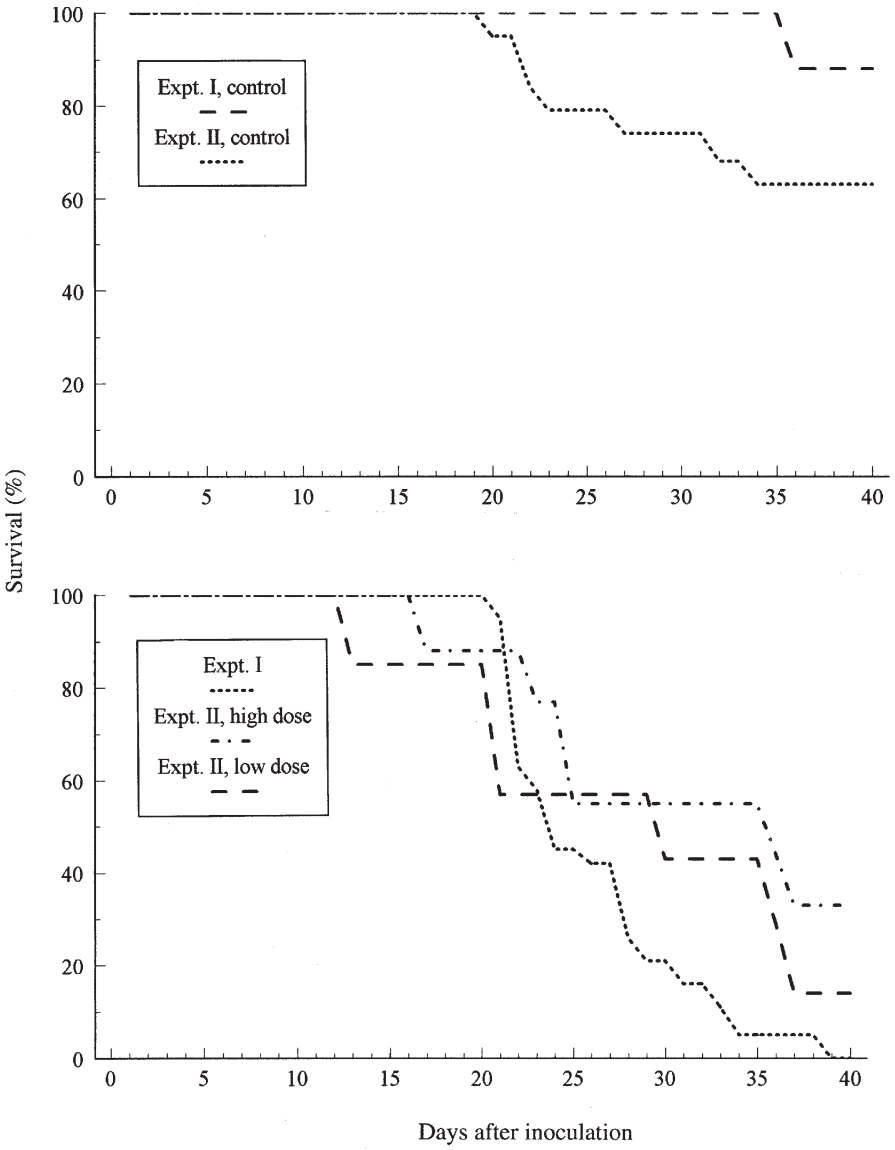
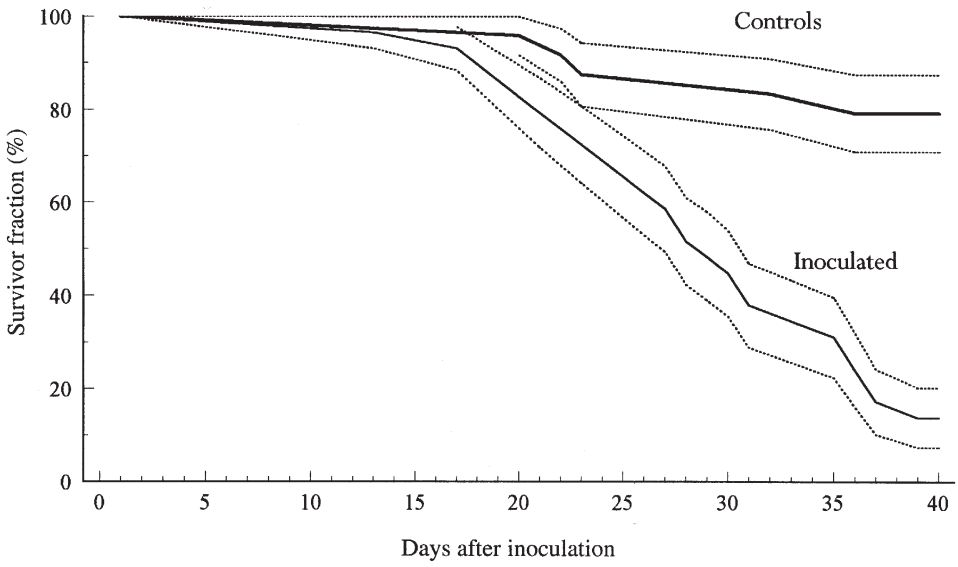


Figure 2

Survivorship curves for uninfected crabs and crabs infected with *H. perezi* (high dose=10^5 parasites/crab, low=10^3 parasites/crab). Sample sizes were 22 and 8 uninfected controls; 20 (mortality-I experiment), 10 and 10 (high and low dose, mortality-II experiment) respectively.

hemolymph, uninucleate trophonts) and mortality-II (buffer-washed parasites, vermiform plasmodia) experiments (Tarone-Ware, χ^2=1.21 with 1 df, P=0.27), even between different initial doses of the parasite (Fig. 2; Tarone-Ware, χ^2=0.74 with 1 df , P= 0.39). Uninfected crabs (controls) experienced significantly fewer mortalities than did infected hosts (Fig. 3; Tarone-Ware, χ^2=, 19.27 with 1 df , P<0.001). The controls for the mortality-II experiment did, however, exhibit background mortalities (Fig. 2); but the mortality rate was not significantly different from controls in the mortality-1 experiment (Tarone-Ware, χ^2=0.65 with 1 df, P= 0.42). None of the control crabs developed infections with *H. perezi*. Because mortalities within treatments were similar between experiments, data were grouped for further analysis.

The median time to death for infected crabs was 30.3 ±1.5 (SE) days. Because the controls exhibited few mortalities, the median time to death for the uninfected controls could not be calculated. Infected crabs had a significantly higher mortality rate, seven to eight times greater than that of the uninfected con-



VARIABLE	ESTIMATE	SE	χ^2	P
Intercept	2.933	0.106	763.33	0.0001
Uninfected	1.055	0.287	13.50	0.0002
Injected	0	0	.	.
Scale	0.517	0.083		

Figure 3

Survival function (Kaplan-Meier estimation) for uninfected crabs and crabs infected with *H. perezi*. Dashed lines are upper and lower standard errors. Data include all crabs from mortality-I and mortality-II experiments. Sample sizes are given in Figure 1.

trols (Fig. 3; proportional hazards, χ^2=13.50, P<0.001; relative risk=$e^{1.055/0.5174}$). Hemocyte and parasite density were jointly analyzed as covariates in the proportional hazards model. For injected crabs, the decline in ln(total hemocyte density) was significantly associated with mortality (ln *day of death* = 0.875 + 0.145 ln *total hemocyte density* – 0.017 ln *Parasite density* + 0.409 W; χ^2=4.47 with 1 df, P<0.05). Hemocyte density (untransformed), and parasite density (ln, and untransformed) were not associated with mortality (χ^2, P=0.07, 0.61, and 0.47, respectively); thus, decreases in hemocyte density (ln), not parasite density, were associated with imminent death.

Direct observations from crabs used to maintain infections and experimental results indicated that the parasite was detectable in the hemolymph approximately two weeks after injection (Fig. 4). Although the parasite could be detected as early as one week after inoculation, detectability (the percentage of infected crabs exhibiting detectable parasites in the hemolymph) was relatively low (30–35%) after 14 to 18 days, reaching 80–85% after 26 to 32 days, and 100% after 35 days. (Detectability was based solely on inoculated animals that developed infections. The four crabs from the mortality-II experiment that did not present infections, hereafter referred to as "immune" crabs, were excluded from the analysis of detectability.) Proliferation and growth of the parasite followed a similar pattern as detectability, and the two variables were clearly related (Table 1; Fig. 4).

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

Plasmodia were found within the hearts of 93% (n=14/15) of the injected crabs in the early infection experiment. Plasmodia were found in 4 of 5 crabs as early as day 3 (Table 2). Uninucleate trophonts were observed in the heart on and after day 7. Rel-

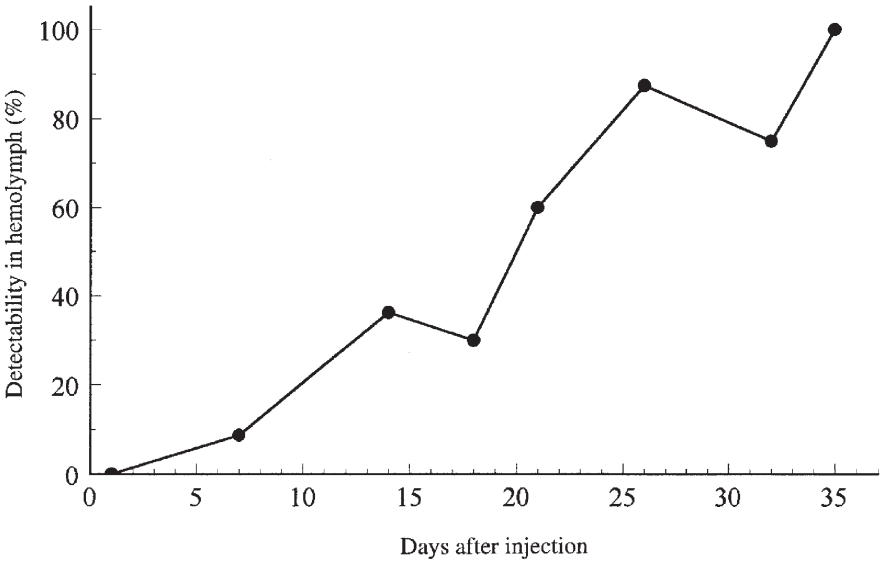


Figure 4

Detectability of parasites in hemolymph of infected blue crabs over the course of infection. Data were combined from mortality-I and mortality-II experiments and include only infected crabs. Samples sizes were 21, 11, 10, 10, 16, 4, and 4 crabs on days 7, 14, 18, 21, 26, 32, and 35, respectively.

Table 1

Parasite intensity ($\times 10^5$ parasites/mL per infected host) in the hemolymph in relation to days after inoculation. Counts combined for mortality-I and mortality-II experiments. N_{plas} = crabs with plasmodia, N_{troph} = crabs with trophonts, $N_{infected}$ = infected crabs exhibiting parasites. See Table 3 for sample sizes for hemocytometry.

		Plasmodia	log(plasmodia)		Trophont	log(trophont)	
Days	N_{plas}	Mean ±SE	Mean ±SE	N_{troph}	Mean ±SE	Mean ±SE	$N_{infected}$
7	1	0.50 ±0.00	4.70 ±0.00	1	0.25 ±0.00	4.40 ±0.00	1
14	4	1.44 ±0.53	5.09 ±0.16	3	1.25 ±0.29	5.06 ±0.11	4
18	1	1.25 ±0.00	5.10 ±0.00	2	1.00 ±0.25	4.99 ±0.11	3
21	5	7.70 ±4.61	5.61 ±0.24	5	8.65 ±4.17	5.87 ±0.18	5
26	11	14.98 ±6.07	5.85 ±0.17	12	8.19 ±3.86	5.40 ±0.22	14
32	2	8.38 ±1.63	5.92 ±0.09	3	4.25 ±1.32	5.59 ±0.14	3
35	4	7.88 ±3.89	5.69 ±0.25	4	49.81 ±36.82	6.35 ±0.31	4

atively more parasites were observed in the heart tissue over time (Table 2); but no effort was made to standardize area in the histological preparations. Growth of the parasite was rapid in the heart. The dosage in the early infection experiment was, however, four times higher than that in the mortality-I and mortality-II experiments; thus, results between experiments were not directly comparable.

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

Hemograms of infected crabs were significantly different from those of uninfected controls (Tables

3 and 4, Fig. 5). Total hemocyte density was significantly depressed in infected crabs (Fig. 5A; 2-way ANOVA by group and day, F=5.03, P<0.001). Total hemocyte density was not significantly different between crabs inoculated with different initial doses (2-way ANOVA, 10^3 vs. 10^5 parasites per crab and day, F=3.19, df=1, 64, P=0.079). Crabs that were injected and did not acquire the infection ("immune" hosts) did not have significant decreases in hemocyte densities (Table 3, Fig. 5; 2-way ANOVA, F=1.46, df = 13, 105, P=0.145). In the early infection experiment, the decrease in total hemocyte densities occurred within three days of inoculation (Table 5).

In addition to a decrease in cell density, the proportions of different host cell types (density of cell type divided by total hemocyte density) in infected crabs shifted to those with proportionally more granulocytes than hyalinocytes (Table 6, Fig. 5, B and D) (2-way ANOVA, F=1.83; df=20, 149, P<0.05). Significant shifts in the population of semigranulocytes were also noted (F=2.51, df =20, 149, P<0.001).

Table 2

Relative intensity of plasmodia in histological preparations of heart sections of mature, nonovigerous female blue crabs from the early infection experiment. Mean intensities represent direct counts of plasmodia and are not standardized by tissue area.

Day	$N_{Infected}/N_{Injected}$	Mean intensity (±SD)	Range
Control	0/4	0.0 ±0.0	—
3	4/5	3.6 ±3.9	1–10
5	5/5	12.0 ±11.0	1–26
7	5/5	55.8 ±26.1	15–74

Table 3

Total mortality-hemocyte densities ($\times 10^6$ hemocytes/mL) in relation to days after inoculation. Hemocyte counts were combined from mortality-I and mortality-II experiments. (-- = not done, no infected crabs survived to day 40).

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

Table 4

Total and differential hemocyte densities (mean ±SE; $\times 10^6$ hemocytes/mL) in naturally infected male and female blue crabs in relation to severity of infection (light < 4.0×10^5 parasites/mL; moderate = 4.0×10^5 to 2.0×10^6 parasites/mL; heavy > 2.0×10^6 parasites/mL; -- = not done).

Severity	n	Hemocyte density	Granulocyte density	Semigranulocyte density	Hyalinocyte density
Mature males					
Light	4	16.16 ±2.67	5.46 ±1.41	7.69 ±2.07	3.02 ±0.61
Moderate	6	7.46 ±1.74	1.92 ±0.63	4.10 ±1.10	1.45 ±0.46
Heavy	16	6.66 ±2.53	1.34 ±0.53	3.39 ±1.35	1.93 ±0.76
Mature females					
Light	--	--	--	--	--
Moderate	5	22.41 ±11.25	9.32 ±5.15	5.44 ±2.85	7.65 ±3.66
Heavy	5	14.37 ±7.37	5.98 ±3.09	4.01 ±1.63	4.39 ±2.89

"Immune" crabs exhibited a fluctuation in cell types with significantly higher proportions of granulocytes to semigranulocytes during the first five weeks after inoculation (F=4.35, df =5, 18, P<0.01). By day 40, the hemograms of "immune" hosts were virtually identical to those of the uninfected controls (Table 7, Fig. 5, C and D).

In the early infection experiment, hemocyte populations shifted within the first three days of infection (Tables 5 and 6; ANOVA, log hemocytes, F=9.16; df =3, 31, P<0.01); the proportion of granulocytes in infected crabs increased significantly compared with the proportion of semigranulocytes (ANOVA, F=4.39, P<0.05). Uninfected crabs exhibited minor fluctuations in the proportion of granulocytes to that of hyalinocytes but the proportions were similar to those observed in the mortality-I and mortality-II experiments (Tables 6 and 7).

Discussion

In laboratory experiments, *Hematodinium perezi* caused significant mortality to infected mature, nonovigerous blue crabs. Infections were not always fatal (four crabs survived inoculation without developing infections), but the overall mortality to laboratory-inoculated crabs was high at 86% over 40 days. The proportional hazards model indicated that infected crabs were seven to eight times more likely to die than uninfected crabs. Infections in Tanner crab, *Chionoecetes bairdi*, and Norway lobster, *Nephrops norvegicus*, are frequently fatal to the host (Meyers et al., 1987; Field et al., 1992). The mortality of naturally infected Tanner crabs held in aquaria for 97 days was 67% (n=11) and hosts survived from 20 to 158 days in the laboratory. Uninfected Tanner crabs experienced no mortality during the course of the experiment (Meyers et al., 1987). Naturally infected Norway lobsters suffered mortality rates of 86% to 100% over 27 d and 75 d, respectively, and had mortality rates 2–4 times higher than uninfected lobsters, and most of the deaths occurred early in the course of the experiment (Field et al., 1992).

During epizootics, juvenile blue crabs have a higher prevalence of *H. perezi* than do mature hosts (Messick, 1994). Male blue crabs have a prevalence of infection similar to that for females along the

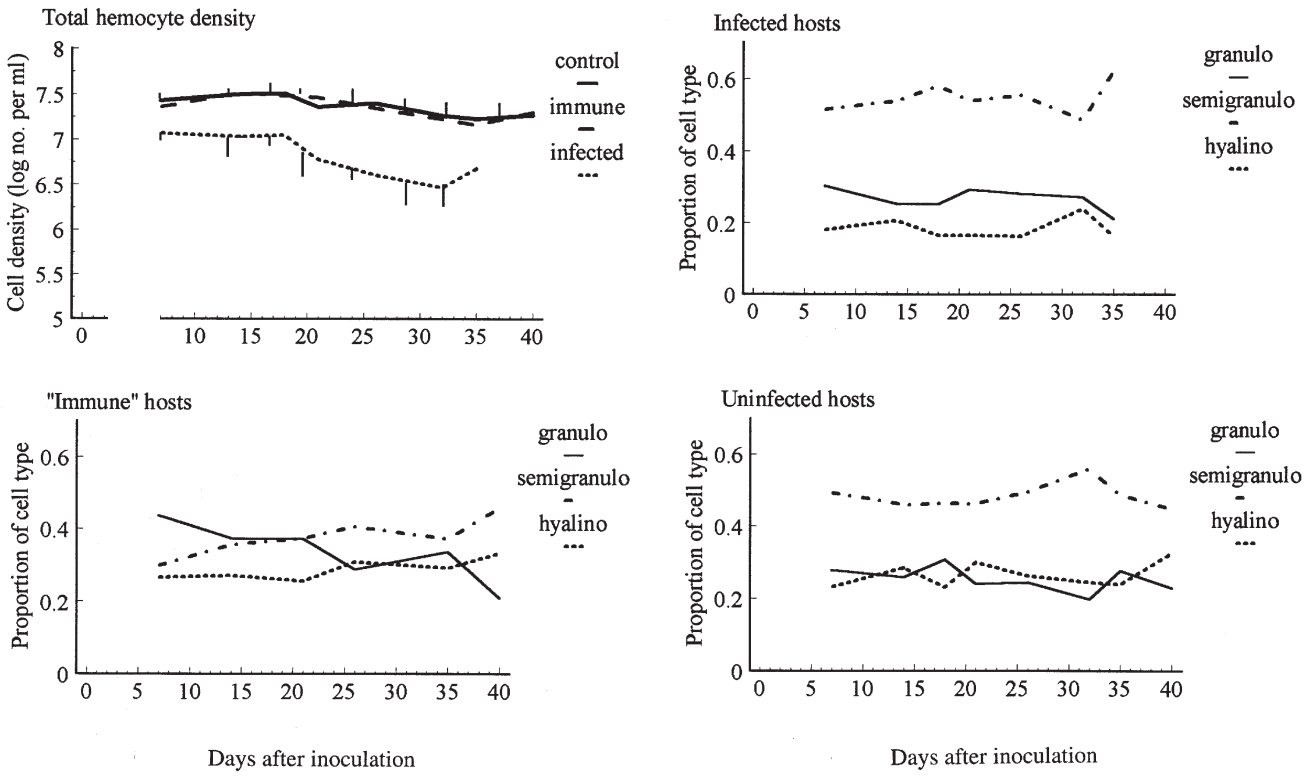


Figure 5

Total hemocyte densities and proportions of host cell types in uninfected, infected and "immune" crabs. Data combined from mortality-I and mortality-II experiments. Bars = SE. Standard errors (not shown) for proportion of host cell types were low (0.02–0.05). "Immune" crabs were survivors from mortality-II experiment that never developed infections. Sample sizes given in Table 3.

Table 5

Differential densities of hemocytes ($\times 10^6$ hemocytes/ml) in relation to days after inoculation for crabs in the early infection experiment. Sample sizes are given in Table 2.

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

Delmarva Peninsula (Messick, 1994; Messick and Shields[4]) and show significantly greater changes than females in certain blood parameters.[5] Infected blue crabs apparently die before acquiring the bitter flavor found in infected Tanner and snow crabs.

Survival analysis indicated that parasite density was not associated with mortality. Similarly, survival time of Norway lobsters did not show a significant relationship with severity of infection, but host mortality did increase with the progression of the disease (Field et al., 1992). In blue crabs, absolute declines in ln(total hemocyte density) were associated with host mortality. Hence, the cellular defensive response of the host appeared seriously compromised by infection. Anecdotal evidence from hosts used to maintain the parasite suggests that infections established with plasmodia are more pathogenic than those established with trophonts; this may explain the similar mortality curves for the high and low doses of plasmodia in the mortality-II experiment. Observations on naturally and experimentally infected crabs indicate three possible outcomes to the disease:

1 Crabs with acute infections, such as those reported here, show rapid mortalities, typically dying within 40 d. Acute infections rarely lead to heavy infections (10^{7+} parasites/mL), and may not lead to the development of dinospores.

Table 6

Total hemocyte densities ($\times 10^6$ hemocytes/mL) in relation to days after inoculation (-- = not done) in the early infection experiment.

	Uninfected control crabs		Inoculated, infected crabs	
		Hemocyte density		Hemocyte density
Days	*n*	Mean ±SE	*n*	Mean ±SE
Presample	5	15.91 ±1.25	20	23.28 ±2.06
3	--	--	5	12.11 ±0.50
5	--	--	5	9.39 ±2.94
7	--	--	5	11.40 ±0.96
10	5	23.98 ±4.87	--	--

2 Crabs with chronic infections (observed in very cases, n=4) endure the acute stage, survive for longer periods (up to 90 days), and develop infections that lead to massive numbers of dinospores (Fig. 6).
3 Some crabs successfully resist the parasite or are refractory to the infection. Preliminary experiments (not shown) suggest that resistant crabs (n=10) may become refractory to further inoculations with *H. perezi*.

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

[4] Messick, G. and J. D. Shields. 1999. The epizootiology of the parasitic dinoflagellate *Hematodinium perezi* in the blue crab, *Callinectes sapidus*. Oxford Cooperative Laboratory, 904 S. Morris St., Oxford, MD 21654. Unpubl. data.

[5] Shields, J. D. 1999. Mortality and pathophysiology studies of blue crabs infected with the parasitic dinoflagellate *Hematodinium perezi*. Saltonstall-Kennedy Program. NOAA/National Marine Fisheries Service. Final Report.

Table 7

Differential densities of hemocytes ($\times 10^6$ hemocytes/mL) in relation to days after inoculation. Hemocyte counts were combined from mortality-I and mortality-II experiments (-- = no data, no infected crabs survived to day 40). Sample sizes are given in Table 3.

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

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

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

Hematodinium perezi was successfully transmitted to blue crabs by injection. Transmission experiments

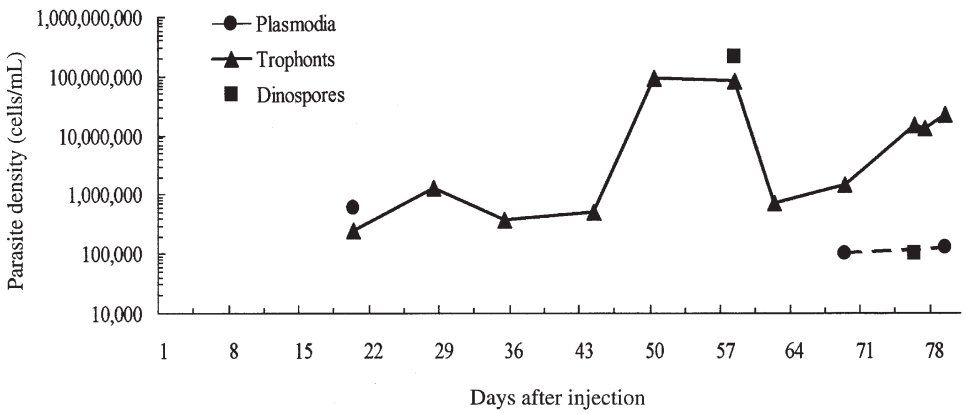


Figure 6

Crab no. 3977 presenting a chronic, experimentally induced infection of *H. perezi* (plasmodia, circles; trophonts, triangles; dinospores, boxes). The crab died after 80 days.

with the parasite in Tanner crabs and Australian sand crabs (*Portunus pelagicus*) have been partially successful. Parasites from primary cell culture (with sterile hemolymph) were successful in establishing infections in Tanner crabs, but inoculation with vegetative stages did not produce infections (Meyers et al., 1987). Injections of trophonts (vegetative stages) were successful in producing infections in the sand crab, but other stages were not investigated (Hudson and Shields, 1994). Infection experiments with Norway lobster have not been reported. Transmission with cultured dinospores has yet to be achieved (Appleton and Vickerman, 1998). Sporulation is a rapid event with *H. perezi,* presumably occurring over several hours instead of several days or weeks as reported for *Hematodinium* sp. from Tanner crabs (Meyers et al., 1987, 1990). At lower temperatures and salinities, *H. perezi* apparently ceases to grow or slows its proliferation in naturally infected blue crabs.[6]

Densities of circulating hemocytes declined rapidly in infected blue crabs. The decline occurred within the first three days and progressed to a 48% decrease in total hemocyte densities within the first week of infection. After three weeks, absolute declines of up to 80% were noted for total hemocyte densities. The loss of cells was evident early in the infection even though the parasites were not detectable in hemolymph. Declines in hemocyte densities have been reported for starved lobsters (33–60% loss) (Stewart et al., 1967), *Aeromonas*-infected lobsters (80–84% loss) (Stewart et al., 1983), *Fusarium*-infected brown shrimp, *Penaeus californiensis* (approximately 88% loss) (Hose et al., 1984), and *Vibrio*-infected *Cancer irroratus* (95% loss) (Newman and Feng, 1982). Reductions in hemocytes were noted for Norway lobster, *N. norvegicus*, infected with *Hematodinium* sp. (Field and Appleton, 1995), and for blue crabs, *C. sapidus*, infected with *Paramoeba perniciosa* (Sawyer et al., 1970), but the degree of loss, and differential counts were not quantified. Declines in hemocyte counts occur quickly in crayfish, *Pacifastacus leniusculus,* (10 min) and are associated with loss of resistance to *Aphanomyces* infections; the declines are dependent upon the stimuli (yeast vs. zymosan vs. buffers), and are evident over the course of several days (Perrson et al., 1987).

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

[6] Messick, G. 1998. Oxford Cooperative Laboratory, 904 S. Morris St., Oxford, MD 21654. Personal. commun.

Sparks, 1980), changes in mitotic stimuli of hemopoetic tissue (Hose et al., 1984), or sequestration of specific cell types in defense of the infection (e.g. nodule formation, Johnson, 1976, 1977). Hyalinocytes and semigranulocytes are the major phagocytic hemocytes in crustaceans (Bachau, 1981; Hose et al., 1990). Such hemocytes form nodules in bacterial, amoebic, and *Hematodinium* infections in blue crabs, *Hematodinium* infections in *N. norvegicus,* and gaffkemia infections in *Homarus americanus* (Johnson, 1976, 1977; Johnson et al., 1981; Messick, 1994; Field and Appleton, 1995) are thus removed from circulation, and may account, in part, for the observed declines. In fungal infections of crayfish, *P. leniusculus*, hemocytic nodules do not dissociate in the presence of zymosan, a yeast derivative, and may last several days (Perrson et al., 1987). In *Aeromonas*-infected lobsters, the hyalinocytes increase in proportion to the other cell types, presumably by increased mitotic activity in hemopoeitic centers, but there is a significant decline in hemocytes after five days of infection (Stewart et al., 1983).

Lastly, several blue crabs (n=4) successfully fought off the infection. These "immune" crabs exhibited significant sustained levels of granulocytes, did not suffer hemocytopenia, their hemolymph clotted normally, and they did not exhibit gross changes in morbidity. Histological preparations of heart, hepatopancreas, muscle, and hemopoeitic tissues were negative for latent infections in the "immune" animals. The relative increase in semigranulocytes and sustained densities of hyalinocytes over time (Fig. 5) suggests an increase in mitotic activity in hemopoetic tissue in response to the infection. This increase may not be sufficient to counter the parasite in susceptible hosts. In *N. norvegicus* infected with *Hematodinium* sp., the hemopoeitic tissues show marked increases in activity. Although changes in host cell densities were not quantified, apparent stem cells were present in the active nodes (Tables 1 and 2 in Field and Appleton, 1995). The role of the granulocytes in the defense against *Hematodinium* infections and the underlying mechanisms leading to refractory hosts warrant further study.

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