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ASPECTS OF THE PATHOPHYSIOLOGY OF BLUE CRABS, *CALLINECTES SAPIDUS*, INFECTED WITH THE PARASITIC DINOFLAGELLATE *HEMATODINIUM PEREZI*

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ABSTRACT

Blue crabs, *Callinectes sapidus,* infected with *Hematodinium perezi* frequently show signs of weakness and lethargy and die when stressed by handling or capture. Radical changes to the hemolymph of heavily infected crabs are obvious by reduced clotting ability, discoloration, and a 50% to 70% decline in total hemocyte density. Few other signs of infection are associated with infections and the resulting mortalities of blue crabs. To assay physiological changes in infected crabs, we measured serum proteins, hemocyanin, serum acid phosphatase, various hemolymph enzymes, hemagglutination activity, and tissue glycogen levels in relation to intensity of infection with *H. perezi.* Serum proteins and hemocyanin levels were lower in infected versus uninfected males, but not in infected versus uninfected females. Acid phosphatase activity was directly related to infection by the parasite. Acid phosphatase activity in the hemolymph was below the detection limit in uninfected crabs, but was detectably high in lightly, moderately and heavily infected crabs. Hemagglutination, possible indicator of innate humoral defense activity, was not affected by infection. Glycogen levels in the hepatopancreas of infected crabs decreased by 50% in females and 70% in males compared to controls. Infection by *H. perezi* caused significant alterations to the hemolymph chemistry and metabolism of the crab. Changes in serum proteins, hemocyanin, and glycogen levels in heavy infections indicate that crabs probably die from metabolic exhaustion.

Hematodinium perezi is a parasitic dinoflagellate that proliferates in the hemolymph of brachyuran crabs. In the American blue crab, *Callinectes sapidus,* the parasite is highly pathogenic, and occurs in recurrent epizootics along the eastern seaboard of the USA (Messick and Shields, 2000). The disease occurs in blue crabs in saline waters (>12‰) from Delaware to Florida (Newman and Johnson, 1975) and into the Gulf of Mexico (Couch and Martin, 1982; Messick and Shields, 2000). Since 1992, prevalences have reached 70% to 100% in crabs from coastal bays in Maryland and Virginia with lower prevalences (0.1–10%) in eastern portions of lower Chesapeake Bay (Messick, 1994; Messick and Shields, 2000). Prevalence shows a sharp peak in autumn with juveniles having a higher prevalence and intensity of infection than adults (Messick, 1994). The parasite may overwinter in infected crabs (Messick et al., 1999). Experimentally infected blue crabs typically die over 14 to 40 days post inoculation and experience a mortality rate of 86% (Shields and Squyars, 2000; Shields, 2001, in press).

At present only two species of *Hematodinium* have been described*: H. perezi* Chatton and Poisson 1931, and *H. australis* Hudson and Shields 1994. Newman and Johnson (1975) and MacLean and Ruddell (1978) identified the species from the blue crab as *H. perezi* based on distinct morphological features. Whereas this diagnosis may be questioned, until comparisons are made with the type species, we follow the convention of Newman and Johnson (1975) and MacLean and Ruddell (1978).

Blue crabs support the largest commercial fishery within Chesapeake Bay, with annual harvests ranging from 80–100 million pounds (Kirkeley, 1997; Johnson et al., 1998). *Hematodinium perezi* may cause losses of as much as $1.5 million during major epizootics with annual losses approaching $250,000 to $500,000 in Virginia alone (based on simple estimates from annual landings, Shields unpubl.

data). Fishing pressure is often cited for the recent declines in blue crab stocks, but disease and environmental processes have not been well examined. *Hematodinium*-like dinoflagellates have impacted other fisheries. Parasitic dinoflagellates cause bitter crab disease in the Tanner and snow crab fisheries (*Chionoecetes bairdi, C. opilio,* Meyers et al., 1987; Taylor and Khan, 1995), as well as severe, costly mortalities to the Norway lobster (*Nephrops norvegicus,* Field et al., 1992) and velvet crab fisheries (*Necora puber,* Wilhelm and Miahle, 1996). The effects of *Hematodinium*-like species on other large fisheries, and our current data (Messick and Shields, 2000), indicate that *H. perezi* has a significant impact on the coastal blue crab fisheries along the Atlantic seaboard of the U.S.A.

Unfortunately, background mortalities due to *Hematodinium* are often difficult to assess as dead crabs quickly deteriorate and become undiagnosable. Thus, our primary objectives were to examine selected biochemical and physiological markers to gauge aspects of the pathophysiology of the infection and to investigate underlying causes of death in crabs infected with *H. perezi.* We examined total serum proteins, hemolymph acid phosphatase activity, the presence of selected enzymes in the hemolymph, hemagglutination ability, and glycogen concentrations in the hepatopancreas between infected versus uninfected crabs. The influence of host sex was examined for several of the biochemical constituents.

METHODS

CRAB COLLECTION.—Crabs were collected with commercial crab pots from two reference locations on the Delmarva Peninsula: Red Bank and Hungars Creeks, Virginia. Additional samples were obtained from crab pots and trawls in Wachapreague Creek and Wachapreague Inlet, Virginia. Crab pot samples were biased towards mature crabs. Sampling within lower Chesapeake Bay was done in conjunction with the VIMS Trawl Survey and the VIMS Blue Crab Dredge Survey as described in Shields and Squyars (2000). Crabs were chilled on ice for transportation to the laboratory. In some cases, crabs were held in static 5 gal. (19 liter) aquaria for further observations. For the tissue analyses, intermolt blue crabs were collected in autumn (primarily October) with some minor exceptions.

HEMOLYMPH ANALYSES.—Crab hemolymph was removed from the axillae of the 5th leg (swimmer) using a 27 ga. needle with a 1.0 ml tuberculin syringe. The hemolymph was examined as a wet smear with an additional smear being processed through a Harris hematoxylin and eosin staining procedure as in Messick (1994). Wet smears were observed at 400×, prepared smears with oil immersion at 1,000×. Parasite intensities (number of parasites per 100 host cells) were obtained by direct counts of at least 300 cells.

COLLECTION OF HEMOLYMPH AND HEPATOPANCREAS.—Hemolymph samples were drawn with a sterile syringe and placed in a microcentrifuge tube. Serum samples were obtained by clotting fresh hemolymph on ice for 30 min, macerating the clot with a tissue grinder, centrifuging (4,000 rpm, 10 min, room temperature, Fisher Scientific Micro 7), and freezing the supernatant at −80°C. Whole hemolymph was obtained by drawing 1.5 to 2.0 ml fresh hemolymph into a syringe, aliquoting into microcentrifuge tubes on ice, and freezing immediately at −20°C followed by transferring to −80°C. Portions of muscle and hepatopancreas were dissected, wrapped in heavy duty aluminum foil, placed in plastic freezer bags, and stored at −80°C for later analyses.

HEMOLYMPH CONSTITUENTS.—Total proteins were analyzed with the biuret method as in Lynch and Webb (1973). Serum samples were processed using a standard kit (Sigma #541), and read with a spectrophotometer (Genesys 5) at 540

nm. Controls consisted of diluted samples of bovine serum albumen (BSA), and hemolymph samples spiked with known quantities of BSA. Hemocyanin assays with sera were measured with a veterinary-grade serum protein refractometer (Westover RHC-200). Acid phosphatase in whole hemolymph was measured using p-nitrophenyl phosphate as substrate (Andersch and Sczypinski, 1947) with a commercial kit (Sigma #104), and read at the absorbance maximum of 420 nm. Acid phosphatase activity was determined from a standard curve constructed using p-nitrophenol and expressed as concentration (units/ml of serum). Controls were applied as per kit instructions. For statistical analyses, samples that were below the minimum detectable level (0.1 SU/ml) were conservatively assigned a value of half the minimum detectable level.

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

HEMOCYANIN.—Hemocyanin measurements were modified from Engel et al., 1993. Briefly, hemolymph serum samples were diluted 1:10 (in some cases to 1:30) with buffer (50 mM Tris, 2.5% NaCl, 10 mM EDTA, pH 10.0) in 1.5 ml cuvettes, and absorbances measured with a spectrophotometer at 338 nm. The concentration of hemocyanin was calculated as $E_{338nm} = 2.33$ for intact undissociated hemocyanin (Nickerson and Van Holde, 1971). Hemocyanin concentration was calculated as (dilution × absorbance)/2.33 expressed as g/100 ml.

HEMAGGLUTINATION ASSAY.—Activity levels of serum hemagglutinins were quantified for individual blue crabs. Aliquots of 50 μl crab serum were serially diluted on a 96-well microtiter plate to 1:1,028 using a Tris-NaCl dilution buffer (50 mM Tris, 0.15 M NaCl, pH 7.5). Freeze-dried sheep (Sigma R3378), rabbit (Sigma R1629), or chicken (Sigma R0504) red blood cells (RBC) were centrifuged and resuspended through three changes of Tris-NaCl buffer, adjusted to a final preparation of 2% RBCs (v:v) and added as 50 μl aliquots to each microtiter plate. Each plate was gently mixed, incubated, and examined at 2 and 24 h for hemagglutination. Controls consisted of a well containing 50 μl of RBC suspension with 50 μl buffer. The titer for a given sample was expressed as the reciprocal of the maximum dilution exhibiting hemagglutination.

TISSUE GLYCOGEN.—The anthrone oxidation method was used to assay for glycogen (modified after Van Handel, 1965; Baturo et al., 1995). Aliquots of hepatopancreas (0.5 g, triplicate samples) were individually placed in 4.0 ml de-ionized water mixed with 1.0 ml 30% potassium hydroxide solution (w:v) and boiled for approximately 20 minutes. The samples were cooled on ice for 2–3 min, then homogenized (Power Gen 125, 7 × 195 mm generator). The homogenates were mixed with 6.0 ml 100% ethanol and 100 μl saturated sodium sulfate solution (1 g Na_2SO_4 in 3.6 ml deionized water), and boiled for 1 min. Upon removal, the samples were centrifuged at 2,000 g for 20 minutes. The supernatant was removed from each tube and the precipitates were dried at 60°C overnight or until dry. The precipitates were then resuspended in 500 μl deionized water, mixed with 3.0 ml 0.15% anthrone reagent (in 70% reagent grade H_2SO_4, made fresh daily) and heated for 10 to 12 minutes in a 90°C water bath. The samples were cooled to room temperature and read on a spectrophotometer at 620 nm. The amount of glycogen in each sample was calculated based upon the absorbances of the standards. Dilutions and tissue weights of the samples were considered when calcu-
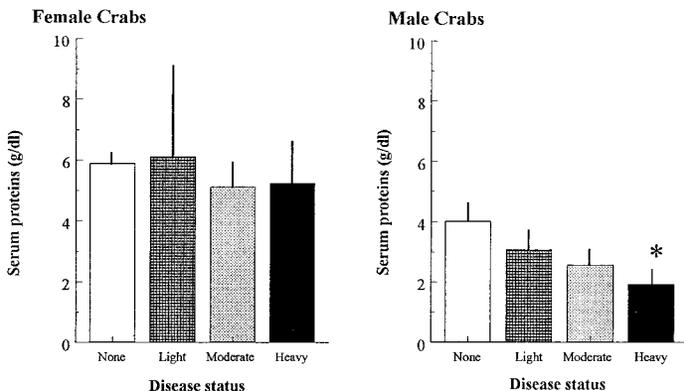
Figure 1.   Total serum proteins (biuret method) in blue crabs infected with *Hematodinium perezi* (* *P* < 0.05, ANOVA, Tukey's HSD, n = 12 per category except n = 6 lightly infected female crabs). Intensity levels were uninfected (white bar), lightly (hatched bar, 0.3–3 parasites/100 host cells), moderately (gray bar, 3.3–10 parasites per 100 host cells), and heavily (solid bar, 10+ parasites per 100 host cells) infected crabs.

lating quantities in tissues. For each daily series, a stock solution of oyster glycogen (1 mg/ml) was prepared and standards (1,000, 500, 250, 125, 62.5, 31.25, and 0 μg/ml solutions) were treated exactly as described above. In some cases, samples were spiked with known quantities of glycogen to serve as additional controls.

Statistical analyses included regression, ANOVA, contingency tables, and *t*-tests (Sokal and Rohlf, 1981; Wilkinson, 1997). A probability level of *P* < 0.05 was considered significant. Relationships between sex, level of infection (categorical: uninfected, light, moderate, heavy) and various constituents were examined with one- and two-way ANOVA and Tukey's HSD. Proportional differences in frequencies of responses for acid phosphatase assays were examined with contingency tests (Chi-square). Relationships between intensities of infection (number of parasites per infected host) and levels of each constituent were analyzed with Pearson product-moment correlations.

RESULTS

BASIC HEMOLYMPH CONSTITUENTS.—Total serum protein levels and hemocyanin concentrations exhibited significant changes in heavily infected crabs. Serum protein levels were 36% lower in heavily infected versus uninfected males (Figs. 1 and 3, ANOVA, *P* < 0.05 and *P* < 0.001 for biuret method and refractometer, respectively). Infected females did not, however, have lower serum protein values than their uninfected counterparts. The intensity of infection was negatively correlated with total serum proteins (refractometer) in infected males (Fig. 2; $r = -0.488$, untransformed; $r = -0.404$, $\log_{10}$-transformed, n = 40, *P* < 0.01), but not in infected females ($r = -0.254$, untransformed; $r = 0.082$, $\log_{10}$-transformed, n = 26, *P* > 0.05). Serum protein levels varied from 0.6 to 6.7 g/100 ml in uninfected males, and from 0.0 to 6.7 g/100 ml in infected males (biuret method). Protein levels varied from 3.6 to 10.4 g/100 ml in uninfected females, and from 1.1 to 12.1 g/100 ml in infected females (biuret method). As expected, total serum protein levels and hemocyanin values were highly correlated, but the relationship was more variable in females ($r = 0.725, 0.526$; n = 40, 26 for males, females, respectively). Lipoproteins, associated with oogenesis and vitellogenesis and mo-
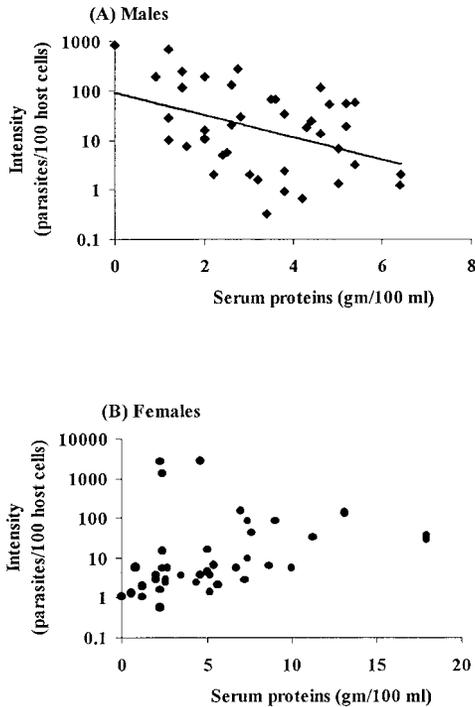
Figure 2.   Total serum protein (veterinary refractometer) in relation to intensity of infection with *H. perezi* in (A) male (regression, y = 91.967e$^{-0.5176x}$, $r = -0.404$, $P < 0.01$) and (B) female blue crabs ($r = 0.082$, $P > 0.1$). Note change in scale between sexes for serum values.

bilized in the hemolymph during reproduction, were not considered in the analysis, nor were gonadal indices measured.

Hemocyanin concentrations also declined in relation to the level of infection in male but not female crabs (Fig. 3). Hemocyanin was 42% lower in heavily infected males compared to uninfected males (ANOVA, $P < 0.001$), and the trend was apparent but not significant (due to high variance) in lightly and moderately infected males. Infected females did not, however, have lower hemocyanin values than uninfected females (ANOVA, $P > 0.50$). Hemocyanin values varied from 0.8 to 11.5 g/100 ml in uninfected males, and from 0.5 to 6.4 g/100 ml in heavily infected males. Hemocyanin levels varied from 0.8 to 9.7 g/100 ml in uninfected females, and from 1.9 to 9.1 g/100 ml in infected females. Intensity of infection was not correlated with hemocyanin levels for males nor for females ($r = -0.233$, -0.320; n = 40, 26, respectively).

ACID PHOSPHATASE AND OTHER ENZYME CONSTITUENTS.—Acid phosphatase activity in the hemolymph varied significantly with infection in the host (Table 1, Fig. 4). Uninfected crabs were significantly less likely to have detectable acid phosphatase activity in whole hemolymph (Chi-square = 19.03, df = 3, $P < 0.001$). Diseased crabs had much higher acid phosphatase activity in their hemolymph (up to 4.6 SU/ml), and significantly more infected crabs had detectable acid phosphatase activity (ANOVA, Tukey's HSD, $P < 0.001$, df = 3). In a moderately infected crab, whole hemolymph had high acid phosphatase activity (0.789 ± 0.051 SU/ml) while cell-free serum had no detectable activity. That is, acid phosphatase was located intracellularly in the parasite, and was not measur-
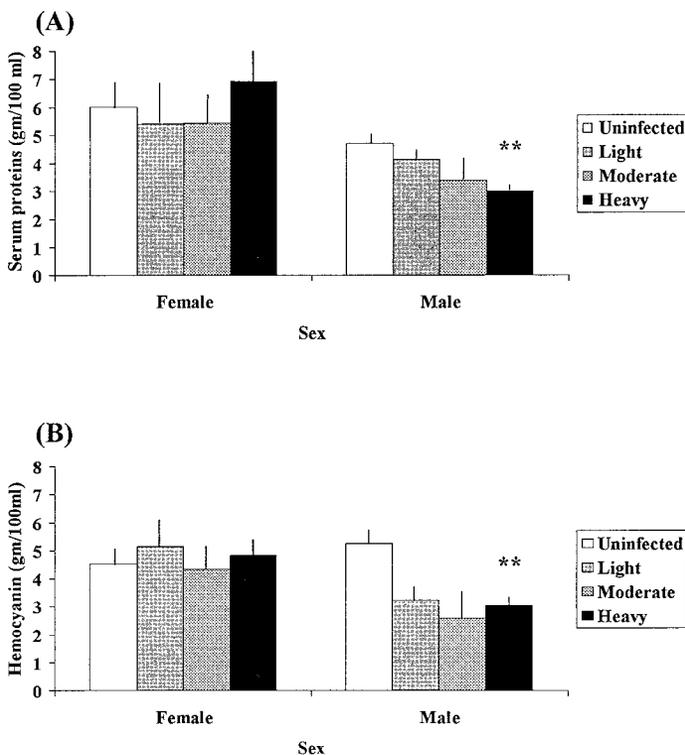
Figure 3.   (A) Total serum protein levels taken with a veterinary refractometer, and (B) hemocyanin levels in blue crabs infected *H. perezi*. (B) Hemocyanin levels in blue crabs infected with *H. perezi* (** $P < 0.01$, ANOVA, Tukey's HSD, n = 35, 4, 9, 19 females, and 75, 11, 5, 31 males, respectively).

able in the serum of at least one infected host. In general, enzyme activity was negligible in control animals (Fig. 4).

Sample sizes for the apiZYME enzyme analyses were small (9 uninfected vs. 8 heavily infected females) and exploratory in nature. Enzyme test strips showed several differences in enzyme levels between uninfected and infected sera ($P < 0.05$, $t_{0.05,\text{two-tailed}} = 1.753$). Infected sera showed significantly higher acid phosphatase ($t = 3.04$), napthol AS-BI phosphohydrolase ($t = 2.89$), and β-galactosidase ($t = 3.54$) activities. Uninfected hemolymph showed significantly higher ($t = 2.99$) α-fucosidase activity. β-glucuronidase was the only enzyme to show a potential proportional difference with 2 of 9 uninfected crabs vs. 7 of 8 infected

Table 1. Acid phosphatase activity in hemolymph of blue crabs infected with *Hematodinium perezi*. Infection level was defined as uninfected (no parasites in hemolymph), lightly (0.3–3 parasites/100 host cells), moderately (3.3–10 parasites per 100 host cells), and heavily (10+ parasites per 100 host cells) infected crabs.

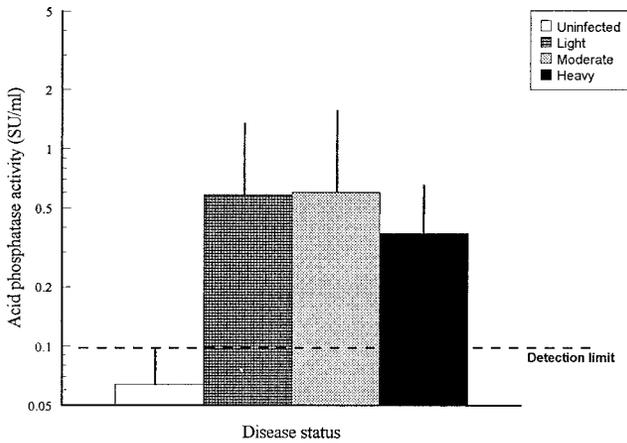| Status	Below detection (0.1 SU/ml)	Above detection
Uninfected	16	3
Light	4	4
Moderate	5	11
Heavy	2	13

Figure 4. Acid phosphatase activity (log scale) in the whole hemolymph of blue crabs infected *H. perezi*. Key to infection levels given in Table 1 and Figure 1; dashed line, detection limit for activity level.

crabs exhibiting noticeable acitivity of the enzyme. [NB: with a larger sample size, Chi-square may show this to be significant, but the means were not significant by *t*-test ($t = 1.49$) due to a high variance in enzyme activity.]

HEMAGGLUTINATION.—A large, replicated series of hemagglutination tests were performed using rabbit, chicken and sheep red blood cells (RBCs) (Tables 2 and 3). No significant differences in proportions (Chi-square), or means (as exponents, ANOVA) were apparent between levels of infection (uninfected, light, moderate, and heavy infections). Infected and uninfected sera reacted poorly to sheep RBCs, but both reacted highly with chicken RBCs. High titers (64+) were observed in some infected animals but there were no differences in the frequencies of animals showing high titers.

TISSUE GLYCOGEN.—Glycogen showed significant declines in the hepatopancreas of infected blue crabs (Fig. 5). Infected males and females showed major declines in glycogen levels in the hepatopancreas (2-way ANOVA with interaction, $P < 0.001$). In uninfected females, glycogen ranged from 4.93 to 10.54 mg/gm, and in infected females, from 2.57 to 5.02 mg/gm. In uninfected males, glycogen ranged from 6.33 to 10.68 mg/gm, and in infected males, from 1.51 to 5.63 mg/gm. The magnitude of the difference between infected males and females was also significant (interaction term, $P < 0.01$); that is, infected males exhibited a greater decline (70%) than infected females (50%). Glycogen levels were not different between uninfected males and females ($P > 0.05$). Glycogen in the muscle was not assessed.

Table 2. Proportion and percentage of individual hemolymph samples reacting with vertebrate red blood cells.

Infection status	Rabbit	Sheep	Chicken	n
Infected	50/68	31/68	59/68	36 male
	73.5%	45.6%	86.8%	32 female
Uninfected	17/24	9/24	24/24	12 male
	70.8%	37.5%	100%	12 female
RBC reactivity	Moderate	Low	High	

Table 3. Frequencies of serum dilutions at which agglutination occurred in uninfected, lightly (0.3–3 parasites/100 host cells), moderately (3.3–10 parasite per 100 host cells), and heavily (10+ parasites per 100 host cells) infected crabs.

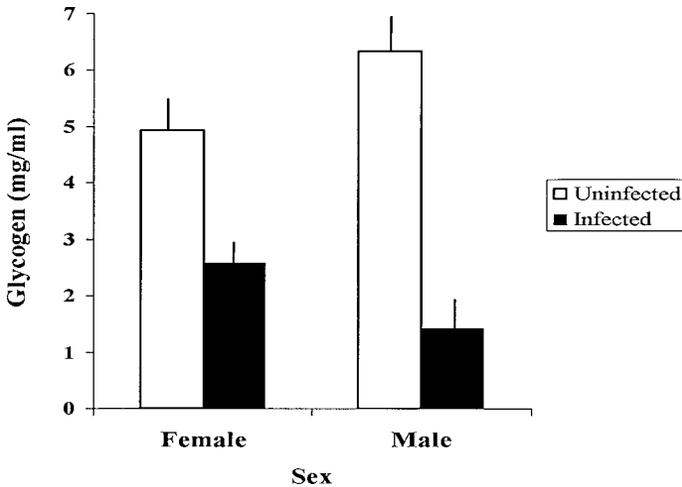| Serum dilution	Rabbit				Sheep				Chicken			
	Uninf	Light	Mod	Heavy	Uninf	Light	Mod	Heavy	Uninf	Light	Mod	Heavy
None	7	5	9	4	15	13	12	12	0	6	1	2
1:1	2	1	6	5	7	1	6	5	12	4	6	10
1:2	5	3	2	2	1	1	2	0	3	2	12	3
1:4	1	3	3	2	0	4	1	0	2	3	3	1
1:8	2	3	3	3	1	2	2	1	1	3	1	1
1:16	3	5	0	2	0	0	0	1	2	4	0	3
1:32	4	2	1	1	0	1	1	3	3	0	0	2
1:64	0	0	0	3	0	0	0	0	1	0	0	0
1:128	0	0	0	0	0	0	0	0	0	0	1	0

Figure 5.   Glycogen levels in the hepatopancreas of uninfected and heavily infected blue crabs (standard error shown, n = 12, 12, 12, 9, respectively with triplicate sampling for each individual).

DISCUSSION

Hematodinium infections elicit distinctly different pathophysiologies between sexes of the blue crab, *Callinectes sapidus.* Serum proteins, hemocyanin, and tissue glycogen were all more heavily altered in infected male versus infected female crabs. Given such pathophysiological differences, we speculate that the survival rate for infected males should be lower than that for females. A sex-biased pathogenicity should present as either a lower prevalence of the disease in males due to preferential mortality, or a higher prevalence in males due to a more rapid and observable fulmination of infection. In most cases, however, males have similar prevalences to females (Messick, 1994; Messick and Shields, 2000). Because female blue crabs show less alteration in serum proteins and hemocyanin, future pathophysiological studies of chronic diseases should assess host sexual differences.

BASIC HEMOLYMPH CONSTITUENTS.—Serum protein levels in several crustaceans are altered when infected by disease agents (Table 4). Based on available data, parasites found in the hemolymph and connective tissues (*H. perezi, P. perniciosa,* Gram negative bacteria, *Aerococcus viridans*) cause significant declines in serum proteins during heavy or late-stage infections. Concurrent declines are expected for hemocyanin given that it comprises up to 90% of the serum proteins. Conversely, in most cases, tissue-dwelling parasites that lead to chronic disease (microsporidans, rhizocephalans, acanthocephalans) cause elevated levels of serum proteins and hemocyanin (Table 4). Pathological processes may explain these patterns. In hemolymph infections the logarithmic proliferation of the pathogens coupled with their metabolic needs during rapid growth, and the resulting host lethargy, drain the protein and carbohydrate constituents of the host. Hemolymph glucose levels decline rapidly in such infections, in some cases reaching zero (Stewart and Arie, 1973; Pauley et al., 1975; Spindler-Barth, 1976). Starvation may be coupled with these disease processes as heavily infected crustaceans become lethargic or cease feeding (Stewart and Arie, 1973; Taylor et al., 1996; Shields, unpubl. data). Starvation can cause marked declines in serum protein and hemocyanin levels (Uglow, 1969a, 1969b; Stewart et al., 1972). In longer, chronic

Table 4. Changes in serum proteins, hemocyanin, glycogen and lactate in relation to disease agent for several crustaceans. (Expressed as the percentage differences from tissues in normal, uninfected hosts.)

Host	Parasite/disease	Serum proteins (%)	Hemocyanin (%)	Glycogen (%)	Hemolymph lactate (%)	Reference
Callinectes sapidus	*Hematodinium perezi*	M: −36* F: 0	M: −42 F: 0	M: −70 F: −50	nd	This study
C. sapidus	*Paramoeba perniciosa*	M: −79* F: −49*	—	nd	nd	Pauley et al., 1975
C. sapidus	*Ameson michaelis*	+133–200**	nd	mus: −18*** hp: 0***	+556	Findley et al., 1981
C. sapidus	*Loxothylacus texanus*	0	+	nd	nd	Manwell and Baker, 1963
Carcinus maenas	*Thelohania maenadis*	−24	nd	mus: −80 hp: 0	+367	Vivarès and Cuq, 1981
C. maenas	*Sacculina carcini*	0	0	0	nd	Uglow, 1969c
	Sacculina carcini	+37–42 +20		hp: −22		Smith, 1911, 1913; Damboviceanu, 1932; Drilhon, 1936; Drilhon and Pora, 1936
C. mediterraneus	*Sacculina carcini*	−23				Sanviti et al., 1981
Pachygraphsus marmoratus	*Sacculina carcini*	+13				Sanviti et al., 1981
Paralithodes platypus	*Briarosaccus callosus*	0	0	nd	nd	Shirley et al., 1986
P. camtschaticus	*Briarosaccus callosus*	+41	+29	nd	nd	Shirley et al., 1986
Lithodes aequispina	*Briarosaccus callosus*	+62	+118	nd	nd	Shirley et al., 1986
Uca pugilator	Gram negative bacteria	0	−54	mus: −90 hp: −65	nd	Spindler-Barth, 1976
Homarus americanus	*Aerococcus viridans*	0	−50†	mus: −55 hp: −73	+31	Stewart and Arie, 1973, Rittenburg et al., 1979
Nephrops norvegicus	*Hematodinium* sp.	nd	−35%†	nd	+1000	Taylor et al, 1996
Gammarus pulex	*Pomphorhynchus laevis*	+84	+38	nd	nd	Bentley and Hurd, 1993, 1995

0, no change; ++, noticeable increase, not quantified; —, noticeable decrease, not quantified; nd, not done.
* Values are from heavy infections. M: male, F: female.
** As ninhydrin positive substances (i.e., includes free amino acids).
*** As carbohydrates, mus = muscle, hp = hepatopancreas.
† As reduction in oxygen binding capacity.

infections where parasites such as rhizocephalans infect other tissues, host behavior is altered, and crabs exhibit behaviors associated with egg grooming (e.g., Bishop and Cannon, 1979). Such behavioral effects may elicit mild hypoxia as a metabolic effect of parasitism due to tissue destruction (e.g., Taylor et al., 1996), or make the host more susceptible to fouling organisms which then interfere with respiration (Gannon and Wheatly, 1992, 1995), or lead to increases in hemocyanin levels as a host response (see Engel et al., 1993). In addition, several tissue-dwelling parasites lyse or alter the surrounding tissues causing increases in free amino acids, protein constituents and enzymes (Vivarès and Cuq, 1981; Stentiford et al., 1999).

The respiratory physiology of Norway lobster, *Nephrops norvegicus,* is seriously affected by *Hematodinium* infections. Copper levels in the hemolymph of infected Norway lobster were 35% lower and oxygen carrying capacity of the hemocyanin was 43% lower than uninfected controls (Field et al., 1992; Taylor et al., 1996). In addition, oxygen consumption in heavily infected lobster is similar to that of lobsters undergoing exercise, and is probably related to large numbers of parasites in the hemolymph (Taylor et al., 1996). Serum proteins levels were not altered in *Aerococcus viridans* infections in *Homarus americanus* (Stewart et al., 1969), yet the oxygen binding capacity of hemocyanin was impaired by 50% (Rittenburg et al., 1979), albeit hemocyanin levels were not assessed. The reduction in carrying capacity of the hemocyanin may explain the decline in tissue glycogen and ATP activity (Stewart and Arie, 1973), and suggests a lack of intracellular oxygen rather than nutrient depletion as the ultimate cause of death in infected lobsters (Rittenburg et al., 1979; see also Stewart, 1980). The reduction of serum proteins, hemocyanin, and glycogen levels in heavily infected male blue crabs suggests that nutrient depletion is the primary cause of death (see below). Regardless, the disruption or dysfunction of the hepatopancreas, the site of hemocyanin synthesis (Rainer and Brouwer, 1993) and glycogen storage, has negative consequences to respiration and lipid metabolism.

Phosphatases have been reported from the hemocytes of crabs (Roche and Latreille, 1934), but little work has examined their role in blood chemistry in crustaceans. Parasite-derived acid phosphatase apparently inhibit the production of host-derived superoxide anions (Remaley et al., 1984). In trypanosomes, acid phophatase is secreted extracelluarly (Lovelace et al., 1986) or is distributed on the cell surface (Gottlieb and Dwyer, 1981). The acetosporan oyster parasite, *Bonamia ostreae,* produces acid phophatase at levels similar to those for *Leishmania* spp. (Hervio et al., 1991). Similarly, the protozoan parasite *Perkinsus marinus* produces high levels of acid phosphatase activity; such parasite-derived extracellular enzymes may inhibit the cellular defenses (as oxygen intermediates) and phosphoproteins of the oyster host (Volety and Chu, 1997). Thus, the high levels of acid phosphatase observed in cells of *Hematodinium* may inhibit innate host defenses such as superoxide-mediated cell death. Alternatively, acid phosphatase, beta-glucuronidase and N-acetyl-beta-glucosaminidase may indicate high levels of phagocytic or pinocytic activity by the parasite (Beckman et al., 1992); yet, phagocytosis of host hemocytes is rarely observed with *H. perezi* (Shields, pers. obs.). Other studies have examined enzyme levels in blue crabs, but not in relation to disease (Walsh and Henry, 1990; Najafabadi et al., 1992).

HEMAGGLUTINATION AND HOST DEFENSES.—In crustaceans, agglutinins have been found against vertebrate erythrocytes, bacteria, invertebrate sperm, protozoans, and other cells (for review, Smith and Chisholm, 1992; Söderhäll and Cerenius, 1992). Agglutinins generally have low titers in crustaceans when compared to other invertebrates and show specificity to n-acylaminosugars which are

common constituents of bacterial cell walls. At least three lectins (serum-bound and cell-bound) are known from the blue crab (Cassels et al., 1986). More recently, a bacteriolytic killing factor, callinectin, a small peptide, has been found in the sera and on the hemocytes of blue crabs (Noga et al., 1994; Khoo et al., 1996). Decreased levels of callinectin are associated with shell disease in the Pamlico River, North Carolina.

There is surprisingly little correlative evidence on the role of agglutinins or other humoral factors in the disease resistance of crustaceans. Foreign bodies have been shown to induce an increase in agglutination titers in the blue crab (Pauley, 1973). Rabbit and chicken RBCs induced a short, but weak rise in the titers of agglutinins over 2 days. Lobster sera did not agglutinate *Aerococcus viridans,* but *Cancer irroratus* and *Chionoecetes opilio* showed varying levels of agglutination, but it was not correlated with infectivity of the pathogen (Cornick and Stewart, 1975). Pooled sera of *Peneaus japonicus* agglutinated horse, sheep, chicken and human RBCs (Muramoto et al., 1995). In oysters, serum agglutinin levels did not change in *Crassostrea virginica* infected with *Perkinsus marinus,* or with *Haplosporidium nelsoni* (Chintala et al., 1994). Serum agglutinins did, however, increase markedly in *C. gigas* exposed to *P. marinus,* but declined to zero in moribund *C. virginica* (La Peyre et al., 1995). In blue crabs, hemagglutination in individual serum samples did not correlate with infection by *H. perezi.* Several infected crabs did, however, show relatively high titers of activity (1:64). Opsonin-like activation by agglutinins probably occurs because hemocytes form nodules in response to bacterial and *Hematodinium* infections (Johnson, 1976; Field et al., 1992; Messick, 1994; Field and Appleton, 1995).

Bacterial and protozoal diseases commonly lead to reduced hemocyte densities in crustaceans. In blue crabs infected with *H. perezi,* hyalinocyte densities decline with infection, and declining hemocyte densities are correlated with host mortality (Shields and Squyars, 2000). Loss of clotting is probably dependent on the decline in hyalinocytes in infections, and may be associated with mortality. Clotting is effected by the hyalinocytes and possibly semigranulocytes (Stang-Voss, 1971; Johnson, 1980; Hose et al., 1990). Hyalinocytes dehisce to release products that catalyze coagulogen, the main clotting protein (Bachau and DeBrouwer, 1974; Ghidalia et al., 1981; Hose et al., 1990). Since blue crabs have Type C clotting, or explosive cytolysis (Clare and Lumb, 1994), a rapid decline in hyalinocyte densities coupled with changes in serum proteins leads to the loss of clotting ability.

ENERGY METABOLISM AND GLYCOGEN.—Glycogen is the main storage substrate in many invertebrates, providing energy for several physiological processes including chitin synthesis (Gabbot, 1976). Winget et al. (1977) reported glycogen at 8.4 ± 0.5 (SE) mg/gm from the hepatopancreas of blue crabs collected in autumn from Delaware Bay, a value slightly higher than that reported here. In *C. maenas,* glycogen levels show marked variability with season, sex, starvation, tissue sampled, and molt cycle (Heath and Barnes, 1970). In *Chasmagnathus granulata,* glycogen levels show seasonal patterns in hemolymph and gill, but not in muscle or hepatopancreas (Nery and Santos, 1993). The utilization of glycogen was apparently different between summer and winter with glycogen being used for energy metabolism during summer (with lipid storage), followed by glycogen storage over winter (with lipid metabolism) (Kucharski and Da Silva, 1991; Nery and Santos, 1993).

Glycogen reserves decrease with infections in decapod crustaceans (Table 4). As with serum constituents, hemolymph-dwelling pathogens (Gram negative bacteria, *A. viridans, H. perezi*) are associated with declines in glycogen in the he-

patopancreas over time. The rapid decline in glycogen in infected hosts indicates a severe metabolic drain due to the pathogens. In lobsters, *H. americanus,* infected with *A. viridans,* glycogen declined precipitously over two to four days in the hepatopancreas and muscle, but took over one week to decline in the heart (Stewart and Arie, 1973). The declines in the hepatopancreas were as great as 70% over four days, and were likely due to a bacterial predilection for that organ. Experimentally infected lobsters stopped feeding two days after infection by *A. viridans* (Stewart and Arie, 1973) and experimentally infected blue crabs showed reduced feeding activity 14 days after inoculation with *H. perezi* (Shields, unpubl. data); hence, the decline in glycogen may be exacerbated by cessation of feeding.

In contrast, tissue-dwelling microsporidans (*A. michaelis, T. maenadis*) apparently only had a local impact on the glycogen in the surrounding muscle; glycogen reserves in the hepatopancreas were not affected (Findley et al., 1981; Vivarès and Cuq, 1981). In addition, lipid levels did not change in infected crabs (Vivarès and Cuq, 1981). Glycogen and lipids increased in the hepatopancreas of *C. maenas* infected by *Sacculina carcini* (Smith, 1911, 1913) and lipid reserves did not change in hermit crabs infected by a bopyrid isopod (Reinhard et al., 1947). The high metabolic drain associated with these parasitic castrators may result from mobilization and synthesis of proteins rather than use of lipids and glycogen reserves.

Glycogen is a precursor for chitin synthesis, and large quantities can be found in the epidermis and underlying connective tissues prior to ecdysis (Travis, 1955; Johnson, 1980). Juvenile green crabs, *C. carcinus,* store glycogen in the hepatopancreas prior to molting while large, adult crabs (anecdysial) do not store large quantities (Heath and Barnes, 1970). Juvenile snow and Tanner crabs, *Chionoecetes opilio* and *C. bairdi,* with advanced stages of *Hematodinium* sp. may not successfully molt (Meyers et al., 1987). Glycogen is depleted by 50% in these animals (Shields and Taylor, unpubl. data). While it is unclear if heavily infected snow crabs survive ecdysis, chitin deposition would likely be hindered during the process. However, lightly infected blue crabs have successfully molted in the lab (Shields, unpubl. data). Since there is no difference in the prevalence of *H. perezi* in postmolt, intermolt, and premolt crabs (Messick and Shields, 2000), the reduction in glycogen may not be apparent in lightly infected blue crabs.

Several pathogens interefere with glucose metabolism. Hemolymph glucose levels were 40% to 60% lower in blue crabs infected with *P. perniciosa*; indeed, some infected crabs had no detectable quantities of glucose (Pauley et al., 1975). Similar findings were reported for lobster, *H. americanus,* infected with gaffkemia (Stewart and Cornick, 1972). Blue crabs parasitized by the microsporidan, *Ameson michaelis,* show markedly increased levels of lactate and decreased levels of blood glucose in the hemolymph, thoracic muscle and hepatopancreas (Findley et al., 1981). Increases in lactate in hemolymph and muscle were also noted for *C. mediterraneus* infected with *Thelohania maenadis* (Vivarès and Cuq, 1981). High lactic acid levels in the hemolymph may result from the use of muscle tissue as an energy source by the parasites or from parasite-induced stress and indicate that the affected tissues are undergoing anaerobic metabolism in response to hypoxia (Findley et al., 1981; Taylor et al., 1996).

The processes leading to host death in *Hematodinium* infections are complex. Clearly, respiratory dysfunction is evident by the decline in hemocyanin levels (Field et al., 1992; this study), the loss of oxygen binding capacity of the hemocyanin (Taylor et al., 1996), and the magnitude of parasitic congestion and disruption of the gills, and other tissues (Meyers et al., 1987; Field et al., 1992; Hudson and Shields, 1994; Messick, 1994). Ischemia and focal necrosis are im-

portant factors in mortality of blue crabs infected with *Vibrio* spp. (Johnson, 1976), and may contribute to mortality in blue crabs infected with *H. perezi*. The chronic nature of *Hematodinium* infections indicates that exhaustion of metabolic reserves may occur over long periods and likely results from proliferation of the parasite (Meyers et al., 1987; Shields and Squyars, 2000), and reduced feeding activity associated with lethargy (Taylor et al., 1996; Stentiford et al., 2000; Shields, unpubl. data). Lastly, low hemocyte densities are correlated with imminent host death (Shields and Squyars, 2000), and may facilitate lethal secondary bacterial infections (Meyers et al., 1987; Field et al., 1992), or lead to loss of clotting ability with death ensuing from loss of hemolymph.

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